

Research Article

Biosecurity implications of the highly invasive carpet sea-squirt *Didemnum vexillum* Kott, 2002 for a protected area of global significance

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Abstract

Loch Creran on the west coast of Scotland supports the most expansive reefs of the serpulid (*Serpula vermicularis*) in the world. It also supports flame shell (*Limaria hians*) and horse mussel (*Modiolus modiolus*) reefs, thereby ensuring this loch received designation as a European Special Area of Conservation in 2005. In 2015, environmental DNA of the invasive, non-native colonial tunicate *Didemnum vexillum*, which has a worldwide distribution, was detected in Loch Creran. *Didemnum vexillum* was confirmed as being present at an oyster farm in the sea loch, using the cytochrome oxidase I gene (COI), following rapid intertidal and dive surveys in early September 2016. The abundance and distribution range assessment carried out at the farm indicated an early-stage *D. vexillum* invasion. The follow up dive surveys and wider loch intertidal surveys carried out in 2017 and 2018 confirmed that the *D. vexillum* presence continues to be associated exclusively with the oyster farm. This is the first time that such a highly invasive species has been found within a protected area of global significance and it has significant biosecurity and policy implications regarding how to manage such invasive species.

Key words: COI, invasive species, marine protected area, rapid assessment, *Serpula vermicularis*, tunicates, ascidian, Pacific oyster

Introduction

Loch Creran, a small semi-enclosed sea loch on the west coast of Scotland, was designated as a marine Special Area of Conservation (SAC) under the Habitats Directive (92/43/EEC) in 2005 to protect its unique biogenic reef features. These reefs were formed by the serpulid polychaete *Serpula vermicularis* Linnaeus, 1767 and the horse-mussel *Modiolus modiolus* Linnaeus, 1758. The former has created the most extensive *S. vermicularis* reefs in the world (Moore et al. 2009). In addition, this sea loch also contains flame shell *Limaria hians* (Gmelin, 1791) reefs, which led to the

additional designation of this area as a Natural Conservation Marine Protected Area (Loch Creran MPA) in 2014.

The colonial tunicate Didemnum vexillum Kott, 2002 has been identified as a high risk species which could have a "disastrous effect on species diversity" (GBNNSS 2011). Invasions by D. vexillum can lead to changes in habitat complexity, through competition with native species, and ultimately to changes in ecosystem function (Cordell et al. 2013). It has spread extensively throughout the world and successfully established in the cool-temperate waters of New Zealand, the west and east coasts of the United States and Canada and northern Europe (Lambert 2009; Stefaniak et al. 2009; Vercaemer et al. 2015; Fletcher et al. 2018). More recently, D. vexillum has spread to the warmer waters of the northern Mediterranean Sea (Ordóňez et al. 2015; Tagliapietra et al. 2012). In the UK and Ireland, D. vexillum has been reported since 2006 at marina sites (Griffith et al. 2009; Minchin and Nunn 2013; Minchin and Sides 2006) and on farmed mussel longlines and oyster bags on the west coast of Ireland in 2007 (McKenzie et al. 2017). Populations have been also found along the southern English coast and further east on the north coast of Kent (Bishop et al. 2015). In Scotland, the first sighting of D. vexillum was in the Firth of Clyde, south-west Scotland (approximately 80 km south of Loch Creran) in 2009 (Beveridge et al. 2011).

Didemnum vexillum can colonise both horizontal as well as suspended vertical surfaces and depending on the colonised surface and tidal exposure, *D. vexillum* can take two different forms, either extensive encrusting mats with short lobes or long tendril clonal growths (Lambert 2009). The fast colony growth and highly mobile behaviour of *D. vexillum* colonies, coupled to the capability for transient inter-colony fusion and chimerism, contribute to the species' bioinvasive success (Fidler et al. 2018). As a consequence, extensive colonies of *D. vexillum* can occur within a few years after initial introduction, for examples forming a carpet-like growth form over a total of 473 km² of seabed in the Georges Bank, northeastern U.S. (Valentine et al. 2007a). In addition, *D. vexillum* fouling on aquaculture equipment and shellfish stocks has been causing significant economic losses due to increased husbandry costs, increases in shellfish mortality and movement restrictions imposed on stocks (Fletcher et al. 2013).

