Ministry for Primary Industries
Manatū Ahu Matua

Optimising settlement arrays for surveillance of non-indigenous biofouling species

Results and recommendations following settlement array field trials

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Executive Summary

The introduction to New Zealand and spread of marine non-indigenous species (NIS) has the potential to affect a range of valued ecological, economic, social and cultural resources. Although preventing the introduction of NIS to New Zealand is the preferred option for management, measures are needed to detect the early arrival of new NIS to facilitate their eradication or control.

The goal of this report is to provide recommendations for the design, and protocol for use of passive sampling devices ('settlement arrays') to complement the current Marine High Risk Site Surveillance (MHRSS) programme. The primary goal of the MHRSS is to detect several target species (*Asterias amurensis*, *Carcinus maenas*, *Caulerpa taxifolia*, *Eriocheir sinensis*, and *Potamocorbula amurensis*), with the current survey methodologies designed for these species. Settlement arrays targeting biofouling species have little utility for sampling the primary species, but have the potential to meet both of the secondary objectives of the MHRSS:

1. To detect incursions of new to New Zealand non-indigenous or cryptogenic species not listed on the Unwanted Organisms Register at high risk sites throughout New Zealand.
2. To detect incursions (i.e., range extensions) of established non-indigenous or cryptogenic species that exhibit characteristics of pests and diseases.

This report describes the results of field trials of the settlement arrays undertaken in Westhaven Marina, Waitemata Harbour, Auckland. Three separate deployments of the settlement arrays were made: (i) October 2014 to January 2015, (ii) June 2015 to October 2015, and (iii) November 2015 to February 2016. The design of the field trials was based on a related review of scientific literature on the use settlement plates to sample biofouling assemblages and marine NIS.

The field trials tested elements of array design to determine their effect on the total species richness of biofouling NIS sampled, and the relative incidence of NIS and indigenous species (IS). Experimental treatments examined the influences of settlement plate orientation (undersides of horizontal plates vs. vertical plates), surface texture (smooth vs. rough) and the presence of thin-layer antifouling coatings (non-biocidal primer vs. two thicknesses of antifouling paint) on the biofouling assemblages. The antifouling coatings applied were intended to simulate aged coatings on vessels where the levels of biocide are depleted (i.e., antifouling coating thickness were equivalent to one third, two thirds, and the full recommended application thickness, respectively). These plates were deployed to target NIS that are tolerant of commonly used antifouling biocides. On each occasion, 10 arrays, each containing a single replicate of the 12 orthogonal treatment conditions, were deployed for a period of three months. Upon retrieval, the identity (to lowest taxonomic unit) and percent cover of each biofouling organism observed on the plates was recorded.

In total, 19 NIS were detected on settlement plates, including the Mediterranean fanworm *Sabella spallanzanii*, the ascidians *Styela clava* and *Ciona intestinalis*, and several bryozoans (*Bugula neritina*, *Bugula flabellata*, *Watersipora subatra*, *Amathia verticillata*, *Celleporaria umbonatoidea*). Only a subset of the total NIS detected were found attached to biocidal plates (i.e., 8 out of 19 NIS), by comparison across the three deployments the non-biocidal control plates had two to three times more NIS, and four to five times the IS richness. While biocidal plates were much faster to process, there was no evidence of higher NIS detection on these

plates compared to controls for the same unit effort. Overall, the following observations were made:

- More NIS were sampled by non-biocidal plates than biocidal plates for the same unit effort.
- Compositionally different assemblages of NIS were sampled by non-biocidal plates that were oriented vertically and horizontally.
- No evidence of increasing separation of community composition with increasing distance between arrays, but there were significant differences in the biofouling assemblages on control plates at different sites.
- Greater numbers of NIS were sampled in deployments over summer than in winter, although a single NIS (the bryozoan *Tricellaria inopinata*) was only found during the winter deployment.
- Compositionally different assemblages of NIS were sampled by non-biocidal plates that were pitted.
- Higher incidence of NIS on horizontal plates with several species occurring only on horizontal plates (e.g., *S. clava, C. intestinalis*).

The provision of shaded habitat has been associated with the establishment and spread of NIS in countries including Italy and Australia. The use of shaded settlement surfaces is potentially an effective means of detecting incursions of several non-indigenous biofouling species, such as *Eudistoma elongatum*, *S. clava, C. intestinalis* and *S. spallanzanii*. Providing both shaded habitat and habitat receiving sufficient light for photosynthetic organisms will help sample a broader taxonomic range of biofouling species.

Spatial and temporal separation of settlement arrays was associated with large variations in community composition, and shifts in communities between years and across seasons. Only one unique NIS was detected on settlement plates during winter compared to summer deployments, and several IS were observed only during a single deployment. Spatial separation of arrays within marina environments will influence the ability to sample some of the inherent variability of biofouling communities, this is likely related to physical gradients and stochastic recruitment dynamics.

Settlement arrays detected two of the secondary target species of the MHRSS (i.e., NIS already present in New Zealand), *S. spallanzanii* and *S. clava*. They also detected the bryozoan, *Celleporaria umbonatoidea*, during the first array deployment (summer 2014-15), representing a range extension of this species. These findings suggest that settlement arrays may help contribute to both secondary objectives of the MHRSS (i.e., detecting new to New Zealand organisms, and detecting range extensions of established NIS). However, range extensions of *S. spallanzanii* and *S. clava* have been regularly detected at relatively early stages of incursions using other surveillance methods (particularly diver surveys). Given the high densities of *S. spallanzanii* and *S. clava* in Westhaven Marina and the relatively low capture rates on settlement plates, it is unlikely that settlement plates would sample these species better than visual surveys.

Passive sampling methods using settlement arrays should be considered as complementary to the current MHRSS activities increasing the capacity to detect non-target NIS. They also sample juvenile life-history stages of NIS. Settlement arrays, however, do not necessarily enhance the detection of secondary target NIS (i.e., *S. spallanzanii* and *S. clava*) already present in New Zealand compared to other survey methods. Settlement arrays have potential as an outlet for community engagement initiatives and may help increase the awareness of NIS, as evidenced by overseas programmes. The following recommendations are provided for incorporating passive sampling methods into the national MHRSS programme:

- Deployment of 10–20 arrays per site, each consisting of 8-12 plates (80-160 plates) to decrease detection thresholds for rare species.
- Plates deployed both horizontally and vertically in equal numbers.
- All plates are pitted to increase species richness.
- Plates should be made of dark (grey or black) PVC with no paint treatments.
- Arrays should be distributed 10's to 100's of metres apart to sample distinct species assemblages.
- Plates should be deployed for three to four months (depending on season) to allow biofouling to grow to an observable size, assisting visual taxonomic identification.
- Deployments should target the spring-summer period (October–January) to sample NIS spawning during warmer conditions, and the autumn-winter period (April-July) to sample NIS potentially arriving from the northern hemisphere ready to spawn (i.e., following the Northern Hemisphere spring).

Construction, deployment, retrieval and processing settlement arrays would cost an estimated ~NZ$19 496 for 80 plates, ~NZ$27 388 for 120 plates, and ~NZ$35 280 for 160 plates per annum for two deployments at a single location.

While settlement arrays can contribute to the secondary objectives of the MHRSS programme (i.e., sampling non-target NIS), the author's believe that the high cost of deploying and processing large numbers of settlement plates would be better spent on other methods, such as, diver surveys by trained taxonomists and parataxonomists. It is noted, however, that as the issues related to the sensitivity and specificity of high throughput sequencing (HTS) methods are overcome, the combination of settlement arrays and metagenomic sequencing could increase the efficiency of plate processing, enabling the deployment of larger numbers of plates for the same processing cost. These gains in efficiency would need to be reassessed as continuing research is done to operationalise HTS methods for biosecurity surveillance.

Definitions

Biofouling: the accumulation of aquatic organisms on surfaces immersed in, or exposed to, the aquatic environment.

Biosecurity: the exclusion, eradication or effective management of risks posed by pests and diseases to the economy, environment and human health.

Biosecurity surveillance: the collection, collation, analysis, interpretation and timely dissemination of information on the presence, distribution or prevalence of risk organisms and their adverse effects on New Zealand's environments.

Cryptogenic: species that are not demonstrably indigenous or non-indigenous to the New Zealand biogeographic region.

High Throughput Sequencing (HTS): Simultaneous processing of multi-species genetic samples.

Marine High Risk Site Surveillance (MHRSS): a nationwide programme of targeted surveillance for high risk marine pest species funded by the Ministry for Primary Industries (MPI).

MPI: Ministry for Primary Industries.

New to New Zealand: non-indigenous or cryptogenic species that have not previously been recorded from New Zealand waters.

Non-indigenous species (NIS): species that are known or suspected to have been introduced to New Zealand as a result of human activities. Synonymous with 'alien', 'adventive', 'exotic', 'introduced' and 'non-native'.

Passive sampling methods: where an environmental or biological sample is obtained by exposing a surface or medium to the ambient environment over a fixed period of time.

Settlement plate: standardised unit of artificial substratum for detecting settled biofouling species.

Settlement array: collection of settlement plates containing various experimental treatments for sampling biofouling communities.

1 Introduction

1.1 BACKGROUND

Early detection surveys are an important component of biosecurity surveillance. Lags in the discovery of new non-indigenous species (NIS), or of range extensions by NIS already present in New Zealand, provide them with a chance to establish, proliferate, spread and cause harm. Successful eradication or management of NIS often hinge upon the ability to detect new populations when they are relatively small and easily contained and treated (Tobin et al, 2014).

Since 2002, the Ministry for Primary Industries (MPI) has funded a nationwide programme of targeted surveillance for high risk marine pest species at a selection of New Zealand's ports and marinas ("Marine High Risk Site Surveillance", MHRSS). The primary objective of the MHRSS is to detect incursions of new to New Zealand NIS listed on the Unwanted Organisms Register at high risk sites throughout New Zealand.

The MHRSS is conducted to maximise the likelihood of early detection and successful eradication or management of any high risk pests and diseases. Two secondary objectives for the programme are:

i. to detect incursions of new to New Zealand non-indigenous or cryptogenic species not listed on the Unwanted Organisms Register at high risk sites throughout New Zealand, and

ii. to detect incursions (i.e., range extensions) of established non-indigenous or cryptogenic species that exhibit characteristics of pests and diseases.

The MHRSS was designed primarily to achieve early detection of seven NIS that are listed on the New Zealand Register of Unwanted Organisms (i.e., to achieve the primary objective; Inglis et al, 2006a):

- Northern Pacific seastar, *Asterias amurensis*,
- European shore crab, *Carcinus maenas*,
- Aquarium weed, *Caulerpa taxifolia*,
- Chinese mitten crab, *Eriocheir sinensis*,
- Asian clam, *Potamocorbula amurensis*,
- Mediterranean fanworm, *Sabella spallanzanii*, and
- Asian kelp, *Undaria pinnatifida*.

The MHRSS uses a variety of sampling methods, including visual diver surveys, baited and un-baited traps, benthic sleds, and shore-based visual searches that were chosen with the aforementioned species in mind (Inglis et al, 2006a, Seaward et al, 2015). MPI is considering expanding the sampling methodologies of the MHRSS programme to enhance the detection of biofouling species, should they enhance the efficacy of detection, or enhance sampling efficiencies.

Vessel biofouling is a major pathway for NIS to enter New Zealand waters (Inglis et al, 2010, Bell et al, 2011). The MHRSS currently relies on visual searches (underwater by divers and above-water) for detecting biofouling NIS in selected ports and marinas. Safety concerns can

limit the range of sites that are accessible by divers during the surveys, and the effectiveness of visual searches may be compromised during periods of low visibility, with the associated risk that target species (especially juvenile forms) may be overlooked (Hayes et al, 2005, Gust et al, 2006). Integration of passive sampling methods, such as settlement arrays, into the New Zealand MHRSS may complement current sampling methods, and enhance detection of biofouling NIS at the early stages of an incursion or range extension.

Settlement plates have been used extensively in marine ecological research to sample the juvenile stages of sessile marine organisms (Keough, 1983, Butler, 1986, Nandakumar et al, 1993, Glasby and Connell, 2001, Johnston et al, 2002). They are increasingly being used to study the population dynamics of biofouling NIS (Martin et al, 2011, Sephton et al, 2011) and for biosecurity surveillance in marine environments (Marshall and Cribb, 2004, DeRivera et al, 2005, Labowitch and Cribb, 2006, McDonald and Travers, 2008, Ruiz et al, 2009, Bridgwood and McDonald, 2010, Muñoz and Bridgwood, 2012, Northern Territory Government, 2014). In a surveillance context, they have been used to detect particular 'high risk' species (e.g., the bivalve, *Mytilopsis* sp.; Bridgwood and McDonald, 2010) and to monitor changes in the distribution and abundance of a range of biofouling NIS (DeRivera et al, 2005, Ruiz et al, 2009). Settlement plates can be deployed and retrieved relatively easily in areas that may be hazardous for divers, require little specialist expertise to deploy and collect, can sample early-stage biofouling assemblages and provide a standardised sample that is integrated over the period of deployment (i.e., "time-averaged"). There have, however, been relatively few assessments of the efficacy of settlement plates for different types of biosecurity surveillance (Floerl et al, 2012, Tait and Inglis, 2016).

Other than providing a suitable settlement surface, settlement plates, like other passive sampling devices, do not typically have any specific form of attractant, rather they rely instead on the rate at which planktonic stages of an organism encounter the surfaces, settle and grow to an observable size. The suitability of the surface for larval settlement has an important influence on larval behaviour (Keough and Downes, 1982), and manipulation of some variables (e.g., surface texture, light environment) may encourage the recruitment of some species to artificial settlement surfaces. The relationship between the presence of an NIS within the environment and its detection on a settlement surface is complex as it is influenced by the size of the local population, the seasonal abundance of planktonic life stages and patterns of water movement (the "supply side" of the encounter rate; Johnston et al, 2009) as well as the design of the monitoring programme, including the size, type, number, and spatial arrangement of the surfaces and the duration of their deployment (Floerl et al, 2012, Tait and Inglis, 2016).

1.2 PURPOSE OF THIS REPORT

The objective of this project was to recommend a design and sampling protocol for the use of settlement arrays to detect a range of biofouling NIS. The project consisted of two components:

1. A review of existing literature on the utility of settlement surfaces for sampling marine biofouling organisms to guide their design for complementing the MHRSS programme.
2. A field test of settlement plate surfaces and modes of array deployment recommended by the literature review to determine the optimal configuration for detecting the largest number of biofouling NIS.

The literature review made specific recommendations for the design of the arrays used in the subsequent field test and methods for their deployment (summarised in Section 2.1 of this report; Tait and Inglis, 2016). This report describes the design, implementation and results of

the field tests of the arrays and provides recommendations regarding the integration of passive sampling methods into the national MHRSS.

2 Methodology

2.1 DESIGN OF THE SETTLEMENT ARRAYS

2.1.1 Recommendations from the literature review

Recommendations made in the literature review for the experimental design of the field tests are summarised in Table 2-1. Key features included:

- Use of a single, standardised type of material for the settlement plates (polyvinyl chloride (PVC)).
- Deployment of the plates on PVC or steel frames at a single depth (~2 m), suspended from floating structures.
- Three deployments, each for a period of three months, to incorporate two spring-summer and a single winter-spring season.
- A minimum of 10 arrays per deployment each containing a single replicate of each level of the following experimental treatments:
  - plate orientation (vertical vs. the underside of horizontally-oriented plates),
  -
  - predator exclusion cages (caged and un-caged),
  - presence of antifouling coating (non-biocidal control, thin antifouling top coat and moderate antifouling top coat), and,
  - surface rugosity (rough only).

Vertical and horizontal plates were recommended because previous studies had shown that they sampled compositionally distinct biofouling assemblages (Glasby, 2000, Knot et al, 2004). Similarly, predator exclusion was recommended because research highlighted major differences in the species composition of assemblages on settlement plates that have been caged to exclude predators and those that have been left uncaged. Biocidal treatments incorporating thin coatings of antifouling paints were proposed to simulate the degraded coatings observed on in-service vessels. The purpose was to determine if the presence of the coatings preferentially favoured recruitment by NIS over IS, and to define the thickness of coating that sampled the largest ratio of NIS relative to IS. The full rationale behind these recommendations is provided in the companion report (Tait and Inglis, 2016).

Slight modifications to the proposed design were implemented prior to and following the first deployment and discussions with the MPI project team. These are summarised in Table 2-1 and described in detail in the following sections. The most significant change was the removal of the predator exclusion treatment and its replacement with a treatment that compared recruitment on abraded and smooth PVC plates (Table 2-1). Predator exclusion cages were not incorporated due to the potential for biofouling on the cages to affect recruitment to the settlement plates, the need to include an additional cage-control in the experimental trials, and the time and resource costs associated with maintaining biofouling-free cages (Lavender et al, 2014). Surface rugosity was identified as an alternative treatment to predator exclusion cages in the event that a caged treatment was deemed inappropriate for practical reasons (Tait and Inglis, 2016). Increasing surface heterogeneity has been shown to increase the survival of early recruits through predator avoidance (Walters and Wethey, 1991, Walters and Wethey, 1996), thereby providing similar outcomes as a caged treatment. Following the first

deployment, pitting replaced the sand-blasting treatment due to lack of significant surface modification by sand-blasting (particularly after paint application) and antifouling coating thickness was reduced to enhance the recruitment of biofouling species.

Table 2-1 Experimental treatments recommended in the literature review (Tait and Inglis, 2016) and used in the three deployments of settlement arrays.

		Treatment levels			
Experimental treatment	No. levels	Tait and Inglis (2016)	Summer 2014-15	Winter 2015	Summer 2015-16
Material	1	Plastic (PVC)	Plastic (PVC)	Plastic (PVC)	Plastic (PVC)
Depth	1	~2 m	~2 m	~2 m	~2 m
Orientation	2	Vertical Horizontal (undersides)	Vertical Horizontal (undersides)	Vertical Horizontal (undersides)	Vertical Horizontal (undersides)
Surface abrasion	2	Smooth Rough (sand-blasted)	Smooth Rough (sand-blasted)	Smooth Rough (pitted)	Smooth Rough (pitted)
Antifouling coating	3	Non-biocidal control thin top coat moderate top coat	Non-biocidal control ~75 µm top coat (A1) ~150 µm top coat (A2)	Non-biocidal control ~40 µm top coat (A0.5) ~75 µm top coat (A1)	Non-biocidal control ~40 µm top coat (A0.5) ~75 µm top coat (A1)
Consistent paint treatments		n/a	Non- biocidal control ~75 µm top coat (A1)	Non- biocidal control ~75 µm top coat (A1)	Non- biocidal control ~75 µm top coat (A1)
Predator exclusion	2	Uncaged Caged	-	-	-
Plates per array		12	12	12	12
No. of arrays		10	10	10	10
Total No. of plates per deployment		120	120	120	120
Total materials cost (per array)		NZ$335-385	NZ$300-350	NZ$300-350	NZ$300-350

2.1.2 Experimental design used in the three field tests

The settlement plates were made of 4.5 mm thick sheets of polyvinyl chloride (PVC) cut into 145 x 145 mm squares (Figure 2-1, Figure 2-2). Half of the 120 plates in the initial (summer 2014-15) deployment were roughened by abrasive sandblasting while the other half were left smooth. The other, experimental treatments included:

- Antifouling paint (non-biocidal control and two levels of biocidal treatments: ~75 µm topcoat and ~150 µm topcoat, respectively).
- Plate orientation (horizontal and vertical).