This paper presents results of a rapid assessment survey on a Pacific oyster farm located in the intertidal zone of Loch Creran, Scotland, confirming unofficial and unconfirmed reports of presence of the highly invasive species *D. vexillum*.

Materials and methods

Site description

Loch Creran is a small semi-enclosed tidal sea loch (12.8 km long) on the west coast of Scotland (Figure 1). It has a constricted opening into the wider





Figure 1. Map of Loch Creran, showing the locations of the serpulid, *Modiolus* and *Limaria* reefs, the intertidal survey site, key maritime activities in the sea loch and the location where the *D. vexillum* was found.

Lynn of Lorn, with a flushing time of approximately 3 days, equivalent to a 60% exchange of loch volume with coastal waters (Edwards et al. 1986) and mean current speeds, in the middle basin, between 0.05 and 0.08ms⁻¹ (Wilson et al. 2009). The serpulid reefs are the most abundant around the perimeter of the middle basin between depths of 3–10 m (Moore et al. 2009), whereas the horse mussel and flame shell beds are concentrated nearer the loch entrance (Marine Scotland MAPS NMPi (http://maps.marine.gov.uk), with data provided by the Scottish Natural Heritage under the Crown Copyright (http://www.gov.scot/crowncopyright)). The sea loch also supports three oyster farms, a salmon farm, a shore-based salmon processing facility with adjacent access-pier (Marine Scotland MAPS NMPi (http://maps.marine.gov.scot)), three anchorages and swinging moorings for a total of 80 yachts, with an over-wintering, shore-based facility (Argyll and Bute Council 2017).

Intertidal shore survey

At low water spring tides (0.4 m Chart Datum) on 1 September 2016, a lower shore survey was conducted in the middle basin of Loch Creran, over



a three-hour period. The survey started from the shore-access pier of the fish-processing facility and worked eastwards ($56^{\circ}31'16''N$; $5^{\circ}24'4''W$) towards the oyster farm where *D. vexillum* had been suspected (Figure 1). The gradually sloping shore consisted of sandy mud with patches of pebbles and shell. Occasional boulders were present, typically covered with macroalgae (e.g., *Fucus serratus* and *F. vesiculosus*). At the oyster farm those trestle tables and bags at the north western edge of the oyster farm were surveyed (approximately 10% of total farm area). Eighteen of the 29 trestle lines in this area (14–17 m long) were surveyed. Remaining trestles were not surveyed as these were under water at the time of the survey.

The Pacific oysters, *Magallana gigas* (Thunberg, 1793) (alternate genus for *Crassostrea*; http://www.marinespecies.org), were held within plastic mesh bags secured with rubber straps onto the iron framework of the trestle tables arranged in pairs of lines on the lower shore. A further row of bags was suspended beneath some trestle lines to create a double layer with approximately 15–30 cm between the upper and lower bags. In total, the upper and lower surfaces of 787 oyster bags were examined and the occurrence of *D. vexillum* colonies was recorded and photographed.

Didemnum vexillum impact scoring

The Abundance and Distribution Range method (ADR) (Olenin et al. 2007) was used for a rapid scoring of *D. vexillum* impact. The ADR assessment area was scaled to the size of the surveyed farm region. The coverage of *D. vexillum* on individual bags was scored as low (< 5% cover of a single side of one oyster bag), moderate (5–50% cover) or high (> 50% cover). The number of bags infested on each line of trestles gave a distribution score from "local" (1 bag), "several localities" (< 50% of bags), "many localities" (> 50% of bags), to "all localities" (100% of bags). Combinations of abundance and distribution provide a scale that ranges from A to E (see Olenin et al. 2007 for more details).

Dive survey

A dive survey took place on the 2^{nd} September 2016 to visually inspect the sub-tidal seabed for *D. vexillum*, adjacent to where this species had been found during the intertidal survey. Divers were deployed at 56°31′16.27″N; 5°23′59.12″W and the survey was conducted in a search pattern from the starting depth of the serpulid reefs at 7 m to their lower limit at 11 m. Six transects of 75–100 m were completed perpendicular to each other; the survey covered an area of approximately one kilometre. Each reef present along the six transects was visually assessed for *D. vexillum*, especially the larger reefs extending to > 50 cm in height. Drift algae and other detritus was removed prior to each assessment.

Between April and July 2018, numerous subtidal diving surveys to assess the presence/absence of *D. vexillum* were conducted on floating structures and fixed moorings for aquaculture companies operating in Loch Creran; the surveys included the hull inspection of a feeding barge.

Genetic characterisation of D. vexillum

On 1st of September 2016, *D. vexillum* tissue samples were collected from three separate colonies located on two separate trestle lines. Thirty additional separate colonies (at least 1.5 m apart) were collected between autumn 2016 and summer 2018, covering an area of the entire farm. Tissue was preserved in 100% molecular grade ethanol (Sigma) and processed as described in Graham et al. (2015) using universal tunicate primers tun_forward and tun_reverse2 to amplify the partial COI gene target (Stefaniak et al. 2009). The product was purified (illustra ExoProStar, VWR) and sequenced in both directions using the same primers as in the amplification reaction (www.dnaseq.co.uk). Sequences were aligned using Clustal Omega (www.ebi.ac.uk) and consensus sequence (Genbank Accession No. MG833034) was compared to sequences in the NCBI database using the *blastn*.

Results

Shore survey

Colonies of *D. vexillum* occurred mainly on the underside of the oyster bags, the frames of trestle tables and on the rubber straps used to secure oyster bags. No colonies or fragments of *D. vexillum* were seen directly on the seabed, boulders on the shore, nor along the lower shore, between the access-pier of the fish processing facility and the oyster farm.

Pale-yellow pendulous growths hung below some oyster bags and where trestles bore two levels of bags many of these growths lay, and grew across, bags on the lower level (Figure 2A). Pendulous growths were generally thin, extending approximately 40 cm in length. Some were broader towards their terminal end. No clear breakages were observed in these growth forms and no colony fragments were noted on the adjacent substrate. Microscopy did not reveal any brooded larvae, but clear, spicule-free channels could be seen between zooid groups, which are characteristic of *D. vexillum* (Figure 2B) (see Lambert 2009).

Abundance and distribution of D. vexillum in the oyster farm

Eighteen of the 29 lines of trestles tables, located in the surveyed section of the farm, were examined. *D. vexillum* was observed on 8 lines (Table 1). The majority of the affected trestles had a relatively low proportion of contaminated bags ($\sim 10\%$) which were reachable between neap and spring tidal conditions. Overall, the surveyed proportion of the oyster farm had a





Figure 2. A. An example of the extensive pendulous growths of *D. vexillum*, on the underside of an oyster bag, observed during the intertidal survey. Photo \bigcirc D. Minchin, MOI. B. Crosssection of a single pendulous growth of *D. vexillum* (x15). \bigcirc C. Beveridge, SAMS.

low *D. vexillum* coverage, although 50% of the bags on two trestles had a low to moderate *D. vexillum* coverage. Overall, 78 of 787 examined oyster bags (i.e., 9.9%) were found with one or more recognisable colonies of *D. vexillum*, with the affected surface area of a bag side ranging from < 5% to 20% (Table 1).

Dive survey

No colonies of *D. vexillum* were observed during the subtidal survey. The majority of the serpulid reefs consisted of small colonies of living *S. vermicularis*, with the largest reefs (maximum height 0.5 m) found at a depth of 8 m.