The non-biocidal control plate was coated with primer paint (International Primocon™). The biocidal treatment plates (A1 and A2), were coated with the primer and a self-polishing copolymer (SPC) paint that contained copper- and zinc-based biocides (International Micron Extra™, Table 2-2). Since the paints were applied over the surface texture treatments, the total number of coats was standardised between all treatments to ensure consistent depth of abrasions (i.e., control = three coats of primer, A1 = two coats primer + one coat antifouling; A2 = one coat primer + two coats antifouling). The primer and antifouling coatings were applied to the plates using rollers. Coating thickness was measured with digital callipers following each application to ensure consistent thickness. To increase the precision of measurements using digital callipers, the total thickness of three paint coats was measured to estimate the average thickness per coating. A subset of five plates were selected from each paint treatment for measurement, with each plate measured in 5 different places before and

after paint application (exact point of plates marked prior to painting). The average thickness of paint coats was 75 µm ± 10 SD (antifouling paint), 85 µm ± 18 SD (primer paint), and 40 µm ± 9 SD (thinned antifouling paint). Total thickness of three coatings for three treatments (i.e., three coats primer, two coats primer + one coat antifouling paint, and one coat primer + two coats antifouling) were not significantly different ($F_{2,73} = 0.0005$, $p = 0.99$).

For deployment, a single replicate plate of each treatment condition was secured with zip-ties to each of 10 array frames (total 120 plates, $n = 10$ per orientation/surface/paint treatment per deployment). The frames were made from 32-mm thick PVC pipe, joined with elbow connectors and T-connectors into three rows, with four plates secured per row (Figure 2-1). Plates were fitted in alternating horizontal (facing downwards) and vertical orientations, with other experimental treatments randomly assigned throughout the frame.

Following analysis of results from the first deployment (summer 2014-15), two experimental treatments (antifouling coatings and surface texture) were altered in the winter 2015 and summer 2015-16 deployments in an attempt to optimise the settlement and therefore detection of NIS. These changes included replacing the sand-blasted surface treatment with a treatment that had pits routed into the plates (22 x 4.5 mm diameter by 2 mm deep pits in half of the plates; Figure 2-2), and halving the concentrations of the biocides in the antifouling treatments from one and two roller coatings (~ 75 µm topcoat and ~ 150 µm topcoat), to half and one roller coating (~ 40 µm topcoat and ~ 75 µm topcoat, Table 2-1). Paint treatments and labels for each survey were; A1 (~ 75 µm topcoat) and A2 (~ 150 µm topcoat), summer 2014-15; A0.5 (~ 40 µm topcoat) and A1 (~ 75 µm topcoat), winter 2015; A0.5 (~ 40 µm topcoat) and A1 (~ 75 µm topcoat), summer 2015-16.

The pitted treatment was incorporated based on evidence showing that small surface irregularities (< 500 µm) can inhibit the recruitment of a range of biofouling species (Bers and Wahl, 2004), but larger irregularities (> 2 mm) can enhance recruitment by providing refuges (Walters and Wethey, 1991, Walters and Wethey, 1996). The arrangement of pits was intended to spread the treatment evenly across the settlement plates.

The half roller coating was achieved by 50 % dilution of the biocidal coatings using an appropriate thinner (Table 2-2). While thinners are highly volatile and could affect biocide concentration during application (i.e., first plates painted receive low biocide concentration, with concentration increasing throughout application), this treatment was applied in less than one minute to minimise such effects. The initial volume of antifouling paint for this treatment was half that of the A2 treatment. Although thinners are not intended to be used at this ratio due to the effects on the biocidal performance (Table 2-2), the goal of this experimental manipulation was to test the influence of lower than recommended biocidal concentrations on recruitment of copper tolerant NIS.

Settlement array frames were deployed at a constant depth of 2 m, attached to floating pontoons with rope (Figure 2-3, Figure 2-4). To maintain the arrays in a horizontal position, a 1 m length of stainless steel rod was fixed through the centre of the PVC frame, with the length of chain attached to a cinder-block anchor sufficient to hold the frame in place throughout the tidal cycle.

Figure 2-1 Settlement array deployment in Westhaven Marina, Waitemata Harbour, Auckland.

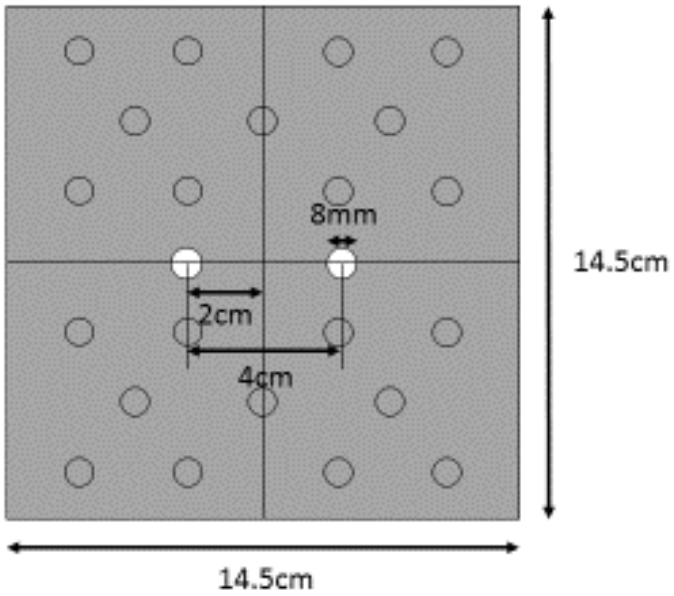

Figure 2-2 Settlement plate layout and dimensions with the two attachment holes (white circles) and the routed pits (grey circles).

Table 2-2 Characteristics of biocidal antifouling paint and non-biocidal primer used to coat experimental plates. Note that coating thickness as specified by the manufacturer was not achievable without the addition of thinner.

	Micron Extra (International®)	Primocon (International®)	Antifouling thinner #3 (International®)
Paint type	Self-polishing co-polymer	Primer	Thinner
Surface preparation	Sanding, primer	Sanding	Sanding, primer
Shelf life	12 months at 25°C	Indefinitely	Indefinitely
Shelf life once applied	6 months	Indefinitely	6 months
Thinning	Not recommended to be thinned by more than 10 %	10-20 %	-
Environmental conditions	> 5 °C	> 5 °C	> 5 °C
Application	Brush (small areas), roller, spray	Roller, brush or spray	Roller or spray
Colour	Grey	Grey	-
Number of coats	3 (180 µm)	1-5 depending on material	-
Thickness per coat (roller)	60 µm	60 µm	-
Biocidal ingredients	Copper(i) oxide, Zinc oxide, Zineb	Nil	Nil
Price ($NZ)	NZ$279.0 (4L, Burnsco)	NZ$189.0 (4L, Burnsco)	NZ$39.99 (1L, Burnsco)

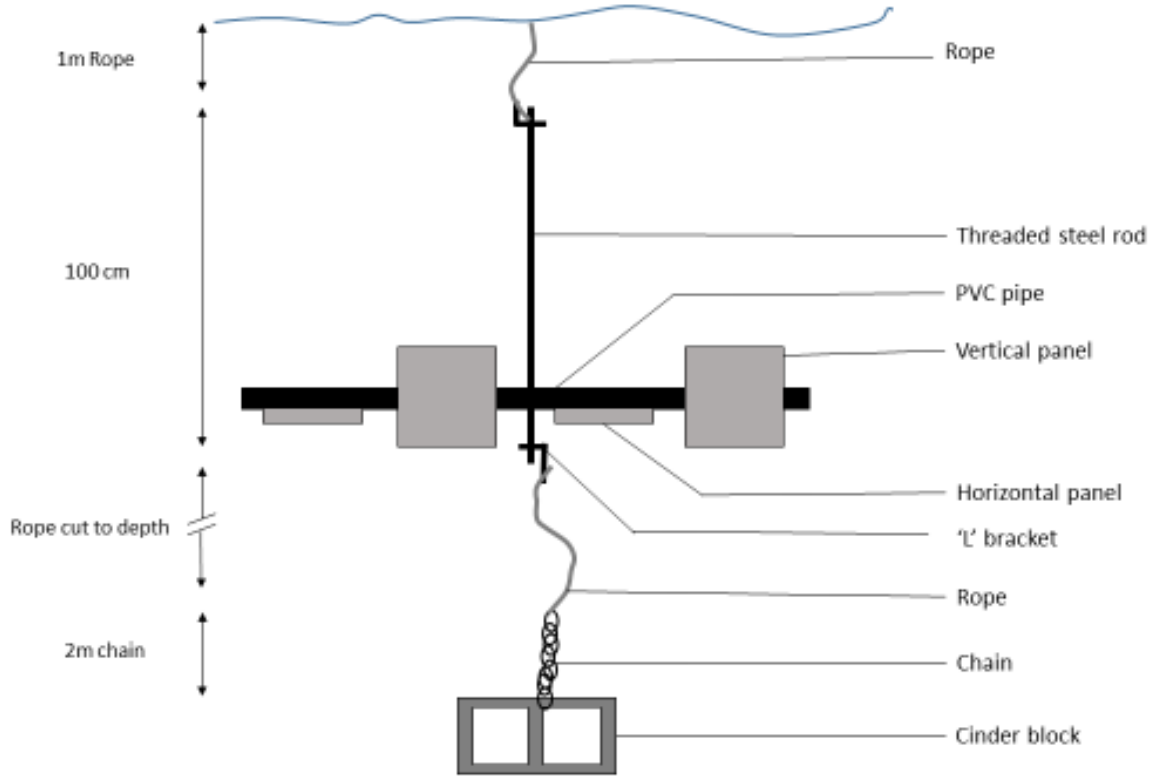

Figure 2-3 Diagram of deployed settlement array, including the mooring to the seafloor.

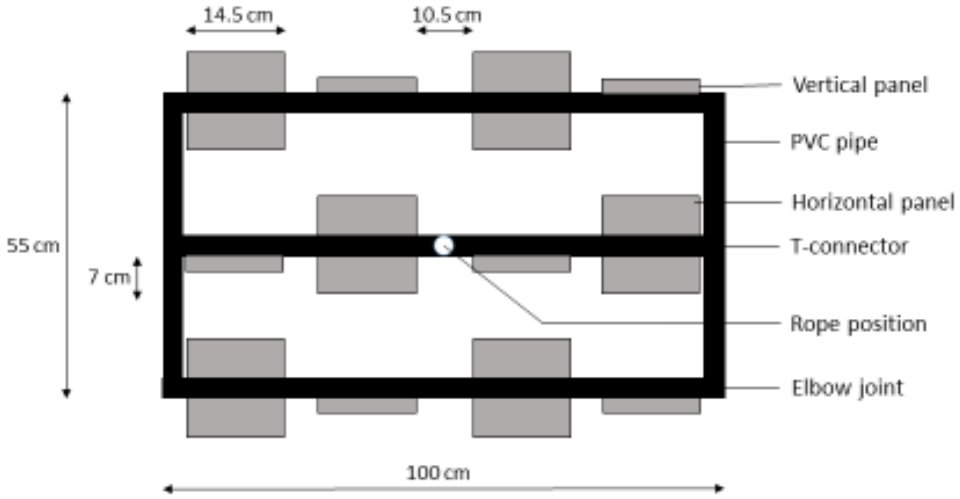

Figure 2-4 Birds-eye view of settlement plate arrangement on settlement array frames.

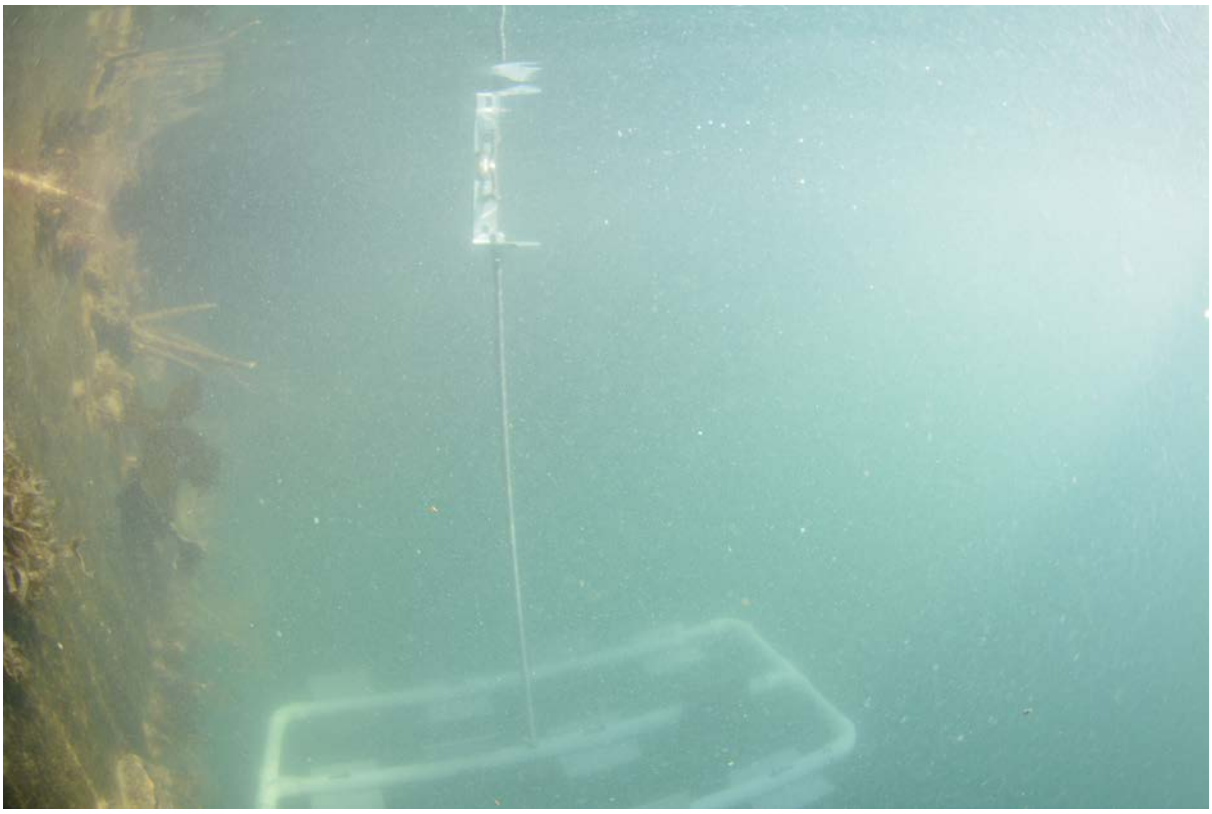

Figure 2-5 Settlement array deployed in Westhaven Marina.

2.2 LOCATION AND TIMING OF THE FIELD TESTS

The field trials were undertaken in Westhaven Marina, Waitemata Harbour, Auckland (Figure 2-6). Settlement arrays were deployed on three occasions on the same pontoons from 20th October 2014 to 29th January 2015, from 23rd June 2015 to 20th October 2015, and from 12th November 2015 to 16th February 2016. The winter (June to October) deployment was extended by approximately one month due to slow growth rates observed on settlement plates. This allowed biofouling to be identified with higher taxonomic resolution. The arrays were deployed in pairs on five rows of pontoons towards the seaward end of Westhaven Marina.

Figure 2-6 Location of Westhaven Marina in Waitemata Harbour, Auckland, New Zealand (inset). Satellite image shows the locations of the ten settlement arrays spread across five pontoons within Westhaven Marina.

2.3 BIOFOULING IDENTIFICATION

After ~3 months deployment, all plates were retrieved and transported to the NIWA Auckland laboratory in individual bags filled with seawater. All plates were photographed and the percentage cover of biofouling and species richness were determined under light microscopy. The percentage cover of each biofouling species was estimated individually using a reference grid (10 x 10 cm, with 1-cm^2 grids). Due to the three dimensional nature of biofouling assemblages it was possible for heavily fouled plates to have biofouling cover exceeding 100%. Biofouling attached to the sides (including the insides of attachment holes in the centre of plates) or backs of plates were excluded from identification and analysis.

The time taken to process each plate was recorded to provide a measure of the labour costs associated with each experimental treatment. Sample processing was performed by the same three parataxonomists (Leigh Tait, Kimberley Seaward and Serena Wilkens) over the duration

of the project. Voucher specimens for species not readily identifiable were preserved (Appendix B), and sent to taxonomic specialists for identification, via the Marine Invasive Taxonomic Service (MITS) provided by NIWA on behalf of MPI. All specimens were identified to the lowest possible taxonomic level, and categorised as either IS to New Zealand, NIS, cryptogenic (undetermined geographic origin), or unresolved/indeterminate (i.e., unable to be identified to species).

2.4 DATA ANALYSIS

Differences in average species diversity and percent cover of biofouling per treatment were analysed separately for each deployment (summer 2014-15, winter 2015, and summer 2015-16) using factorial ANOVA, with the categorical factors, site (site as a random factor), paint treatment, orientation and surface texture (fixed factors). Normality of data was tested using Shapiro-Wilk tests and homogeneity of variances using Brown and Forsythe's test. A site constituted a pair of arrays deployed on the same pontoon (i.e., two arrays on pontoon A were a single site). Arrays were paired by pontoon to examine the effects of distance to the marina entrance, and the associated gradient of flow (i.e., high flow at the entrance or pontoon A and decreasing flow towards the inner marina towards pontoon E). The use of pontoons as sites was also in response to the age of pontoons (or time since cleaned), with differences in the biofouling communities potentially affecting the successional stage of biofouling closest to the settlement plates.

To examine temporal changes over the three deployments, only those treatments that remained consistent across the three deployment periods were analysed (i.e., smooth control plates and the smooth A1 (75 µm) biocidal treatment. Roughened plates (pitted and sand-blasted), and A2 and A0.5 biocidal treatments were excluded from the temporal analysis. There were 10 replicates (n = 10) of each paint, orientation and surface texture treatment for each season. However, a single frame with 12 plates was excluded from analysis during the summer 2014-15 deployment due to divergence in community composition associated with a delayed deployment due to a frame broken during transport (n = 9 per orientation/surface/paint treatment).

Multi-dimensional scaling plots (MDS) and dendrograms (using K-means clustering) were used to visualise variation in compositional structure of biofouling assemblages among treatments and plates. Similarity between plates and experimental treatments in the cluster analysis and MDS was calculated using the Bray-Curtis resemblance measure. Differences in average dispersion between the experimental treatments (antifouling coating, surface orientation and surface texture) were tested using PERMANOVA (using 999 simulation permutations). Monte Carlo simulations were used on factors which had relatively few unique permutations (i.e., less than 100 unique permutations) to enhance the reliability of test statistics. Separate analyses were conducted for each deployment and for the combined analysis of treatments that remained consistent across seasons. All multivariate analyses were performed using the PRIMER-E© and PERMANOVA+ routines.

To determine the relationship between the number of plates deployed and the total diversity of biofouling species sampled by each treatment, sample-based rarefaction (using the statistical package Estimate S©; Colwell, 2013) was used to compare the average cumulative species diversity (and range) on the biocidal plates (A0.5, A1, and A2) and non-biocidal controls. Species incidence data were pooled across orientation and surface texture treatments (n = 40) for each paint treatment due to the low species richness observed on antifouling coatings (A0.5, A1 and A2). Rarefaction curves were used to estimate the expected number of species detected at a given number of samples (Colwell et al, 2004) and extrapolated the rarefaction out to 80 plates to examine the potential for greater numbers of samples to detect a greater

diversity of species (Colwell et al, 2012). These methods were used to examine any potential efficiencies gained by using biocidal coatings to sample species richness. It is noted by Colwell (2013) that it is difficult to interpret extrapolation of rarefaction curves beyond the actual sample number, but in this context it helps to estimate the utility of biocidal coatings to sample more NIS for the same processing time (given the much quicker processing time of biocidal plates). The number of plates of each treatment that could be processed within a standardised time (as a measure of effort) was calculated and compared to the numbers of IS and NIS that would be sampled with each treatment. Confidence intervals (95 %) of rarefaction curves were also plotted (Colwell et al, 2004). Statistical significance of rarefaction curves is not straightforward, but as a general rule of thumb, "non-overlap of 95 % confidence intervals can be used as a simple but conservative criterion of statistical difference" (Colwell, 2013). Curves were plotted separately for IS and NIS.