No colonies of *D. vexillum* were reported in any of the surveys of the aquaculture-related floating and fixed structures.



| | No. of trestle lines sampled | Total No. of bags | No. of bags with D. vexillum | ADR scoring |
|-------|------------------------------|-------------------|---------------------------------|-------------|
| | 10 | 213 | 0 | - |
| | 1 | 67 | 32 | В |
| | 1 | 67 | 20 | В |
| | 1 | 76 | 3 | А |
| | 1 | 58 | 1 | А |
| | 1 | 83 | 7 | А |
| | 1 | 78 | 7 | А |
| | 1 | 73 | 5 | А |
| | 1 | 72 | 3 | А |
| TOTAL | 18 | 787 | 78 | |

Table 1. Occurrence of *D. vexillum* on 1 September 2016 based on scoring system according to Olenin et al. (2007), where A = Low abundance at one or several localities; B = Low abundance at many localities or moderate abundance at one or several localities.

Molecular characterization of D. vexillum colonies

A product of approximately 600 bp was amplified from the three colonies collected in September 2016 and additional 30 colonies collected between 2016 to 2018 at the oyster farm site. All generated sequences were identical and a *blastn* search revealed that all samples belonged to *D. vexillum* haplotype 3. Loch Creran *D. vexillum* sequence was identical to other haplotype 3 sequences obtained from multiple sites in UK (Graham et al. 2015) and worldwide (Stefaniak et al. 2009).

Discussion

The presence of *D. vexillum* was confirmed from an oyster farm in Loch Creran, using molecular analysis of more than 30 colonies collected throughout the farm site. These findings validate the unconfirmed results obtained using environmental DNA in a water sample collected from a site adjacent to the farm by an environmental consultancy in May 2015 (Xelect Ltd 2015) and an unconfirmed sighting, reported by a farm employee, to the Scottish Association for Marine Science in early August 2016.

Rapid detection and identification

Accurate identification of *D. vexillum* either in the field or using a basic microscopy approach in the laboratory is difficult, as this organism can appear in different colours and forms depending on the substrate or tidal exposure (Lambert 2009). Therefore, at present, sequencing of the cytochrome oxidase I gene (COI) of mitochondrial DNA has been demonstrated as the most accurate identification tool for any suspect colonies (Stefaniak et al. 2009). Using the COI marker, up to 23 different haplotypes of *D. vexillum* have been reported worldwide, with the highest diversity recorded in Japan, where 17 unique haplotypes can be found (Stefaniak et al. 2012). The European populations appear to have undergone a recent genetic bottleneck with the most common haplotype 3 representing up to 73.4% of all studied colonies, while in Japan this haplotype accounts for up to 19.6% (Stefaniak et al. 2012).



Over 30 separate colonies of *D. vexillum*, collected on the oyster farm in Loch Creran between 2016 and 2018, were characterized using the COI marker and all colonies appeared to belong to haplotype 3. Therefore, it is highly likely that this invasion resulted from a single introduction from one source population. On the other hand, in the UK the haplotype 3 is the most commonly present haplotype (over 70% of sites) (Graham et al. 2015) and sequences obtained from Loch Creran colonies were identical to other haplotype 3 invasions in UK (Clyde area, south and south-east coast of England, Wales) (Graham et al. 2015; Stefaniak et al. 2012), worldwide (Canada, France, Ireland, Netherlands, New Zealand, USA) as well as, haplotype 3 collected from the native range in Japan (Stefaniak et al. 2009). Therefore, accurate identification of origins of invasion, using a single molecular marker, is not possible and further research is needed to develop more accurate tools to assist in tracing introductions of highly invasive *D. vexillum*.

Conventional rapid assessment surveys in the intertidal zone are a wellestablished monitoring tool for invasive non-native species (Ashton et al. 2006; Minchin et al. 2016; Nall et al. 2014), however, this approach relies on an adequate ability to identify the species on site. In recent years, numerous studies demonstrated the capacity of eDNA to detect non-native species in freshwater environments and a review by Blackman et al. (2018) summarizes well the potentials and pitfalls of this approach. The initial report of D. vexillum in Loch Creran, by an environmental consultancy using eDNA, followed by a stakeholder sighting highlights the importance of both continued stakeholder awareness and the use of novel techniques to deliver accurate and rapid detection of new introductions. Monitoring for D. vexillum using eDNA detection will, however, require further validations, including necessary testing of specificity, sensitivity and estimation of detection probabilities prior to routine applications. Development of robust monitoring tools, which are capable of detecting even low traces of D. vexillum prior to an establishment, should be prioritised in order to initiate rapid responses and help to direct and target resources available for the effective management of this species.

Potential pathways of introduction

From a biosecurity perspective, it is critical to determine all potential pathways for *D. vexillum* introduction in order to mitigate further spread. Activities associated with aquaculture, such as movement of shellfish stock or equipment and transfer of fouling on commercial and recreational vessels have been identified as potential introductory pathways for numerous INNS (Tidbury et al. 2016). Both of these pathways are feasible ways of introducing *D. vexillum* into Loch Creran.

During 2010–2016, the farm site in Loch Creran received multiple imports of juvenile oysters from at least two potential hatcheries in UK



mainland (Kent, Cumbria) and Guernsey. The suppliers would have been certified as "disease free" at the time of these imports, however, there were no legal requirements to declare the status in regards to INNS, of shellfish stocks for export or import. There are published findings from one of the potential hatchery sources in Kent (near Whitstable) and associated shore sites, reporting D. vexillum presence in 2011 (Hitchin 2011). Samples from the Kent shore survey have been sequenced for the partial COI gene and identified as haplotype 1 (Graham et al. 2015), while Loch Creran D. vexillum is shown to be haplotype 3. Didemnum vexillum in the Channel Islands, including Guernsey has not been reported, although with this species distributed along the south coast of England it is highly likely that it will establish in the islands in the near future (Department of Environment, States of Jersey 2017). Two other active shellfish farms operating in Loch Creran at the time of the D. vexillum sighting at the oyster farm were also surveyed in October 2016 (Brown et al. 2018a, b) and August 2018 (Scottish Government, unpublished data). No D. vexillum was observed at these farms.

The accidental transfer of D. vexillum on hulls of recreational boats into Loch Creran should also be considered, as this species has been known to be present in multiple marinas in England and Scotland (Beveridge et al. 2011; Bishop et al. 2015; Griffith et al. 2009). Based on the records of frequency/density of recreational sailing (www.rya.org.uk) Loch Creran appears to be well connected with the Clyde area and for example, D. vexillum was detected in the Largs Yacht Haven, Fairlie and Clydeport Jetties in 2010 (Beveridge et al. 2011). Sequencing of the partial COI region confirmed D. vexillum haplotype 3 present at sites in the Clyde (Graham et al. 2015) and, therefore, there is a possibility that D. vexillum could have been transported to Loch Creran from this region. Lastly, natural dispersion or expansion of habitats of D. vexillum from the sites already affected has to be mentioned, however, this tunicate has a short pelagic larval period (Valentine et al. 2009) and potential spread is in the order of hundreds of meters, depending on the local hydrography (Fletcher et al. 2013). It is unlikely, therefore, that the current-driven natural larval dispersal is the pathway for introduction of *D. vexillum* into Loch Creran.

Overall, the possibility of transfer of *D. vexillum* with either imported oyster stock or with recreational boating cannot be accurately traced and, therefore, neither of the aforementioned introductory pathways can be completely eliminated as a potential source. It must be highlighted, however, that *D. vexillum* can persist in a microscopic, un-identified "resting" state (Valentine et al. 2007b), thus making it extremely difficult to detect by visual observations during stock movements or hull inspections. This case certainly highlights the future need for both stock suppliers and recipients to ensure that effective monitoring and essential treatment is in place to mitigate unwanted transfer of hitchhiker species on transported shellfish stocks. Considering that only a limited number of colonies were screened

from both Kent and Largs sites (1–2 colonies at each site, respectively) and that there is the potential for two or more *D. vexillum* haplotypes to co-exist at a single site (as reported in Graham et al. 2015), haplotype data can only be used as one indication of the source. Thus, further research including the development of additional molecular markers, is essential to allow a more robust determination of a likely source.