Rarefaction curves were also plotted separately for each of the four control treatments (i.e., control-rough-horizontal, control-smooth-horizontal, control-rough-vertical, and control-smooth-vertical) to examine the efficacy of each treatment for sampling IS and NIS. Due to treatment alteration from summer 2014-15 to winter 2015 and summer 2015-16, not all treatments can be reliably compared between deployments, with the exception of smooth plates (both orientations). This was done only for controls due to the low species diversity of biocidal treatments. Species incidence data (n = 10 for each treatment) were used to calculate the expected number of species at a given number of samples (Colwell et al, 2004) and extrapolated the rarefaction out to 20 plates (Colwell et al, 2012) to examine the potential rate of new species discovery per unit effort for a greater number of samples.

3 Results

3.1 TOTAL SPECIES RICHNESS, COVER AND COMPOSITION OF BIOFOULING COMMUNITIES

Average total species richness (IS, NIS and cryptogenic species) per settlement plate differed significantly between control plates and biocidal treatments (A0.5, A1, and A2, Table 3-1, Figure 3-1). Average species richness did not vary significantly between the thinner biocidal treatment (A1 during summer 2014-15, A0.5 during winter 2015 and summer 2015-16) and the thicker coatings on any of the deployments (A2 during summer 2014-15, and A1 during winter 2015 and summer 2015-16). The only exception was the A0.5-pitted-horizontal treatment during summer 2015-16 which was higher than the A1-pitted-horizontal treatment ($t = 3.3$, $p < 0.01$). Plate orientation and surface roughness had no statistically significant effect on total richness (Table 3-1, Figure 3-1).

Non-biocidal control plates also had significantly higher total percent cover of biofouling organisms compared to the biocidal treatments (Table 3-1, Figure 3-2). During the first deployment (summer 2014-15), biofouling organisms covered an average of ~ 105 % (standard error (SE) = 5 %) of the horizontal control plate surfaces and 50 % (SE = 5 %) of the vertical control plate surfaces. By comparison, < 10 % of the surfaces of biocidal plates during summer 2014-15 and winter 2015 were fouled (Figure 3-2 A and B), and < 30 % of the plate surface during summer 2015-16 (Figure 3-2 C). There was a significant interaction between paint treatments and orientation for summer 2014-15 and summer 2015-16 (Table 3-1). This was associated with similar biofouling cover on vertical and horizontal biocidal plates, but large differences in biofouling cover on non-biocidal controls.

There were no significant differences in the average species richness recorded on smooth-control (horizontal and vertical plates) and smooth-A1 (horizontal and vertical) treatments across the three deployments (Table 3-2). However, the total percent cover of biofouling varied among deployments, with biocidal treatments and orientation (significant Season x Paint x Orientation interaction, Table 3-2). Across all treatments, percent cover of biofouling was generally lowest in the winter deployment (Control, ~30 %) with less variation between vertical and horizontal plates (Figure 3-2). The higher percent cover of biofouling during the two summer deployments resolved significant differences in cover on the horizontal control and A1 plates, and their vertically-oriented equivalents (Figure 3-2). There were no significant differences in mean percent cover of biofouling between control and A1 treatments during summer 2015-16 compared to summer 2014-15 and winter 2015 deployments (as shown by the significant interaction between paint treatment and season; Table 3-2). This reflected generally lower biofouling cover on control plates and increased biofouling cover on A1 plates from summer 2014-15 and summer 2015-16, while the winter 2015 deployment had lower biofouling cover across A1 and control plates (Figure 3-2).

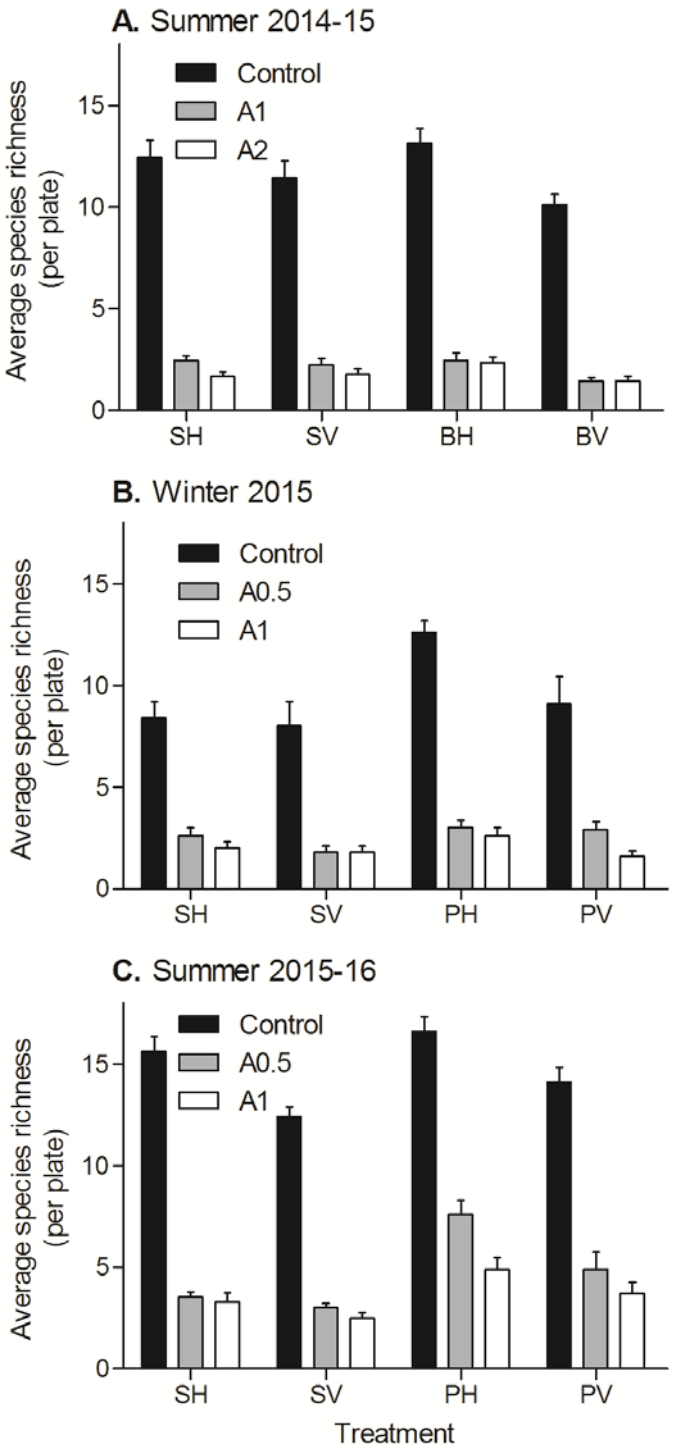

Figure 3-1 Average species richness (± SE) per plate for all combinations of surface treatments. Per plate species richness shown for summer 2014-15 (A), winter 2015 (B), and summer 2015-16 (C). Treatment combinations on x-axis are, smooth and horizontal (SH), smooth and vertical (SV), blasted and horizontal (BH, summer 2014-15 only), pitted and horizontal (PH, winter 2015 and summer 2015-16), blasted and vertical (BV, summer 2014-15 only), and pitted and vertical (PV, winter 2015 and summer 2015-16). Thickness of biocidal treatments were A0.5 (40 µm), A1 (75 µm) and A2 (150 µm). Refer to Table 3-1 for statistical analyses.

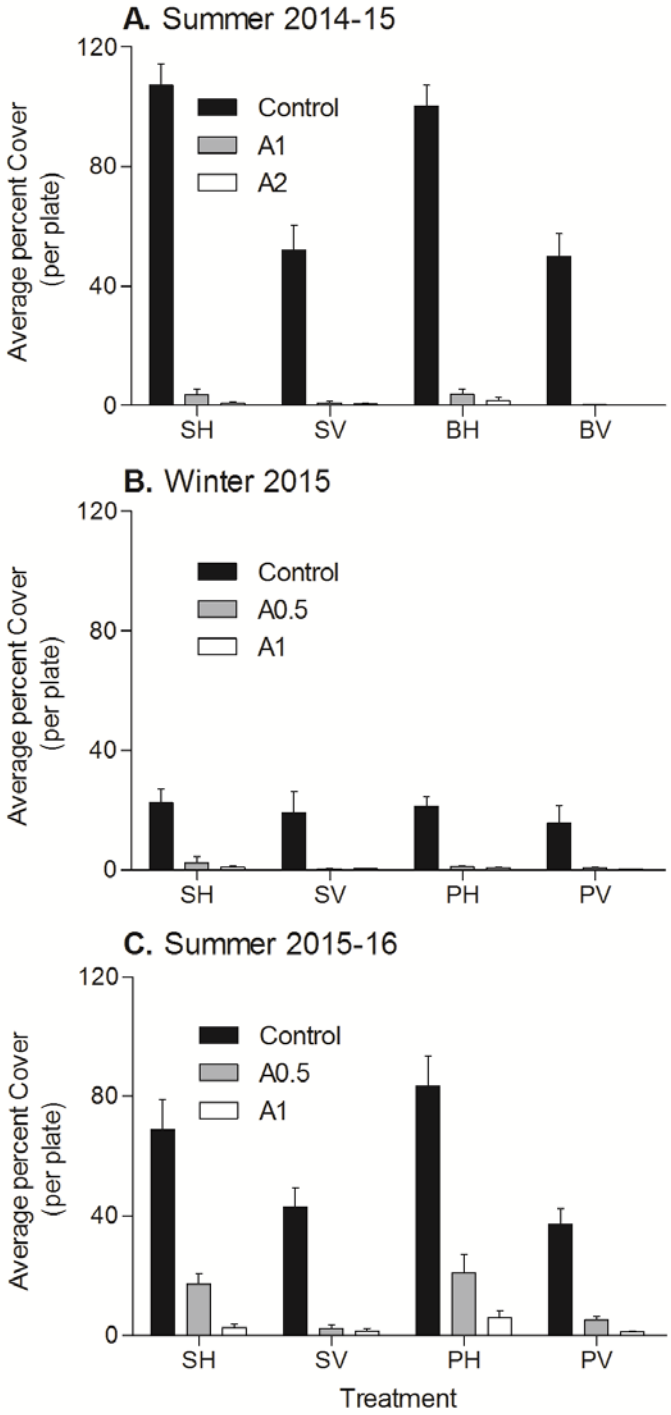

Figure 3-2 Average species cover (± SE) per plate for all combinations of surface treatments. Per plate percent cover shown for summer 2014-15 (A), winter 2015 (B), and summer 2015-16 (C). Treatment combinations on x-axis are, smooth and horizontal (SH), smooth and vertical (SV), blasted and horizontal (BH, summer 2014-15 only), pitted and horizontal (PH, winter 2015 and summer 2015-16), blasted and vertical (BV, summer 2014-15 only), and pitted and vertical (PV, winter 2015 and summer 2015-16). Thickness of biocidal treatments were A0.5 (40 µm), A1 (75 µm) and A2 (150 µm). Refer to Table 3-1 for statistical analyses.

Table 3-1 Analysis of species richness and biofouling cover between experimental treatments for each deployment period. Significant two-way ANOVA analyses are in bold.

	Summer 2014-15		Winter 2015		Summer 2015-16	
Species richness	F	*p*	F	*p*	F	*p*
Paint (P)	$F_{2,96}$ = 35.0	<0.0001	$F_{2,109}$ = 25	<0.0001	$F_{2,109}$ = 22.0	<0.0001
Surface (Su)	$F_{1,96}$ = 0.06	0.8	$F_{1,109}$ = 1.3	0.3	$F_{1,109}$ = 1.5	0.2
Orientation (O)	$F_{1,96}$ = 1.0	0.3	$F_{1,109}$ = 1.3	0.3	$F_{1,109}$ = 1.6	0.2
P * Su	$F_{2,96}$ = 0.03	0.97	$F_{2,109}$ = 0.4	0.7	$F_{2,109}$ = 0.2	0.9
P * O	$F_{2,96}$ = 0.2	0.8	$F_{2,109}$ = 0.4	0.7	$F_{2,109}$ = 0.2	0.8
Su * O	$F_{1,96}$ = 0.3	0.6	$F_{1,109}$ = 0.2	0.6	$F_{1,109}$ = 0.04	0.8
P * Su * O	$F_{2,96}$ = 0.03	0.97	$F_{2,109}$ = 0.3	0.8	$F_{2,109}$ = 0.1	0.9
Biofouling cover						
Paint (P)	$F_{2,96}$ = 65.0	<0.0001	$F_{2,109}$ = 30.7	<0.0001	$F_{2,109}$ = 38.8	<0.0001
Surface (Su)	$F_{1,96}$ = 0.3	0.6	$F_{1,109}$ = 0.2	0.7	$F_{1,109}$ = 0.3	0.6
Orientation (O)	$F_{1,96}$ = 14.1	0.0001	$F_{1,109}$ = 0.9	0.3	$F_{1,109}$ = 10.8	0.001
P * Su	$F_{2,96}$ = 0.3	0.7	$F_{2,109}$ = 0.08	0.9	$F_{2,109}$ = 0.08	0.9
P * O	$F_{2,96}$ = 12.1	<0.0001	$F_{2,109}$ = 0.3	0.7	$F_{2,109}$ = 3.8	0.02
Su * O	$F_{1,96}$ = 0.2	0.7	$F_{1,109}$ = <0.01	0.98	$F_{1,109}$ = 0.6	0.4
P * Su * O	$F_{2,96}$ = 0.2	0.8	$F_{2,109}$ = 0.05	0.95	$F_{2,109}$ = 0.3	0.7

Table 3-2 Analysis of species richness and biofouling cover between experimental treatments for all seasons combined. Only those treatments consistent across deployments were analysed between deployments (i.e., smooth control plates and A1 plates of horizontal and vertical orientation). Significant factorial ANOVA analyses are highlighted in bold and significant interactions reported are in bold (non-significant interactions not reported).

	Species richness		Biofouling cover	
Main effects	F	*p*	F	*p*
Season (Se)	$F_{2,105}$ = 1.9	0.15	$F_{2,105}$ = 8.5	0.0003
Paint (P)	$F_{1,105}$ = 42.3	<0.0001	$F_{1,105}$ = 64.0	<0.0001
Orientation (O)	$F_{1,105}$ = 0.7	0.4	$F_{1,105}$ = 8.6	0.004
Interactions				
Se * P	$F_{2,105}$ = 1.0	0.4	$F_{2,105}$ = 9.1	0.0002
Se * O	$F_{2,105}$ = 0.1	0.9	$F_{2,105}$ = 3.4	0.04
P * O	$F_{1,105}$ = 0.3	0.6	$F_{1,105}$ = 3.4	<0.0001
Se * P * O	$F_{4,313}$ = 0.05	0.96	$F_{4,313}$ = 3.2	0.47

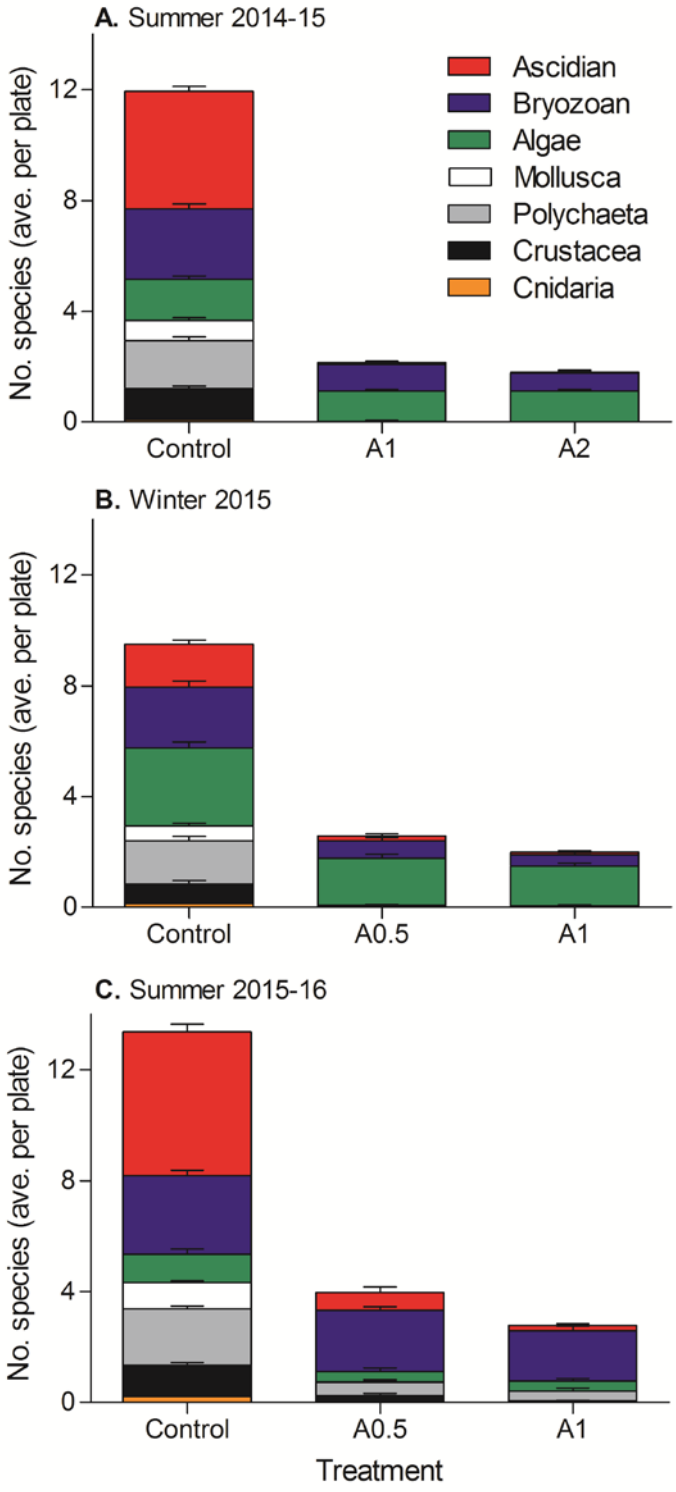

Figure 3-3 Average numbers of species (± SE) per plate separated by taxonomic groups for non-biocidal control and biocidal plates (A1 and A2, summer 2014-15; A0.5 and A1, winter 2015 and summer 2015-16). Taxonomic breakdown shown for summer 2014-15 (A), winter 2015 (B), and summer 2015-16 (C). Thickness of biocidal treatments were A0.5 (40 µm), A1 (75 µm) and A2 (150 µm).

The biofouling assemblages on the plates were dominated by ascidians, bryozoans and algae (Figure 3-3; Appendix C for full list of species detected), with a shift in the prevalence of different functional groups between summer and winter deployments. In particular, the control plates during the winter deployment had fewer ascidian species and more algal species compared to control plates during summer deployments. The biocidal treatment that remained consistent across deployments (A1, 75 µm) was dominated by bryozoans and algae during summer 2014-15 and winter 2015, but other functional groups (particularly ascidians and polychaetes) dominated in summer 2015-16.

There was greater variation in species composition on control plates than on biocidal plates (i.e., A1 and A2, summer 2014-15; A0.5 and A1, winter 2015 and summer 2015-16) in each deployment (summer 2014-15, Figure 3-4; winter 2015, Figure 3-5; summer 2015-16, Figure 3-6, see significant "paint" treatment Table 3-3) as shown by the clumping of biocidal treatments compared to control treatments.

Although there was no evidence of increasing separation of community composition with increasing distance between arrays, there were significant differences in the biofouling assemblages on control plates at different sites (Table 3-3). In comparison, there was very little variation in the biofouling assemblages recorded from biocidal plates (A1 vs. A2, or A0.5 vs. A1) in each deployment ("Paint" treatment, Table 3-3). The similarity in composition of assemblages on these plates is depicted in the dendrograms (Figure 3-4B, Figure 3-5B, Figure 3-6B), with most biocidal plates exhibiting > 90 % similarity in assemblage composition.

Orientation of control plates also affected the composition of biofouling assemblages (Figure 3-7). Significant three-way interactions between season, site and orientation suggest a combination of factors drive the composition of biofouling assemblages. There was a high degree of overlap in species composition between vertical and horizontal plates, but also a range of unique species occurring on each orientation (Table 3-5). Analysis of control and A1 plates across seasons (smooth, horizontal and vertical plates) showed high separation of community composition on control plates between seasons, but very similar community composition on A1 plates across deployments (Figure 3-8, Table 3-4). There were statistically different communities found between sites during each deployment (Table 3-3), but community composition did not vary between sites when analysed across seasons (Table 3-4).

Comparison of species between horizontally and vertically orientated plates showed more unique species (i.e., species not shared between both orientations) on horizontal plates compared to vertical plates (Table 3-5). Seven species were unique to horizontal plates, and two species were unique to vertical plates during the summer 2014-15 deployment; six species were unique to horizontal plates, and three species were unique to vertical plates during the winter 2015 deployment, and; 13 species were unique to horizontal plates and five species were unique to vertical plates during the summer 2015-16 deployment.

Comparison of species on smooth and sand-blasted plates showed that two species were unique to the smooth plates and one species was unique to sand-blasted plates during the summer 2014-15 deployment (Table 3-5). Following the change of the roughening treatment from sand-blasting to pitting, two species were unique to smooth and eight species were unique to pitted plates during the winter 2015 deployment, and five species were unique to smooth and eight species were unique to pitted plates during the summer 2015-16 deployment (Table 3-5).

There were no unique species observed on biocidal plates compared to control plates (Table 3-6).

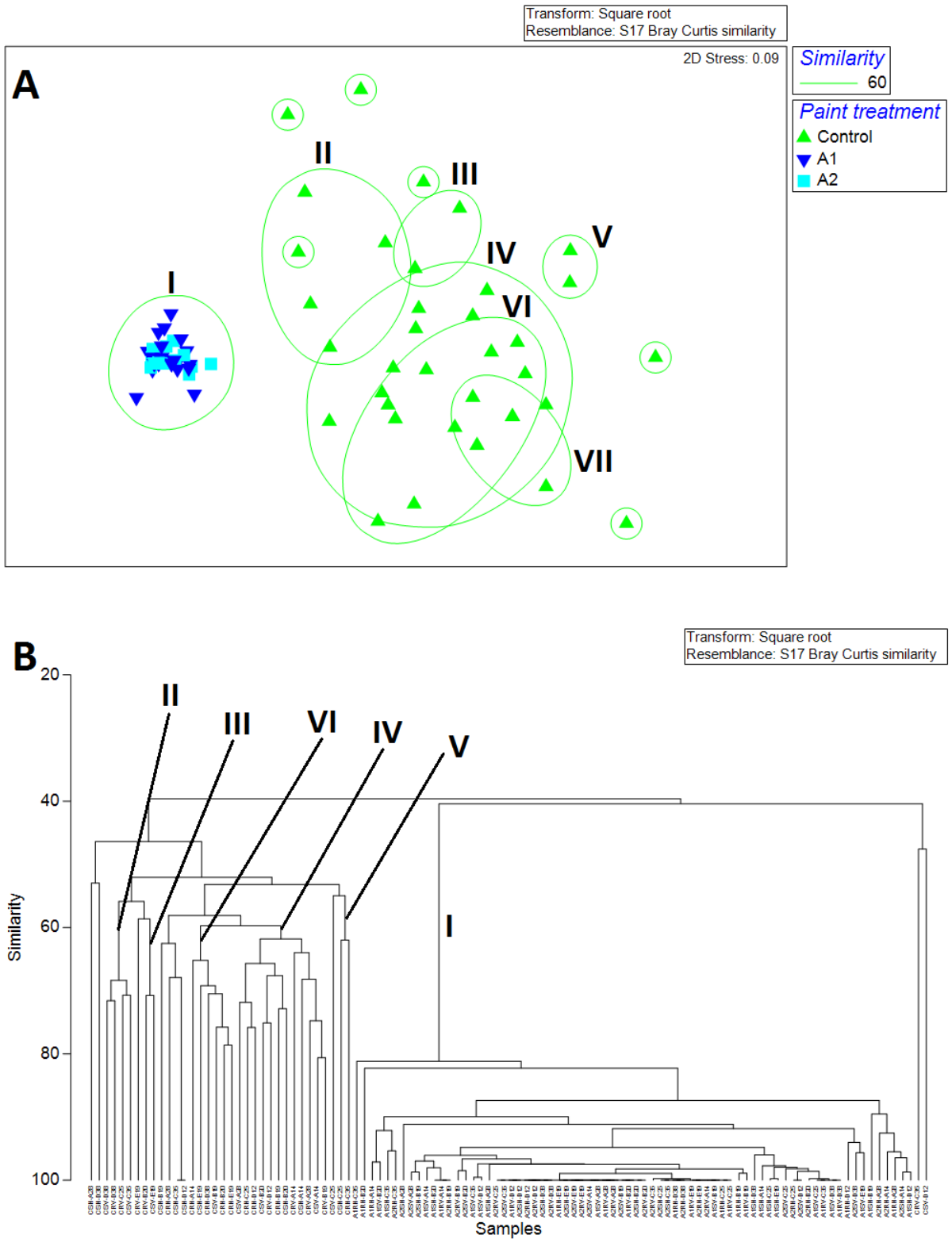

Figure 3-4 MDS (multidimensional scaling plot) plot (A) of community composition for summer 2014-15 presented for control and biocidal plates (A1 and A2), and dendrogram (B) of the similarity between individual plates. Biocidal treatments were A1 (75 µm coating thickness) and A2 (150 µm), respectively. CLUSTER analysis used to compare similarity on the MDS plot (plates sharing 60 % similarity contained within solid green lines). Roman numerals relate clusters (A) to similarity splits in the dendrogram (B). See Table 3-3 for analysis of treatments.

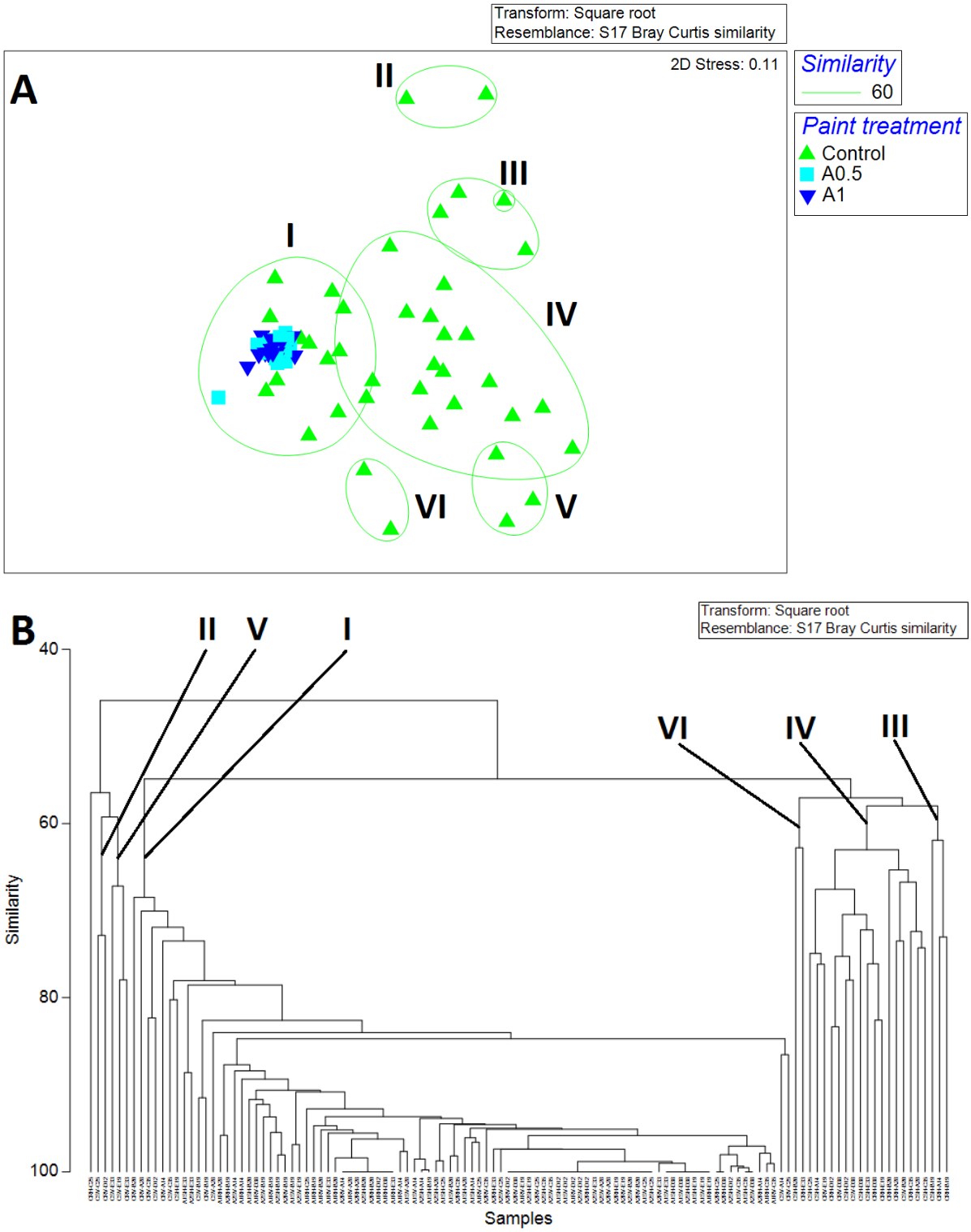

Figure 3-5 MDS (multidimensional scaling plot) plot (A) of community composition for winter 2015 presented for control and biocidal plates (A0.5 and A1), and dendrogram (B) of the similarity between individual plates. Biocidal treatments were A0.5 (40 µm coating thickness) and A1 (75 µm), respectively. CLUSTER analysis used to compare similarity on the MDS plot (plates sharing 60 % similarity contained within solid green lines). Roman numerals relate clusters (A) to similarity splits in the dendrogram (B). See Table 3-3 for analysis of treatments.

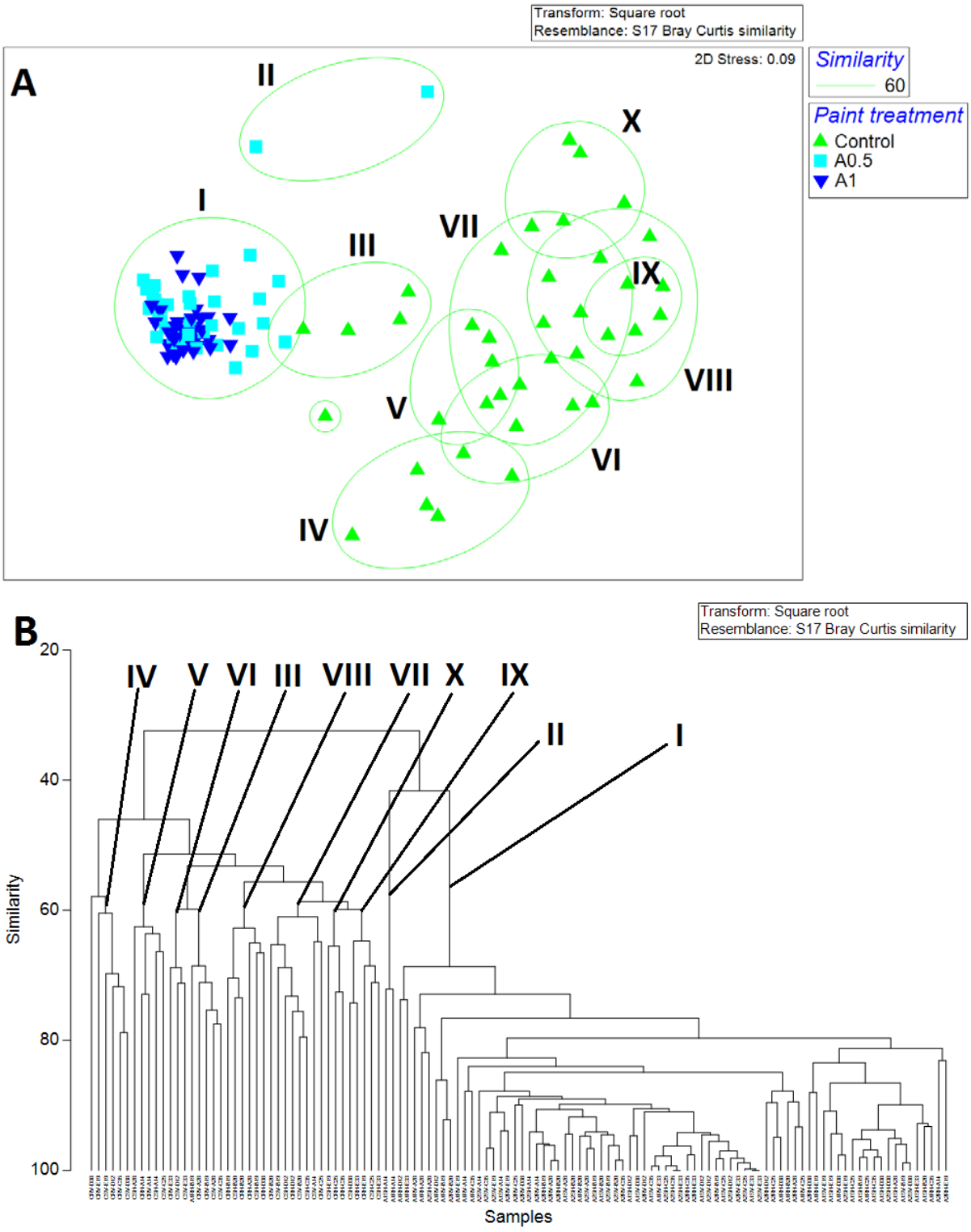

Figure 3-6 MDS (multidimensional scaling plot) plot (A) of community composition for summer 2015-16 presented for control and biocidal plates (A0.5 and A1), and dendrogram (B) of the similarity between individual plates. Biocidal treatments were A0.5 (40 µm coating thickness) and A1 (75 µm), respectively. CLUSTER analysis used to compare similarity on the MDS plot (plates sharing 60 % similarity contained within solid green lines). Roman numerals relate clusters (A) to similarity splits in the dendrogram (B). See Table 3-3 for analysis of treatments.

Table 3-3 Differences in community composition (PERMANOVA) for each deployment. Significant analyses are highlighted in bold.

	Summer 2014-15		Winter 2015		Summer 2015-16	
Treatments	F	*P*	F	*p*	F	*p*
Site (S)	$F_{4,107} = 2.1$	0.006	$F_{4,119} = 1.9$	0.005	$F_{4,119} = 5.3$	0.001
Orientation (O)	$F_{1,107} = 5.4$	0.012	$F_{1,119} = 3.3$	0.035	$F_{1,119} = 7.5$	0.012
Surface (Su)	$F_{1,107} = 0.88$	0.47	$F_{1,119} = 4.1$	0.018	$F_{1,119} = 2.9$	0.027
Paint (P)	$F_{2,107} = 51.2$	0.002	$F_{2,119} = 19.6$	0.001	$F_{2,119} = 56.1$	0.002
Interactions						
S * P	$F_{8,107} = 1.8$	0.005	$F_{8,119} = 2.4$	0.001	$F_{8,119} = 2.2$	0.001
O * P	$F_{2,107} = 4.6$	0.005	$F_{2,119} = 3.8$	0.02	$F_{2,119} = 5.3$	0.001
S * O	$F_{4,107} = 1.1$	0.4	$F_{4,119} = 2.1$	0.001	$F_{4,119} = 1.7$	0.01
S * Su	$F_{4,107} = 0.7$	0.8	$F_{4,119} = 0.5$	1.0	$F_{4,119} = 0.8$	0.7
O * Su	$F_{1,107} = 2.3$	0.1	$F_{1,119} = 1.2$	0.3	$F_{1,119} = 1.2$	0.3
Su * P	$F_{2,107} = 0.3$	0.9	$F_{2,119} = 0.5$	0.9	$F_{2,119} = 1.7$	0.1
S * O * Su	$F_{4,107} = 0.7$	0.8	$F_{4,119} = 0.9$	0.5	$F_{4,119} = 0.8$	0.8
S * O * P	$F_{8,107} = 1.1$	0.3	$F_{8,119} = 1.9$	0.001	$F_{8,119} = 1.0$	0.5
S * Su * P	$F_{8,107} = 0.8$	0.8	$F_{8,119} = 0.6$	1.0	$F_{8,119} = 1.1$	0.2
O * Su * Pt	$F_{2,107} = 1.6$	0.2	$F_{2,119} = 1.1$	0.4	$F_{2,119} = 1.0$	0.5
S * O * Su * P	$F_{8,107} = 0.7$	0.9	$F_{8,119} = 0.9$	0.7	$F_{8,119} = 0.8$	0.9

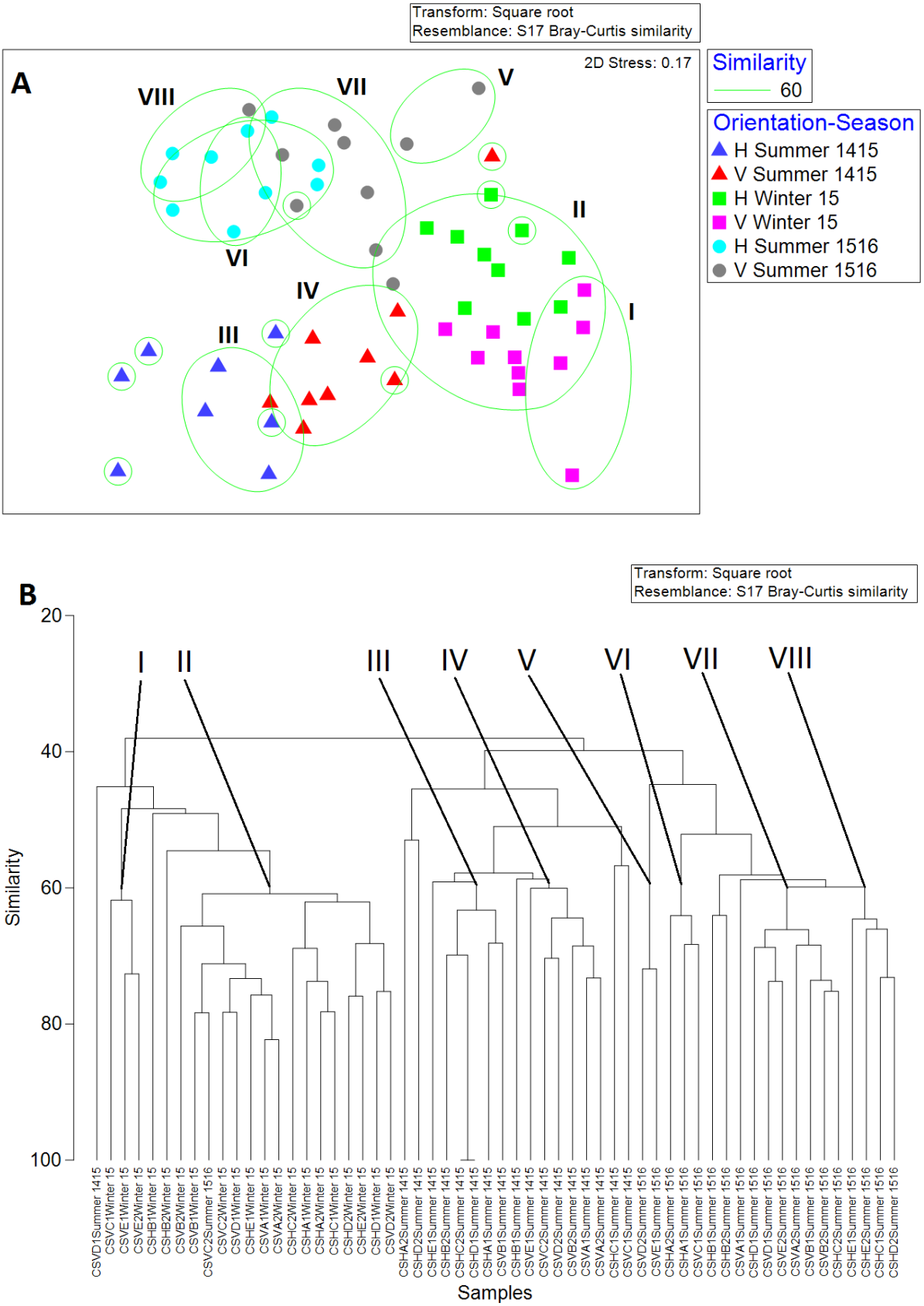

Figure 3-7 MDS (multidimensional scaling plot) plot of community composition presented for control plates only separated by deployments and plate orientations (A), and dendrogram (B) of the similarity between individual plates. Only smooth plates were included in the analysis. CLUSTER analysis used to compare similarity on the MDS plot (plates sharing 60 % similarity contained within solid green lines). For analysis of treatments see Table 3-4.