Management strategies for containment of D. vexillum

A key concern with the introduction of D. vexillum to the Loch Creran marine SAC, is its potential to rapidly overgrow and displace other organisms (Mercer et al. 2009) and thus, it presents a potential serious threat to the protected "reef" structures in the Loch. There is a high likelihood that viable colonies will become dislodged from the active aquaculture site, where bags are moved frequently, and subsequently transferred by water currents to the reef structures. Eradication of D. vexillum, once established, is difficult. To date, D. vexillum eradication attempts from artificial structures in New Zealand and Wales have been unsuccessful and expensive (Coutts and Forrest 2007; Holt et al. 2011; Sambrook et al. 2014). The Scottish Government initiated discussions with the local stakeholders to develop a biosecurity plan to mitigate spread of D. vexillum from the farm into the wider loch environment in 2017. More than 70 different stakeholders, with specific links to the Loch, were identified and a large proportion have been involved in discussions and the preparation of a plan to identify the most practical and efficient management actions and monitoring procedures. All potential mechanisms for D. vexillum spread, associated with each major introductory pathway, have also been analysed and specific actions for each stakeholder and/or advice on effective treatment have been described in the plan (Brown et al. 2017).

Most emphasis to date has been placed on the management of *D. vexillum* at the affected oyster farm. The Wildlife and Countryside Act 1982, as amended by the Wildlife and Natural Environment (Scotland) Act 2011, makes it a criminal offence to release, or cause any animal outwith the control of a person, to be at a place outwith its native range. The legislation also provides relevant bodies with mechanisms for the control of non-native species, including the use of voluntary Species Control Agreements (SCA). SCAs set out what control measures should be implemented. When, and if these are not complied with, an Agreement may be replaced by a Species Control Order. The affected oyster farm in Loch Creran has voluntarily worked with the Scottish Government with regards to the implementation of *D. vexillum* control and containment measures (ICES 2017) and a SCA has been implemented. Conditions under which movement of stocks from the farm was permitted were specified in the SCA. Specifically, a 24 hour freshwater treatment, followed by 48 hour air exposure was indicated as



the most effective treatment taking into account both maximum *D. vexillum* morbidity and minimum oyster mortality (Turrell et al. 2018).

In order to monitor effectiveness of control measures at the farm and to ensure continuing absence of *D. vexillum* in the wider loch environment, intertidal and subtidal surveys, including rapid assessments of human-made structures such as moorings, rafts, piers, pontoons were completed in October 2016 (Brown et al. 2018b), November 2016 (Brown et al. 2018a), October 2017 and August 2018 (Scottish Government, *unpublished data*). In addition, subtidal dive surveys at the adjacent finfish aquaculture installation, carried out between April–July 2018, also concluded the absence of *D. vexillum*. In addition, a number of subtidal dive surveys of the serpulid reef in Loch Creran were conducted between 2017–18 and no *D. vexillum* was found on the reef structures or adjacent sea floor habitats (Scottish Natural Heritage, *in prep.*). All surveys conducted since the sighting of *D. vexillum* on the oyster farm in the Loch have been negative. This suggests that, to date, the containment of *D. vexillum* at the farm site appears to be successful.

In order to safeguard subtidal marine protected areas, there is an urgent need for early warning detection tools for high risk species, in addition to agreed effective management practises, including eradication or containment, followed by efficient monitoring. To mitigate the potential impact of INNS prior to their successful establishment, government, local stakeholders, academic and administrative agencies have to co-operate in reporting these target species and be involved in the regular monitoring in areas of national and international conservation significance.

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