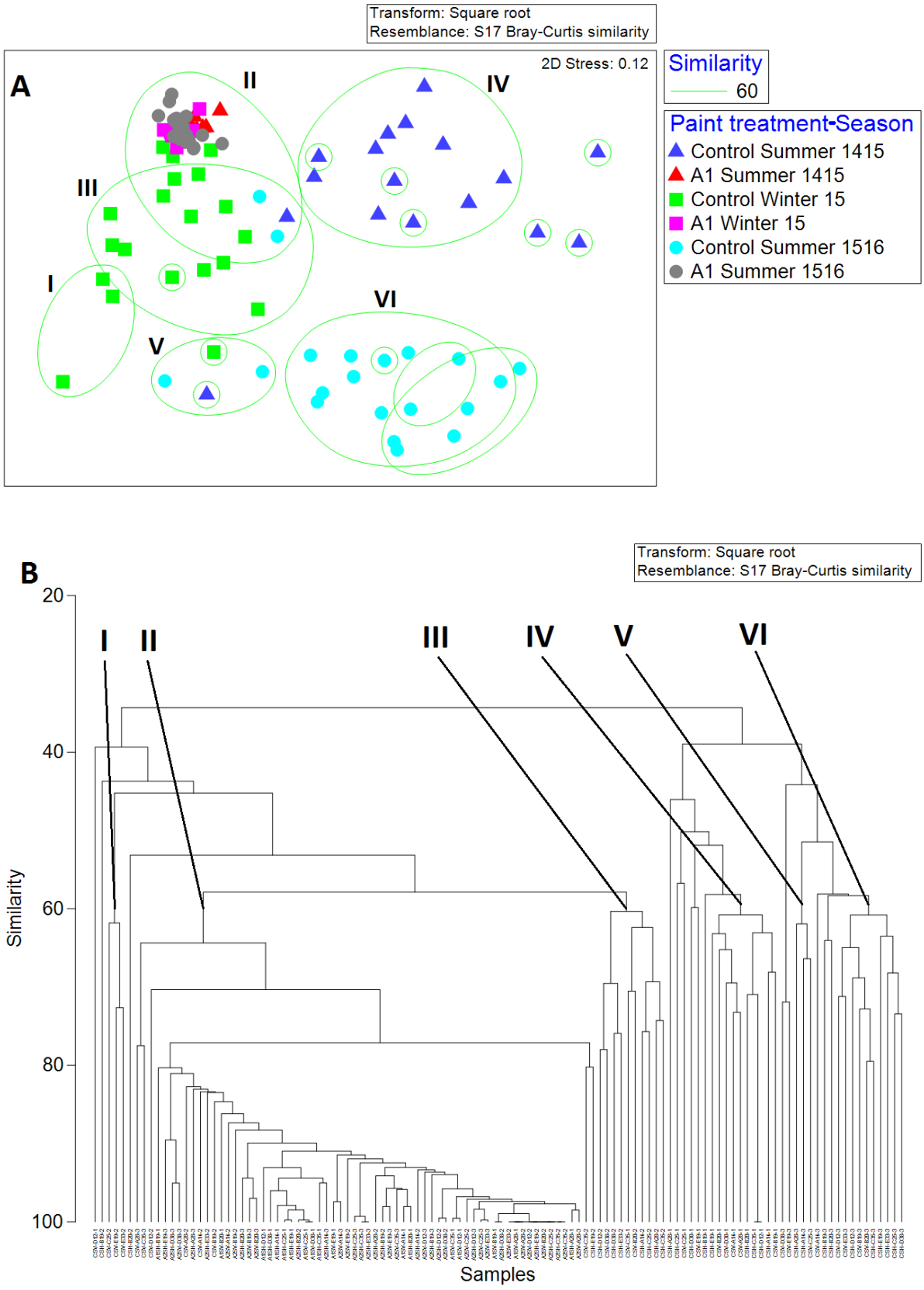

Figure 3-8 MDS (multidimensional scaling plot) plot of community composition presented for control and A1 plates separated by deployments and paint treatments (A), and dendrogram (B) of the similarity between individual plates. Only smooth plates were included in the analysis. CLUSTER analysis used to compare similarity on the MDS plot (plates sharing 60 % similarity contained within solid green lines). For analysis of treatments see Table 3-4.

Table 3-4 Differences in community composition (PERMANOVA) for all deployments. Significant analyses are highlighted in bold (* represents analysis with limited numbers of permutations and re-analysed with Monte Carlo simulations).

	Control only		Control and A1	
Treatments	F	*p*	F	*p*
Season (Se)	$F_{2,57}$ = 21.6	0.001	$F_{2,115}$ = 24.8	0.001
Site (S)	$F_{4,57}$ = 1.3	0.1	$F_{4,115}$ = 1.2	0.3
Orientation (O)	$F_{1,57}$ = 3.3	0.03*	$F_{1,115}$ = 3.3	0.03*
Paint (P)	-	-	$F_{1,115}$ = 6.4	0.002*
Interactions				
Se * S	$F_{8,57}$ = 1.0	0.5	$F_{8,115}$ = 1.5	0.01
Se * O	$F_{8,57}$ = 2.3	0.01	$F_{2,115}$ = 2.2	0.005
S * O	$F_{8,57}$ = 1.2	0.2	$F_{4,115}$ = 1.2	0.3
Se * P	-	-	$F_{2,115}$ = 19	0.001
P * S	-	-	$F_{4,115}$ = 1.4	0.2
P * O	-	-	$F_{1,115}$ = 3.1	0.04*
Se * P * S	-	-	$F_{8,115}$ = 1.3	0.08
Se * P * O	-	-	$F_{2,115}$ = 2.0	0.01
Se * S * O	$F_{8,57}$ = 1.0	0.6	$F_{8,115}$ = 1.1	0.3
Se * P * S * O	-	-	$F_{8,115}$ = 1.2	0.1

Table 3-5 Similarity indices and shared species between control horizontal and vertical plates and control smooth and rough plates. Comparisons between horizontal vs. vertical and smooth vs. rough show the number of species observed on each treatment, the number of species shared between treatments (i.e., between horizontal and vertical, or smooth and rough) and the Chao estimated shared species indices which accounts for un-sampled species.

	Summer 2014-15	Winter 2015	Summer 2015-16
Horizontal vs Vertical			
Species observed horizontal	29	35	45
Species observed vertical	24	32	38
Shared species observed	22	29	33
Chao shared estimated	22	31.7	39.8
Smooth vs Rough			
Species observed smooth	28	27	42
Species observed rough	27	33	45
Shared species observed	26	24	37
Chao shared estimated	28.4	28.2	41.3

Table 3-6 Similarity estimates between paint treatments for each season. ACE (average-cover based indicator ± 95 % confidence intervals) is an estimated diversity assuming not all species have been detected. Chao estimated shared species indices calculates the number of species shared between samples (i.e., control vs. A1 or control vs. A2, summer 2014-15; control vs. A0.5 or control vs. A1, winter 2015 and summer 2015-16). Biocidal treatments were A0.5 = 40 µm (coating thickness), A1 = 75 µm, A2 = 150 µm.

	Summer 2014-15		Winter 2015		Summer 2015-16	
	Control-A1	Control-A2	Control-A0.5	Control-A1	Control-A0.5	Control-A1
Species observed control	29	29	36	36	52	52
Species observed biocide	10	7	14	9	23	18
Shared species observed	10	7	14	9	23	18
ACE control	31 (3.1)	31 3.1	40 (3.8)	40 (3.8)	59 (5.2)	59 (5.2)
ACE biocide	20 (1.3)	9 (0.8)	18 (1.0)	11 (0.8)	25 (2.3)	22 (1.9)
Chao shared estimated	11	7	15.1	11.0	25.2	21.2

3.2 BIOSECURITY STATUS OF BIOFOULING ASSEMBLAGES

Over the course of the three deployments 19 NIS, 6 cryptogenic species and 31 IS were detected. Twice as many NIS were detected on the non-biocidal control plates over the two summer deployments than on any of the biocidal treatments (Figure 3-8). In summer 2014-15, for example, control plates contained an average of 3.5 (± 0.3 SE) NIS, 2.5 (± 0.2 SE) cryptogenic species and 5.5 (± 0.4 SE) IS. By comparison, the biocidal treatments had an average of between 1.5 ± 0.1 (A1, 75 µm thickness) and 1.0 ± 0.1 (A2, 150 µm thickness) NIS and < 1 IS. Few IS recruited to the biocidal treatments over any of the deployments. A consequence was that the ratio of NIS:IS was much greater for the each of the biocidal treatments than the controls (Table 3-7), despite the statistically greater diversity of NIS detected on control plates (Table 3-8).

About half as many NIS and cryptogenic species were detected on control plates in winter 2015 than in either of the two summer deployments (Figure 3-8, Table 3-8). This was associated with the lack of recruitment by several species of ascidians during winter (e.g., *Styela clava and Botrylloides leachii*). By contrast, the average numbers of IS remained relatively consistent, at between five and seven per control plate, over each of the three deployments.

Reducing the thickness of the biocidal treatment in summer 2015-16 resulted in detection of a higher average number of IS and cryptogenic species and a slight increase in the average number of NIS when compared with the previous summer deployment (Figure 3-8, Figure 3-9, Table 3-8).

NIS recovered from the settlement plates included the fanworm, *Sabella spallanzanii*, the ascidians *S. clava* and *Ciona intestinalis*, and several bryozoans (*Bugula neritina, B. flabellata, Watersipora subatra, Amathia verticillata*). Of the 19 NIS detected throughout the study, only eight occurred on biocidal plates. The non-indigenous bryozoan, *Celleporaria umbonatoidea*, was recorded during the summer 2014-15 survey. Its detection in Waitemata Harbour represented a range extension for this species. It had previously been recorded only from Whangarei in 2010 and Opua in 2013. The NIS recorded from biocidal plates during summer 2014-15 and winter 2015 deployments were *B. neritina*, *A. verticillata* and *W. subatra*. *C. umbonatoidea*, *B. flabellata*, *Ascidiella aspersa*, *Botryllus schlosseri*. *Symplegma brakenhielmi* and *Schizoperlla japonica* were also found on biocidal plates during summer 2015-16 deployment (Figure 3-10). The IS found on biocidal plates were the barnacle *Balanus trigonus* and the calcareous tubeworm *Spirobranchus cariniferus*. The occurrence of several NIS on biocidal plates during the summer 2015-16 deployment was highly associated with fouling by tolerant bryozoans (particularly *B. neritina, C. umbonatoidea* and *W. subatra*) with several other NIS (*B. schlosseri, S. brakenheimi*, and *C. intestinalis*) and IS (*Lissoclinum notti*) found growing on the bryozoans, rather than directly on the biocidal coatings (Figure 3-11).

Frequency of NIS occurrence on control plates of horizontal and vertical orientation showed that many NIS were found more consistently on horizontal surfaces than vertical surfaces (Figure 3-12 A, Figure 3-13 A, Figure 3-14 A). Several NIS were found only on horizontal surfaces during each deployment, including *C. intestinalis* and *S. clava*, which were only observed on horizontal treatments across all three deployments. There were few differences in taxa recruiting to smooth and rough surfaces (Figure 3-12 B, Figure 3-13 B, Figure 3-14 B), and no species were found consistently on smooth or rough surfaces alone across the three deployments.

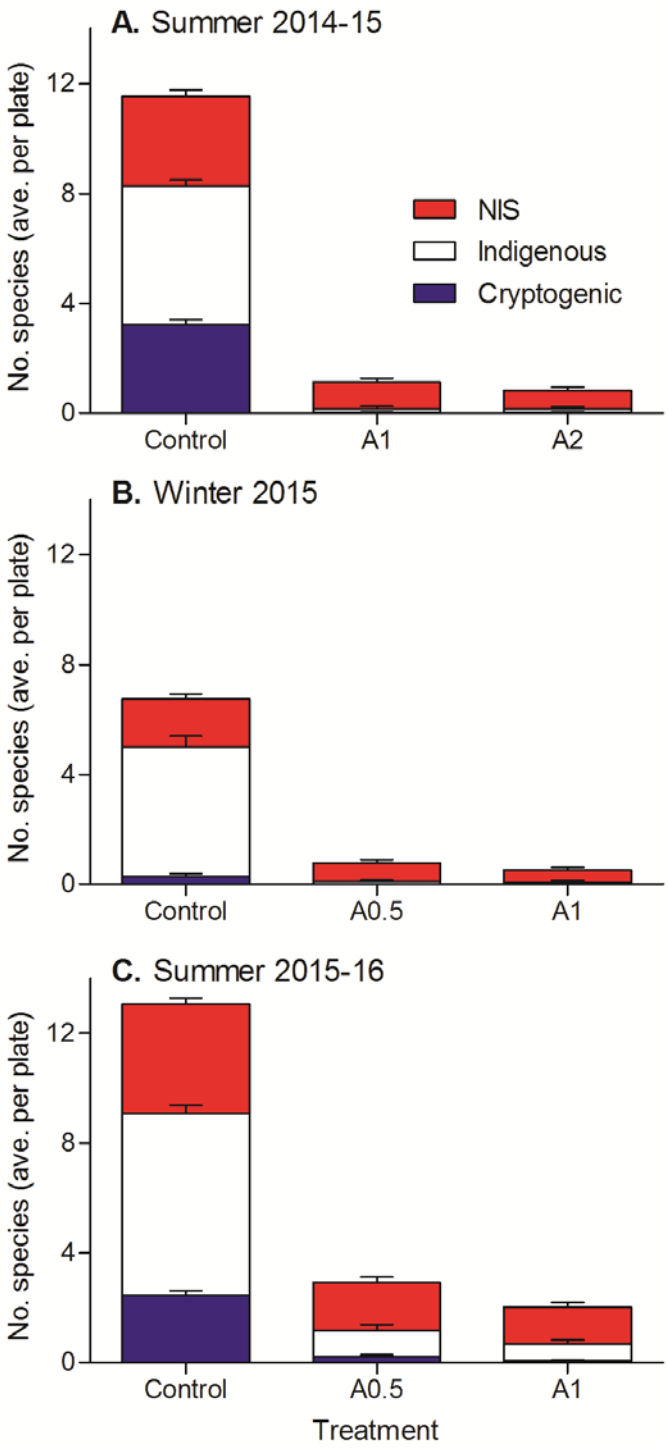

Figure 3-9 Average number of species (± SE) and biosecurity status of biofouling communities across paint treatments. Biocidal treatments for the summer 2014-15 deployment (A) were A1 (75 µm coating thickness) and A2 (150 µm), and for the winter 2015 (B) and summer 2015-16 (C) deployments A0.5 (40 µm) and A1 (75 µm).

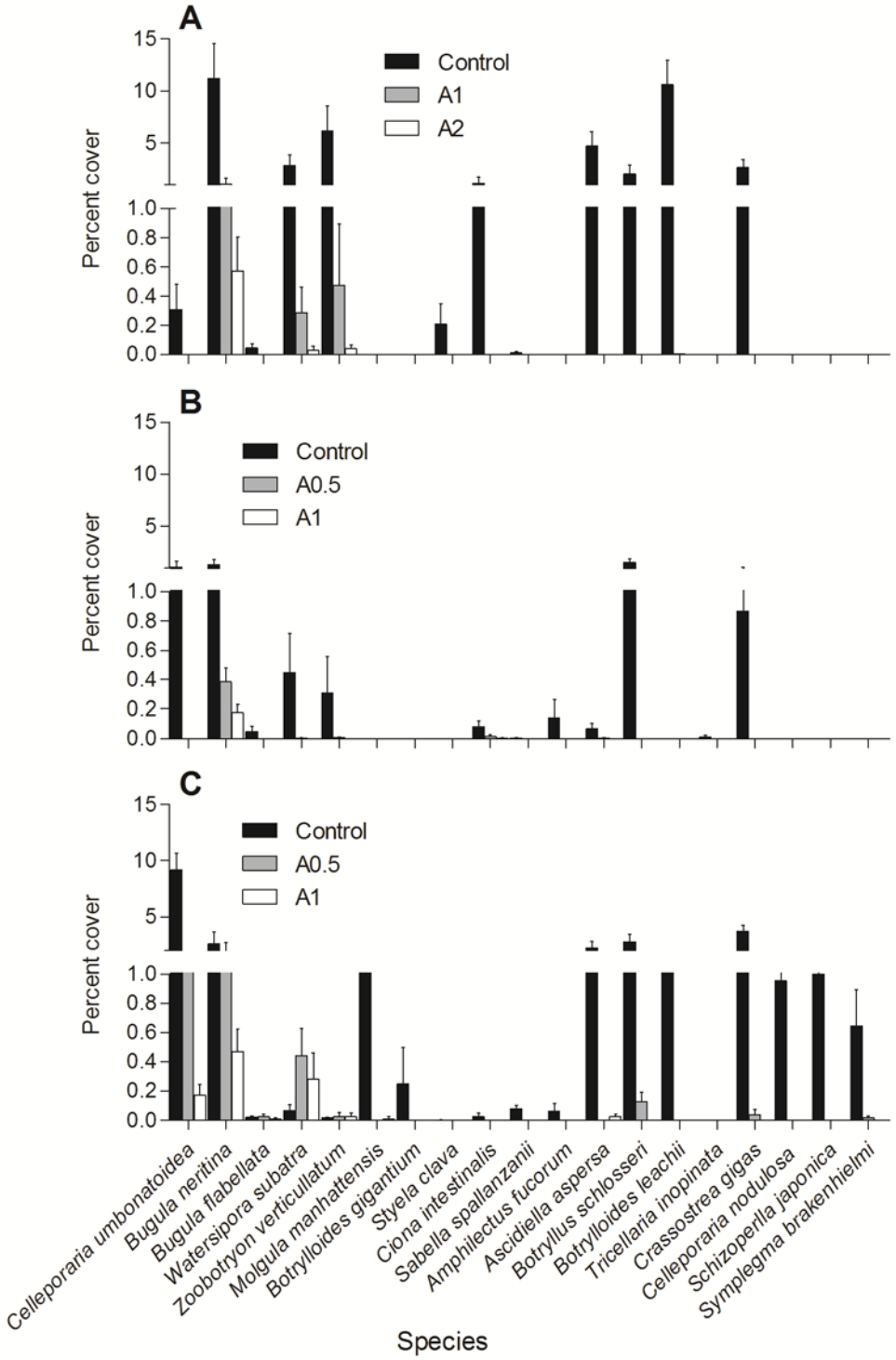

Figure 3-10 Breakdown of percentage NIS cover (± SE) and species richness found on the three paint treatments for summer 2014-15 (A), winter 2015 (B) and summer 2015-16 (C). Biocidal treatments for the summer 2014-15 deployment (A) were A1 (75 µm coating thickness) and A2 (150 µm), and for the winter 2015 (B) and summer 2015-16 (C) deployments A0.5 (40 µm) and A1 (75 µm).

Table 3-7 Ratio of non-indigenous species to indigenous species (NIS/IS) across paint treatments for three deployments.

	Summer 2014-15	Winter 2015	Summer 2015-16
Control	0.7	0.4	0.6
A0.5	n/a	6.8	1.8
A1	7	6	2.2
A2	4.8	n/a	n/a

Table 3-8 Differences in average NIS and IS species richness between season of deployment and control and A1 paint treatments. Variation in average richness analysed with factorial ANOVA.

	Indigenous species		Non-indigenous species	
	F	*p*	F	*p*
Paint	$F_{1,227} = 141.5$	<0.0001	$F_{1,227} = 53.0$	<0.0001
Season	$F_{2,227} = 3.6$	0.03	$F_{2,227} = 8.4$	0.0003
Season * Paint	$F_{2,227} = 1.2$	0.3	$F_{2,227} = 2.4$	0.09

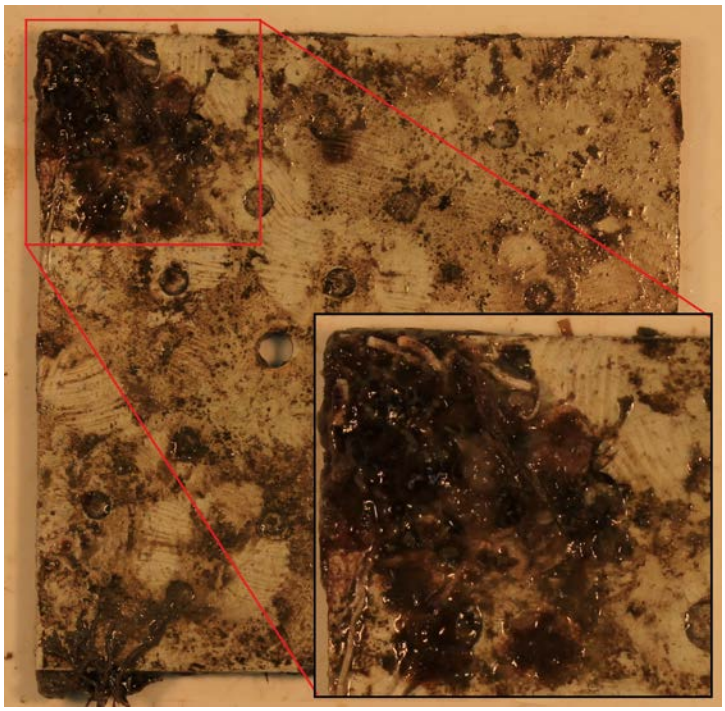

Figure 3-11 Biocide treated settlement plate showing a bryozoan colony over which a range of other species have recruited to (inset).

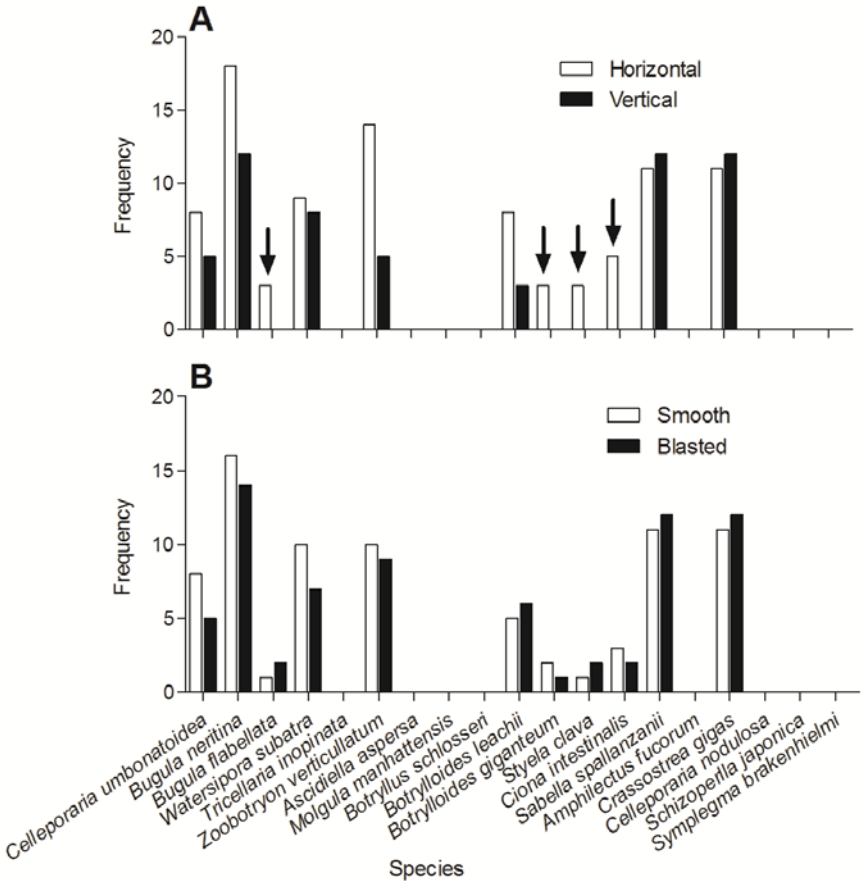

Figure 3-12 NIS detection frequencies per plate on control plates (*n* = 20) separated by horizontal and vertical orientation (A) and smooth and rough (sand-blasted) surface texture (B) for the summer 2014-15 deployment. Arrows refer to species only found on one treatment (per axis).

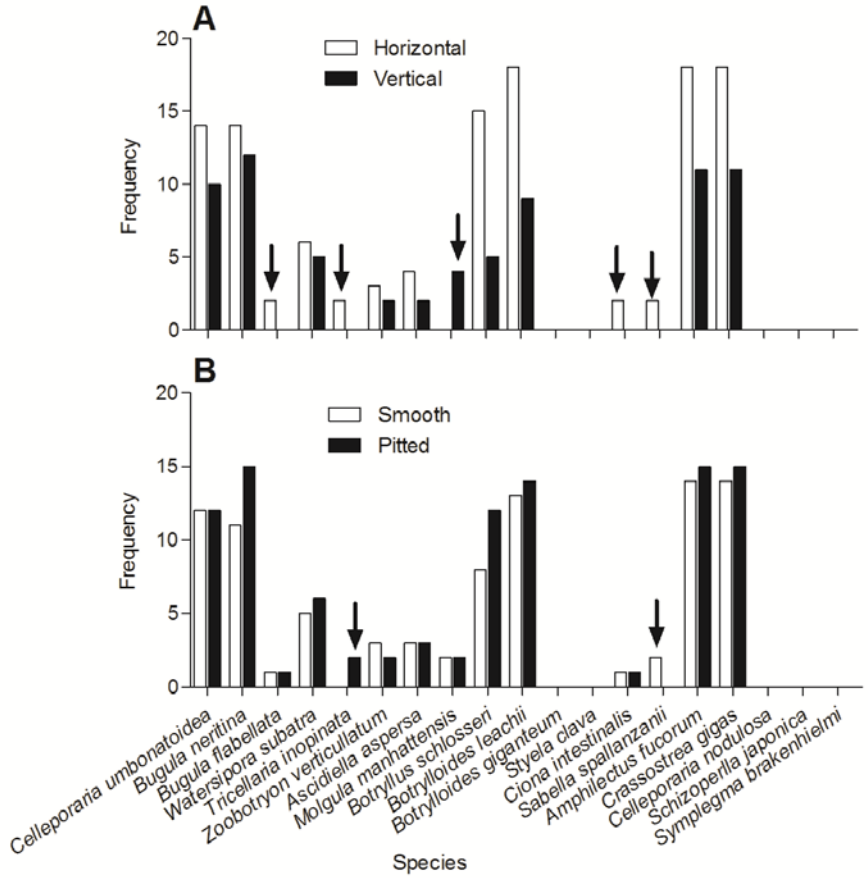

Figure 3-13 NIS detection frequencies per plate on control plates (*n* = 20) separated by horizontal and vertical orientation (A) and smooth and rough (pitted) surface texture (B) for the winter 2015 deployment. Arrows refer to species only found on one treatment (per axis).

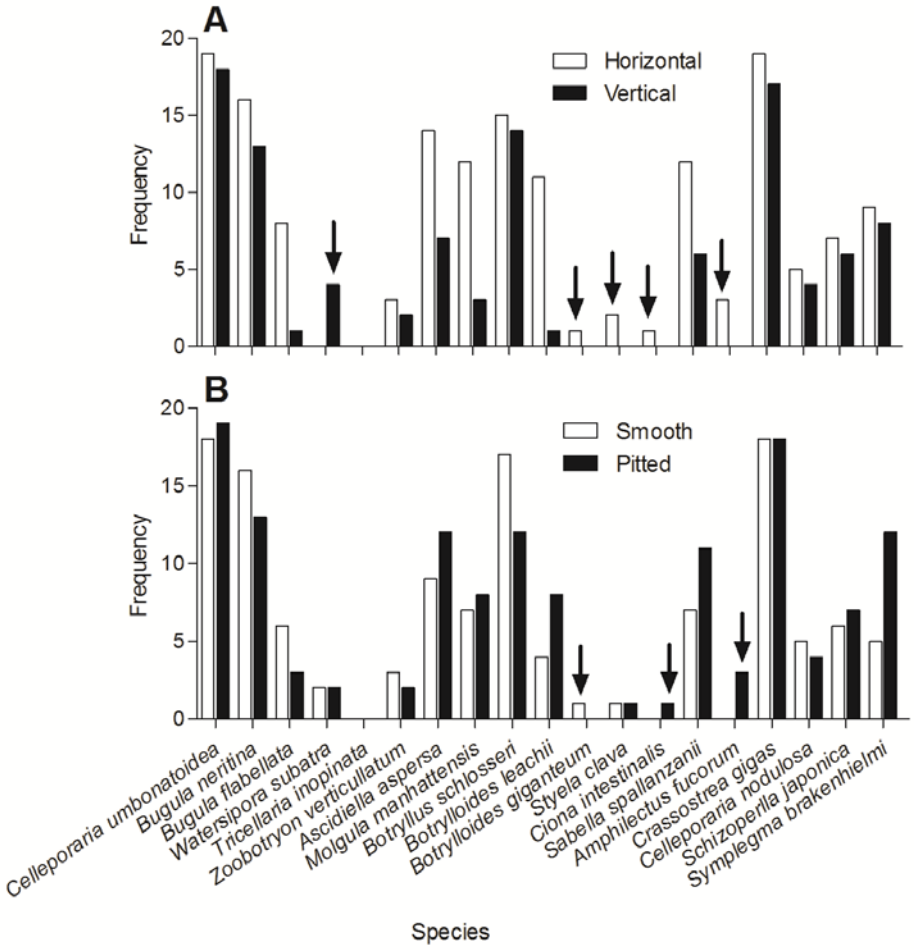

Figure 3-14 NIS detection frequencies per plate on control plates (*n* = 20) separated by horizontal and vertical orientation (A) and smooth and rough (pitted) surface texture (B) for the summer 2015-16 deployment. Arrows refer to species only found on one treatment (per axis).

3.3 EFFICACY OF TREATMENTS FOR THE DETECTION OF NIS AND IS

Horizontal control plates had higher average species richness than vertical control plates throughout the three deployments (Figure 3-15; Table 3-9). There was no difference in NIS richness between seasons despite lower richness on average during winter 2015, and no interaction between season and orientation (Table 3-9).

There was a high degree of overlap in the composition of the NIS assemblages on biocidal (A1) plates between deployments, whereas control plates showed higher separation of NIS composition across deployments (Figure 3-16). This is shown by the significant interaction between season and paint (Table 3-10). Seasonal shifts in NIS composition on control plates may be associated with the increasing cover of *C. umbonatoidea* and reduced cover of *W. subatra* from summer 2014-15 to summer 2015-16 (Figure 3-10). *W. subatra* was the only species observed to have higher cover on biocidal plates compared to controls at any time in the study (Figure 3-10).

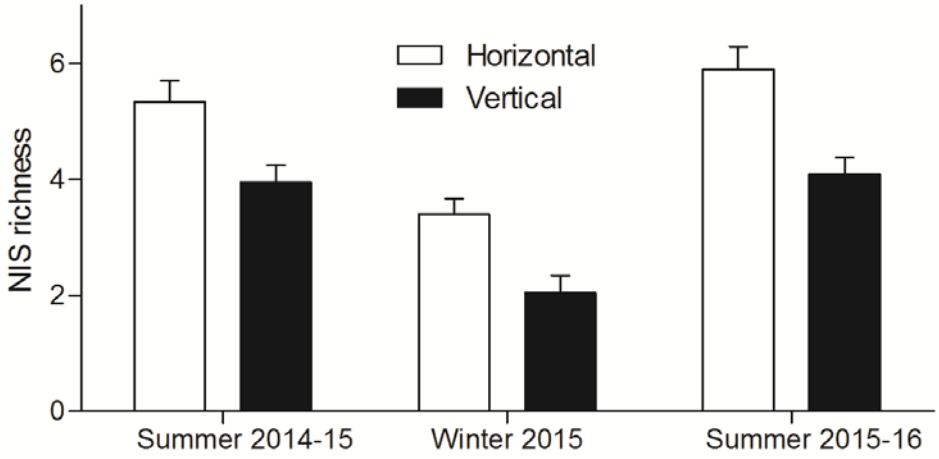

Figure 3-15 Influence of orientation on non-indigenous species (NIS) richness on smooth, non-biocidal plates over the course of three deployments.

Table 3-9 Two-way ANOVA analysis of influence of season and orientation on NIS richness on smooth, non-biocidal controls only.

	F	*p*
Season	$F_{2,111} = 2.2$	0.1
Orientation	$F_{1,111} = 6.8$	0.01
Season * Orientation	$F_{2,111} = 0.2$	0.8

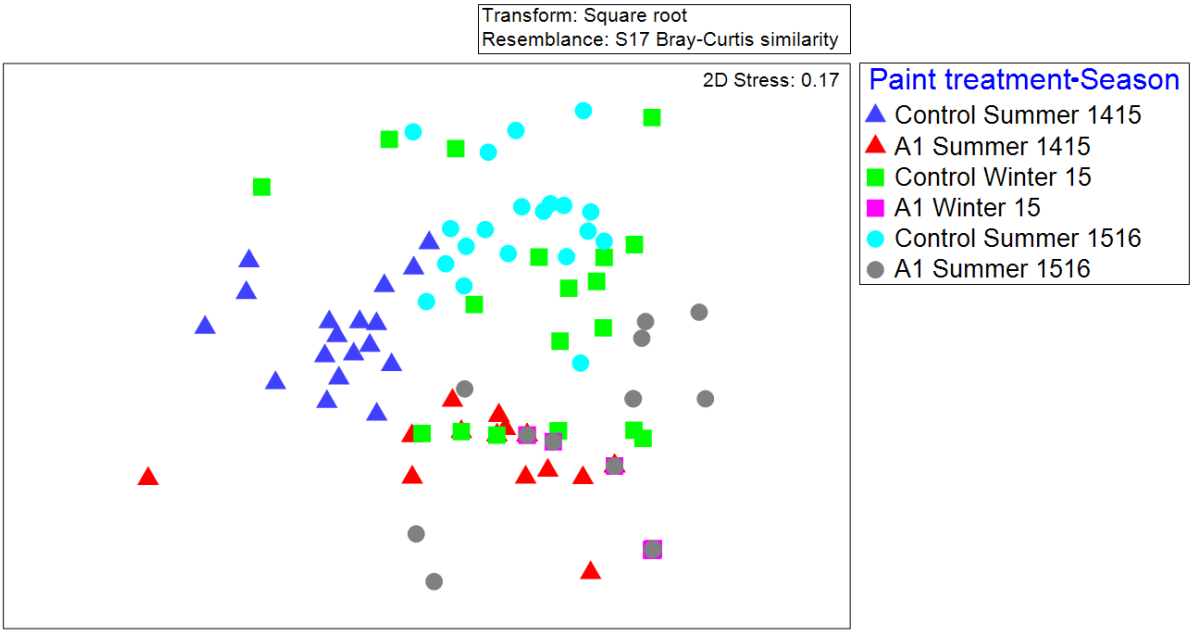

Figure 3-16 MDS (multidimensional scaling plot) plot of non-indigenous species (NIS) composition presented for control and A1 (75 µm) paint treatments across deployments for smooth plates only. For statistical differences between treatments see Table 3-10.

Table 3-10 Analysis of non-indigenous species composition (PERMANOVA) for control and A1 (75 µm) paint treatments across deployments for smooth plates only. Statically significant analyses are highlighted in bold (* represents analysis with limited numbers of permutations and re-analysed with Monte Carlo simulations).

	NIS composition	
Treatments	F	*p*
Season (Se)	$F_{2,115}$ = 11,.7	0.001
Site (S)	$F_{8,115}$ = 0.9	0.6
Orientation (O)	$F_{1,115}$ = 3.2	0.02*
Paint (P)	$F_{1,115}$ = 5.4	0.01*
Interactions		
Se * P	$F_{2,115}$ = 6.6	0.001
Se * O	$F_{2,115}$ = 1.9	0.03
Se * S	$F_{16,115}$ = 1.7	0.008
S * P	$F_{9,115}$ = 0.8	0.6
S * O	$F_{9,115}$ = 1.2	0.3
P * O	$F_{1,115}$ = 4.8	0.01*
Se * P * O	$F_{2,115}$ = 1.6	0.07
Se * S * P	$F_{16,115}$ = 1.8	0.002
Se * S * O	$F_{16,115}$ = 0.8	0.9
S * P * O	$F_{8,115}$ = 0.8	0.7
Se * S * P * O	$F_{16,115}$ = 1.0	0.4

Species rarefaction curves showed that non-biocidal control plates had at least twice as many IS as biocidal plates (Figure 3-16), and twice as many NIS as biocidal plates (Figure 3-17). However, curve extrapolation for summer 2015-16 predicted 75 % of the total NIS that were found on non-biocidal control plates (Figure 3-17 C). In most cases, sampling 40 plates was enough to observe a reduction in species detection rate, with doubling of the number of plates sampled (80 plates) showing diminishing returns on species richness sampled per unit of processing time. Doubling the number of plates (from 40 to 80 plates) would result in the detection of two to three indigenous species on non-biocidal plates, but there was no increase in NIS sampled by 80 non-biocidal plates (Figure 3-18). Species richness sampled by 40 control plates accounted for 90-95 % of the species richness that were potentially sampled by 80 plates.

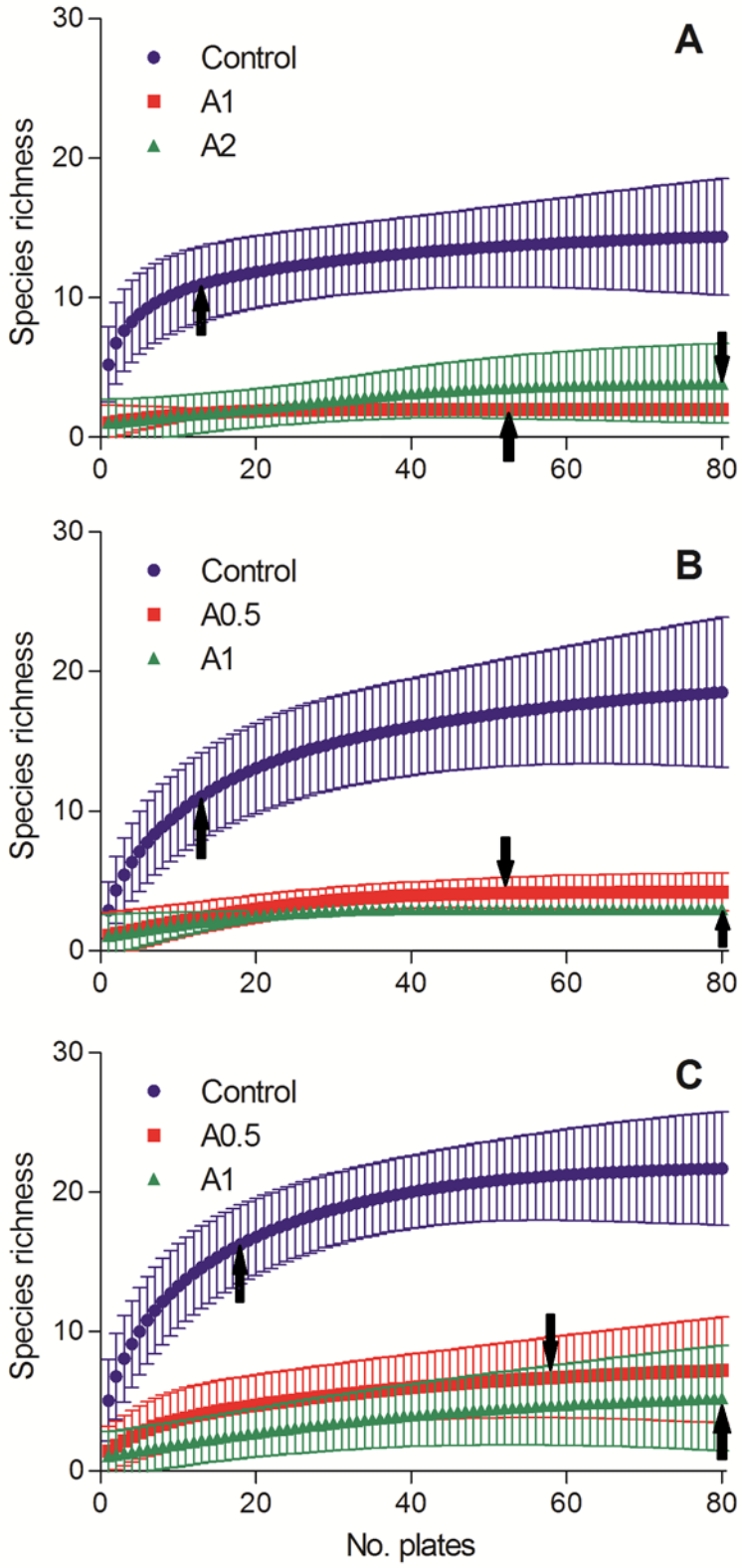

Figure 3-17 Species accumulation (rarefaction) curves for indigenous species across paint treatments (summer 2014-15, control, A1 and A2; winter 2015 and summer 2015-16, control, A0.5 and A1) for summer 2014-15 (A), winter 2015 (B) and summer 2015-16 (C). Error bars show 95 % confidence intervals. Arrows refer to the diversity sampled for equal processing time for each paint treatment (summer 2014-15, control = 12 plates, A1 = 53 plates, A2 = 80 plates; winter 2015, control = 12 plates, A0.5 = 53 plates, A1 = 80 plates; summer 2015-16, control = 18 plates, A0.5 = 58 plates, A1 = 80 plates).

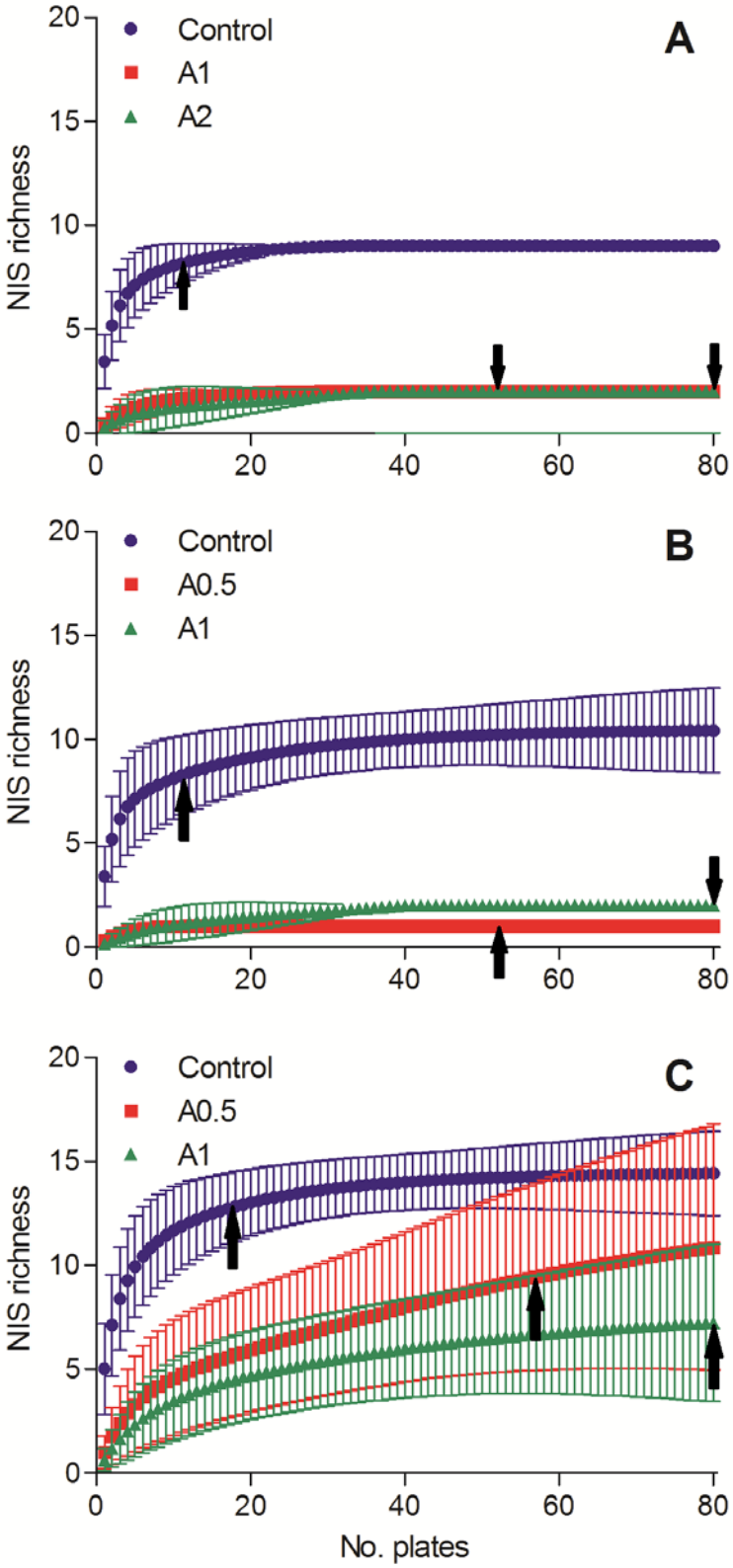

Figure 3-18 Species accumulation (rarefaction) curves for non-indigenous species across paint treatments (summer 2014-15, control, A1 and A2; winter 2015 and summer 2015-16, control, A0.5 and A1) for summer 2014-15 (A), winter 2015 (B) and summer 2015-16 (C). Error bars show 95 % confidence intervals. Arrows refer to the diversity sampled for equal processing time for each paint treatment (summer 2014-15, control = 12 plates, A1= 53 plates, A2 = 80 plates; winter 2015, control = 12 plates, A0.5 = 53 plates, A1 = 80 plates; summer 2015-16, control = 18 plates, A0.5 = 58 plates, A1 = 80 plates).

3.4 RETURN FOR SAMPLE EFFORT

On average, it took 16 minutes (± 7 minutes SD) to identify the species composition of each non-biocidal control plate, this excludes processing time by MITS for species not immediately identifiable to species level, but includes the time of preparing and preserving specimens for MITS. However, it took an average of 24 minutes to process horizontal plates (maximum of 46 minutes) and 8 minutes for vertical plates (maximum of 18 minutes). The average processing time for biocidal plates was approximately four times faster (A1, 4 minutes ± 1.5 minutes) because of the significantly lower number and percentage cover of biofouling species, respectively. In practice, for the summer 2015-16 deployment this meant that an average of 80 A1 plates could be processed in the same time that it took to process 18 control plates. The greater sample size afforded by the quicker processing of biocidal plates did not, however, result in higher detection of NIS relative to the controls. For example, in the summer 2015-16 deployment, 80 A1 plates detected an average of 6 NIS, 25 % less than the average of 8 species recorded on 18 control plates (Figure 3-17). Detection rates of NIS on A1 coatings were much lower for the summer 2014-15 (7 NIS detected on controls compared to 2 on A1; Figure 3-17 A) and winter 2015 deployments (7 NIS detected on controls compared to 2 on A1; Figure 3-17 B).

Faster processing times associated with the lower cover and richness of biofouling species reduced overall processing costs of settlement arrays (i.e., arrays deployed with biocidal plates 40 µm and 75 µm thick; Table 3-11). However, the smaller average number of NIS detected on A0.5 and A1 plates resulted in higher costs per NIS detected compared to control plates (Table 3-11).

Table 3-11 Return of NIS per sampling effort for control, A0.5, and A1 plates. NIS detection on control and A1 coatings averaged across all three deployments for A1 and two deployments for A0.5 (winter 2015 and summer 2015-16). Hourly rate for paratoxonomic processing is set at $150 per hour, and cost of the MITS taxonomic service set at $180 per hour (and fixed to 10 hours for 40 plate deployment and 20 hours for 120 plate deployment).

	Plates processed per hour	Average No. NIS detected	Processing time (hours)	Total processing cost (per deployment)	Processing cost per plate	Cost per NIS detected	Total processing cost for 120 plate deployment
Control	3.75	12	11	$3 400	$85	$284	$10 200
A0.5	15	7	3	$2 200	$55	$315	$6 600
A1	19.6	5	2	$2 106	$53	$422	$6 319

Rarefaction curves for control treatments for the summer 2014-15 deployment showed that combined species richness plateaued at approximately 10 plates, with a doubling of sampling effort only sampling 2-3 (± 2 SD) additional species (Figure 3-18 A). However, during the winter 2015 and summer 2015-16 deployments a doubling of sampling effort could increase the number of species detected by an average of 4 and 6 species, respectively (± 2 SD). Horizontal smooth plates sampled higher average richness of NIS than vertical plates and horizontal sand-blasted plates during summer 2014-15 (Figure 3-18 G). Furthermore, NIS richness during the winter 2015 and summer 2015-16 deployments (Figure 3-18 H & I) was higher on horizontal pitted plates compared to vertical plates and horizontal smooth plates, as shown by the lack of overlap of 95 % confidence intervals. Unlike the rarefaction curves for combined richness for summer 2014-15 (Figure 3-18 A) and winter 2015 (Figure 3-18 B), the summer 2015-16 deployment (Figure 3-18 C) did not plateau, suggesting that these treatments were still sampling new species after 20 plates. However, while the trajectory of these curves varied from rarefaction curves for vertical plates, the overlap between 95 % confidence intervals showed that vertical and horizontal plates were not significantly different.

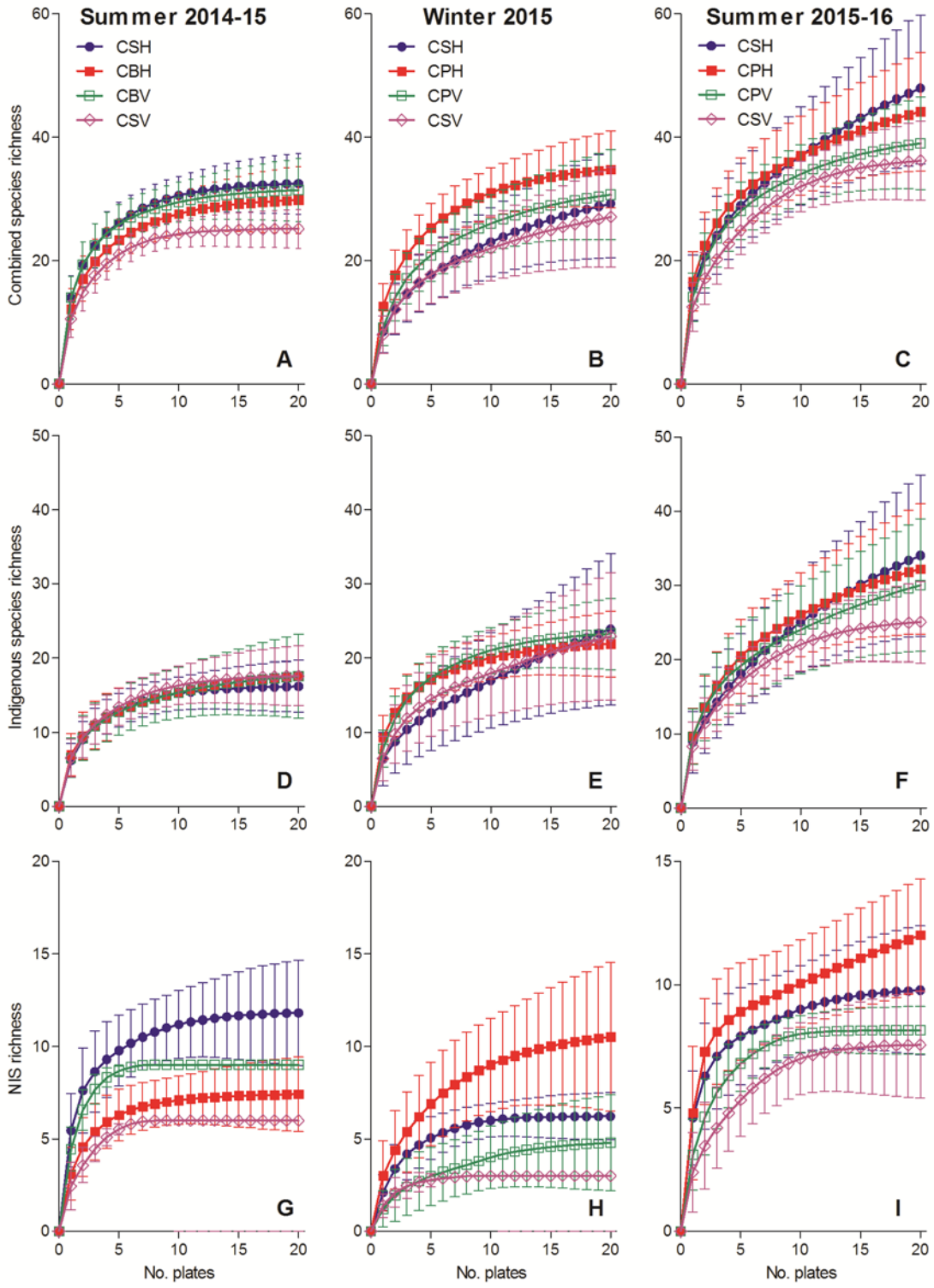

Figure 3-19 Species accumulation curves (rarefaction curves) for control plates in each deployment. Error bars show 95 % confidence intervals. Accumulation curves shown for all species combined (A, B and C), IS (D, E and F), and NIS (G, H and I). Treatments codes are control-smooth-horizontal (CSH), control-blasted-horizontal (CBH, summer 2014-15 only), control-pitted-horizontal (CPH, winter 2015 and summer 2015-16), control-blasted-vertical (CBV, summer 2014-15 only), control-pitted-vertical (CPV, winter 2015 and summer 2015-16), and control-smooth-vertical (CSV). Note that comparison of roughened surfaces between summer 2014-15 (sand-blasted) deployment and the winter 2015 and summer 2015-16 (pitted) is not possible given the change in treatment methodology.

4 Discussion

4.1 INFLUENCE OF EXPERIMENTAL TREATMENTS, LOCATION AND SEASON ON BIOFOULING COMMUNITY COMPOSITION

4.1.1 Effects of temporal and spatial distribution on biofouling composition

Season of deployment had a large influence on the composition of biofouling communities sampled by the settlement plates, including large differences between summer deployments in 2014-15 and 2015-16. Larval recruitment varies considerably with season in temperate ecosystems, with an order of magnitude difference in recruit density observed between summer and winter (e.g., Watson and Barnes, 2004, Broitman et al, 2008, Vaz-Pinto et al, 2014). While many species can spawn year-round in pulses (Watson and Barnes, 2004), most species respond to seasonal changes in the environment (e.g., temperature, day length) and time spawning events with annual seasonal cycles to coincide with food or resource availability (Giangrande et al, 1994). Over the course of this study, significantly higher cover of biofouling organisms was observed during spring-summer deployments than during winter, including significantly higher numbers of NIS during the spring-summer period.

The significant variation in community composition with site and significant variation in treatment effects with site suggests that spatial dispersion has a strong influence in sampling diverse biofouling communities. Sessile biofouling communities are highly variable at a range of spatial scales, from cm to 100s of km (Keough, 1983), with community variability often very high at moderate spatial scales, such as between plates several metres apart (Keough, 1983, Smale, 2013) or plates 100s of metres apart (Keough, 1983, Watson and Barnes, 2004). Entrainment of water within harbour environments has also been shown to influence recruitment dynamics relative to open coastal environments (Floerl and Inglis, 2003), with the potential for variable recruitment across gradients of flow velocity within harbours. Gradients of flow within Westhaven Marina likely occur from the harbour entrance (near pontoon A) and may produce a gradient of propagule exposure with areas of faster flow potentially exposed to greater numbers of propagules (Floerl et al, 2012).

There was no evidence for increasing variation in community composition with increasing distance of array separation, however similar to other studies, community composition varied significantly among spatially separated arrays (Keough, 1983, Smale, 2013). The maximum distance between arrays in this study was less than ~300 m, but greater separation of arrays would likely sample increasingly distinct biofouling assemblages (Watson and Barnes, 2004).

4.1.2 Influence of experimental treatments on biofouling composition

Biofouling community composition was influenced by paint treatment (biocidal and non-biocidal coatings), plate orientation and surface texture. Biocidal paints consistently reduced the percent cover and species richness of IS and NIS relative to non-biocidal controls. Abrasive sandblasting of the plates produced relatively small-scale surface heterogeneity that did not have significant effects on recruitment of biofouling organisms in the summer 2014-15 deployment compared to plates that were left smooth. Creation of more significant surface irregularities (i.e., routed “pits”) in subsequent deployments resulted in significantly different community composition relative to the smooth treatments. Despite this, there was no influence of “pits” on total biofouling cover or on average richness of NIS.

Surface heterogeneity associated with the “pits” likely acts through enhanced survivorship by decreasing physical disturbance (currents) or biological disturbances such as predation (Hunt and Scheibling, 1997). During the course of the deployments, predation by fish (suspected as

Parore, *Girella tricuspidata*; Malcolm Francis, Pers. Comm.) was observed on many of the settlement plates (Figure 4-1). Such disturbances have the potential to dramatically alter biofouling species composition through selective predation. Previous studies have shown that protecting biofouling assemblages from fish predation results in recruitment of more diverse assemblages, particularly of soft-bodied species (Freestone et al, 2010, Freestone et al, 2013, Lavender et al, 2014). Individuals recruiting into "pits" may evade predation long enough to mature beyond vulnerable life-history stages.

Pitted plates did not lead to higher total species richness or higher NIS richness, but they did sample compositionally distinct biofouling assemblages from smooth plates. Although the application of biocidal paint to the pitted treatment could affect paint coverage within pits, there was no significant interaction between paint and surface texture for any of the deployments. The fact that biocidal pitted plates were not statistically different from biocidal smooth plates suggests that paint application to pitted plates did not compromise the effectiveness of antifouling coatings.

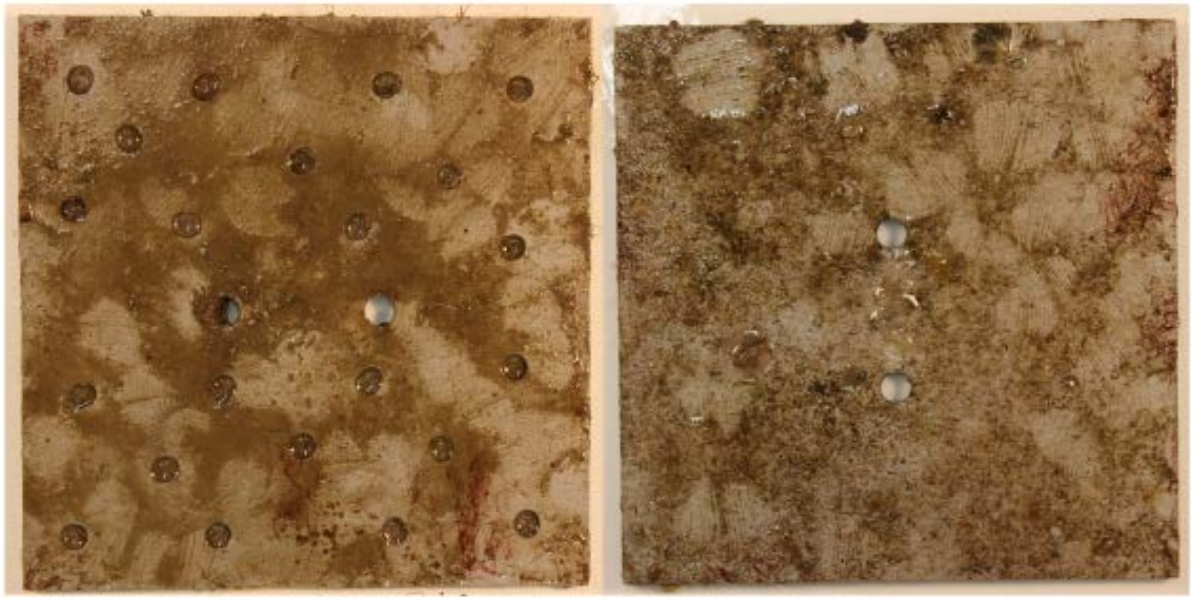

Figure 4-1 Fish scrapings on control plates with and without "pits" (winter 2015 deployment). The "pitted" plate (left) has biofouling within pits untouched by fish grazing which has cleared a large proportion of the biofilm.

Orientation had clear effects on biofouling community composition. Unsurprisingly, higher cover of algal species was observed on vertical surfaces due to the availability of sufficient sunlight (Glasby, 2000, Knott et al, 2004). A higher incidence of some NIS was observed on the underside of horizontal plates, particularly ascidians such as *Ciona intestinalis* and *Styela clava*. Furthermore, total biofouling cover was higher on the horizontal plates compared to vertical plates. This was associated predominantly with high cover of ascidian and bryozoan species (which occurred at much lower densities on vertical plates). Colonial and solitary ascidians are known to recruit preferentially to shaded surfaces (Howes et al, 2007, Martin et al, 2011), such that horizontal (underside surface) plates are necessary for the detection of many ascidian species. Similarly, vertical plates are important for the capture of photosynthetic organisms (e.g., algae). Although no non-indigenous algae were observed throughout the course of the study, many indigenous algal species were found in greater abundance on vertical plates.

The results of this study also showed higher prevalence of NIS on the undersides of horizontal plates compared to vertical plates, in line with observations from other studies, suggesting that shaded environments within marinas can often be dominated by NIS (Dafforn et al, 2015). Shaded environments represent relatively novel habitat in shallow coastal

environments and have been associated with the spread and success of many NIS (Airoldi and Bulleri, 2011). Furthermore, the vectors for biofouling NIS introduction, such as ship hulls and niche areas are predominantly shaded habitats, and the recipient environments (e.g., ports and marinas) typically have a high number of shaded artificial surfaces potentially facilitating the success of shade seeking NIS (Bax et al, 2002).

The historical and continued use of copper-based antifouling paints has resulted in a strong selection pressure for the transportation of copper tolerant biofouling organisms (Piola and Johnston, 2008, Piola and Johnston, 2009). Similarly, resistance to antifouling biocides has influenced macroalgae distributions in the past, whereby the dominant copper-resistant *Enteromorpha* (*Ulva*) sp. were replaced by *Ectocarpus* sp. as the major cosmopolitan fouling alga on ships following the introduction of tributyl-tin containing antifouling paints (Callow, 1986). While it is possible that biocidal coatings could result in active recruitment of tolerant species (McKenzie et al, 2011, McKenzie et al, 2012), the likely mechanisms for higher relative richness of NIS is through release from space competition, with few copper tolerant IS present (Piola and Johnston, 2008). Similar results were observed in this study, with higher ratios of NIS found on biocidal plates compared to controls, but overall smaller numbers of NIS recruited to biocidal plates compared to controls.

In a surveillance context, the reduced abundance and richness of biofouling on biocide treated settlement plates meant that the average time required per plate to identify and process biofouling was significantly lower than that required for more heavily-fouled non-biocidal surfaces. A corollary is that a much larger number of biocidal plates could be deployed and analysed for the same time it took to analyse a relatively modest number of non-biocidal plates. Analysis of the results of this study showed that this larger sample size does not, however, translate into a greater ability to detect NIS, since the average richness of NIS on control plates was generally two to three times greater than that recorded on the biocidal plates. Extrapolation of species accumulation curves (rarefaction curves) showed that increasing the sample size of biocidal plates would not enhance the detection of NIS. The inability of some NIS, particularly soft-bodied species (e.g., *S. clava*, *Sabella spallanzanii* and *C. intestinalis*), to recruit and survive on antifouling coatings greatly reduced the efficacy of biocidal plates to capture the full suite of NIS found on control plates.

Over the course of this study, several species of non-indigenous bryozoans (*Watersipora subatra*, *Celleporaria umbonatoidea*, *Bugula neritina*, *B. flabellata* and *Amathia verticillata*) were regularly observed growing directly on the biocidal coatings. These species provided secondary substratum for the attachment of other species that were not observed growing directly on the paint surface (e.g., soft bodied ascidians), similar to results from Floerl et al, (2004). However, several NIS commonly occurring on control plates (e.g., *S. spallanzanii* and *S. clava*) were not observed on any biocidal plates. Furthermore, the time course required for primary settlement of tolerant bryozoans and secondary settlement of less tolerant NIS suggests that biocidal coatings would not be appropriate for NIS detection for deployments of ~ 3 months. The use of biocidal plates for NIS detection would likely result in the detection of only a subset of the NIS present, with many of these being bryozoans.

4.1.3 Optimising settlement arrays for NIS detection

Analysis of NIS occurring on the settlement plates showed that plate orientation and paint treatment influenced the composition of NIS within the biofouling assemblage. Prevalence of NIS showed that horizontal plates sampled several NIS (e.g., *C. intestinalis* and *S. clava*) not observed on vertical plates throughout the three deployments. Several other species did not occur solely on horizontal surfaces, but occurred at higher frequencies than on vertical plates (*S. spallanzanii*, *Ascidiella aspersa*, *B. flabellata*, *Amphilectus fucorum*, *Botrylloides leachii*). Although vertical plates (non-biocidal) detected several unique species, there were no NIS

consistently sampled by vertical surfaces. To optimise settlement arrays for diverse biofouling communities, incorporating both vertical and horizontal surfaces into settlement arrays would allow for the sampling of shade seeking species, such as ascidians and bryozoans, as well as species which require light (e.g., macroalgae).

Higher numbers of NIS as observed on shaded plates in this study suggest that the use of shaded surfaces is potentially an effective means of capturing similar species, such as *Eudistoma elongatum* (Morrisey et al, 2008). A range of studies have revealed higher densities of NIS on artificial structures compared to adjacent natural substrata (Glasby, 1999, Glasby, 2000, Glasby et al, 2007, Tyrrell and Byers, 2007, Dafforn et al, 2012, Simkanin et al, 2012). These patterns have been related to translocation of biofouling on vessel hulls (predominantly shaded hull and niche areas; Hopkins and Forrest, 2010) and the high density of shaded habitats in developed harbours and marinas. The provision of shade is considered a major factor in the spread and establishment of many problematic NIS (Bax et al, 2002, Airoldi and Bulleri, 2011, Dafforn et al, 2015). Our study showed that horizontal (shaded) surfaces sampled significantly more NIS than vertical surfaces (Table 3-9) making them potentially effective at directly targeting biofouling associated with vessel niche areas. Vessel niche areas are areas on a vessel hull that are more susceptible to biofouling due to different hydrodynamic forces, susceptibility to coating system wear or damage, or being inadequately, or not, painted (Coutts et al, 2003, Coutts et al, 2007, Lee et al, 2007, Davidson et al, 2009, Bell et al, 2011, Frey et al, 2014).

There was no influence of surface texture on the relative prevalence of NIS across the deployments, with no species or taxonomic groups consistently associated with smooth or roughened (pitted or sand-blasted) plates. The use of heterogeneous surfaces, such as pits, may lead to higher recruit survival, particularly if some species are selectively targeted by predators (Freestone et al, 2010, Freestone et al, 2013, Lavender et al, 2014). If the predation observed during the course of this study is non-selective, which may be the case if the predators are feeding on algal biofilms, then pits may allow species to avoid removal long enough to mature past critical life-history stages.

Fewer NIS were found during the winter period than during spring-summer. Although NIS diversity was lower during the single winter deployment from this study, continuous seasonal sampling of settlement arrays would provide better temporal resolution for sampling NIS and potentially enable the sampling of NIS arriving out of sync with local seasonal cycles.

Sufficient spatial arrangement would also increase the potential for species with extended larval durations to recruit to settlement arrays (Floerl and Inglis, 2003), as shown by the significant differences in community composition between arrays separated by 10’s to 100’s of metres during this study.

4.2 Prospects for the use of molecular tools for sample processing

An important constraint on the detection of NIS in complex biological assemblages is the number of samples that can be taken and analysed from within the large areas potentially occupied by the species. Developing molecular technologies hold considerable promise for biosecurity surveillance by enabling cheaper and faster identification of juvenile or larval NIS and, thereby, allowing larger sample sizes for the same cost (Bott et al, 2010). DNA sequencing has been used to determine the phylogenetic identity of several species, confirming non-indigenous status for several species in New Zealand (Smith et al, 2003, D’Archino et al, 2007, Smith et al, 2007), but as yet sequencing methods have not been actively integrated into surveillance activities. New developments in high-throughput sequencing (HTS) and metagenomics allows multi-species samples (environmental DNA samples, eDNA) to be processed simultaneously and screened for NIS. However, the technical challenges associated with detecting NIS in complex samples increases the potential

sources of errors (Darling and Blum, 2007, Darling and Mahon, 2011) and numerous steps are required to ensure quality assurance and control using these techniques. These include:

- proper selection of DNA markers to ensure appropriate level of taxonomic resolution for a range of phyla (Bott et al, 2010),
- alignment of morphological and sequencing approaches for target NIS and closely related species,
- clearly defined monitoring protocols, (i.e., active surveillance for target NIS or passive surveillance to allow the detection of unexpected NIS; Simmons et al, 2016), and
- sufficient replication (this includes biological sample replication and technical replication to correct for intra-sample variation; Zhan et al, 2014) and sampling methods.

Of particular concern for the use of HTS in biosecurity surveillance are the occurrence of false positive and false negative results. Such errors include:

- false positives (type I error, eDNA detected where target species is not present) resulting from eDNA detection from sources other than living organisms (i.e., non-viable material from ballast water discharge, animal excrement or dead organisms),
- false positives resulting from PCR primers and eDNA probes which lack appropriate specificity, allowing the amplification of 'lookalike' non-target DNA (Zhan et al, 2014), and
- false negatives (type II error, eDNA not detected where the target species is present) resulting from lack of sensitivity (Bohmann et al, 2014).

False positives have the potential to lead to high surveillance costs, where a "detection" of NIS from HTS would be followed up with a species specific molecular test (possibly requiring further sampling) to confirm the species identity. Until taxonomic resolution of HTS methods can be confirmed or improved, and international databases of NIS are made available, the use of metagenomics are unlikely to be used as a stand-alone method for NIS detection (Simmons et al, 2016). Therefore, the use of sequencing methods for NIS detection should fall into two categories:

- passive surveillance to screen environmental samples for a range of species (i.e., settlement plate scrapings or water samples combining mixed genetic material), and
- active surveillance to target specific species.

Each surveillance category will likely favour different sequencing techniques, with HTS more cost effective for processing combined species samples (passive surveillance) to locate potential NIS, and the use of species specific molecular probes would provide accurate results for target species (active surveillance; Bohmann et al, 2014, Simmons et al, 2016).

The use of traditional taxonomic methods, while more time-consuming at the identification stage will, at the present time, have a comparatively low rate of false positives compared to HTS, particularly when the circumstances of organism collection are known. For example, specimens collected during the course of biosecurity surveillance (e.g., MHRSS) are sent away with additional information such as where the organism was found and the status of the individual (e.g., specimen found dead in wrack or alive attached to wharf piles). Spuriously amplified sequences is a common problem for HTS methods, with careful management of low abundance sequences required to reveal unique/rare lineages (Zhan et al, 2014). It is noted that 70-80 % of the species identified on settlement plates by Zaiko et al, (2016), have not

been recorded in New Zealand and were likely misattributed because the species was not represented in the sequence database (i.e., the sequence defaulted to the closest attribution).

Studies testing the efficacy of HTS have shown that false negatives stemming from lack of sensitivity are unlikely, with very high detection limits reported (Pochon et al, 2013). However, given the wide range of taxonomic groups targeted by biosecurity surveillance programmes, there is a need to ensure that appropriate primers and probes are used to detect target species, and distinguish them from closely related IS (Darling and Mahon, 2011, Zhan et al, 2014).

Incorporation of HTS methods into biosecurity surveillance are reliant upon the sharing and standardisation of global sequence databases so that both target species and unexpected species can be detected in eDNA samples. Effective use of such databases will also require sufficient sequence data for closely related IS given that sequence database searching will often select the closest relative to a given input sequence (Bott et al, 2010). To properly integrate HTS techniques into the MHRSS programme there will need to be extensive cross-over between traditional taxonomic methods and molecular methods, as this will provide an assessment of the potential rate of false positives using sequencing methods and false negatives using taxonomic methods. In the context of settlement arrays, false negatives during taxonomic identification represent lack of organism detection because the organism was too small to see, or too small to accurately identify to species level. While there are several methodological details that will need to be examined before HTS can be reliably incorporated into marine biosecurity surveillance programmes, the combination of settlement arrays and HTS have the potential to produce a powerful tool for sampling the marine environment for NIS. Processing settlement arrays using HTS has several potential benefits over traditional taxonomic processing:

1. HTS allows for the deployment of higher numbers of arrays for the same processing costs. Although the costs of technical replication should be included into assessments of the biological replication gained from HTS methods.
2. HTS methods can identify larvae, unlike taxonomic methods for many species, potentially reducing the deployment time of arrays required to achieve detection of early colonising biofouling. However, it should also be noted that many biofouling species may require other cues associated with biofilms or conspecifics before they will recruit to settlement plates (Keough and Raimondi, 1995).
3. Reducing the requirement for trained taxonomic/parataxonomic expertise in plate processing.

5 Recommendations

5.1 Use of settlement arrays for marine NIS surveillance

Settlement arrays have the potential to sample a range of biofouling organisms not actively targeted during the marine high risk site surveillance (MHRSS). The primary goal of the MHRSS programme is to detect several high risk species (see Section 1.1), with the sampling methods designed specifically to target those species. The secondary goals of the MHRSS are to detect range extensions of established NIS or cryptogenic species, and to detect new to New Zealand non-indigenous or cryptogenic species not listed on the New Zealand Unwanted Organisms Register.

While there is limited potential for settlement arrays to detect the primary target species of the MHRSS, two of the secondary target species already present in New Zealand (*Sabella spallanzanii* and *Styela clava*) were sampled during this study. Further they have the potential to detect another secondary target species not present in Waitemata Harbour (i.e., *Eudistoma elongatum*). The arrays used in this study also detected a range extension of the bryozoan, *Celleporaria umbonatoidea*. These findings show that settlement arrays may contribute to the secondary requirements of the MHRSS (i.e., detecting new to New Zealand organisms, and detecting range extensions of established NIS). Settlement arrays also provide the ability to closely scrutinise juvenile stages of biofouling communities, making them potentially effective at sampling early life-history stages of non-target biofouling species.

Integration of settlement arrays into the MHRSS should, therefore, consider this sampling method as additional to the primary goals of the MHRSS programme for two reasons:

1. Settlement arrays are not applicable for sampling primary target species.
2. Settlement arrays are not a targeted surveillance method (i.e., they are a generic sampling method to sample a range of unknown biofouling NIS).

Therefore, these passive sampling methods should be seen as complementary to the current MHRSS activities enabling greater capacity to sample non-target, non-indigenous biofouling species. However, the efficacy for settlement arrays to detect incursions at small population sizes (i.e., as seen during the early stages of an incursion) may be limited (Floerl et al, 2012). While this study sampled two secondary target species (*S. spallanzanii* and *S. clava*), these species are present in very high abundance within Westhaven Marina. There is limited evidence to suggest that settlement arrays are more effective than other surveillance methods (e.g., SCUBA surveys) for detecting range extensions of *S. spallanzanii* and *S. clava* or new to New Zealand incursions of NIS. Further to these findings, no non-indigenous ascidian species were sampled by settlement plates deployed in the Alaskan NIS monitoring programme (Doroff et al, 2011). Similarly, after 24 months of settlement array deployment in Raffles Bay (Northern Territory, Australia; Cribb and Marshall, 2005) and 12 months deployment in Darwin Harbour (Northern Territory, Australia; Cribb et al, 2008), no recognised marine pest species were detected. However, it is noted that the Australian programme was designed to confirm the effectiveness of eradication of the non-indigenous *Mytilopsis* sp.

5.2 Optimised array design for NIS detection

To optimise settlement arrays for meeting the secondary objectives of the MHRSS programme (particularly the detection of non-target biofouling species), the arrays should be

capable of detecting a wide taxonomic range of biofouling species. To examine the potential for the settlement arrays to do this means that we must consider the full range of species captured, not only species which are non-indigenous, as no representative NIS were found for several taxonomic groups (e.g., algae and crustacea) and many of the NIS were very abundant in the location sampled. Sampling diverse biofouling communities means trading-off the ability to detect a single taxonomic group well, for sampling many taxonomic groups poorly, which will lead to efficient sampling of abundant species, but poor sampling of rare species. With the goal of passively sampling a diverse range of biofouling the following recommendations are made for settlement array design and deployment to complement the MHRSS programme.

Although there is the potential to incorporate biocidal coatings into the array design for specific purposes (i.e., to target copper-tolerant bryozoan species), the results of this study show that such an approach does not result in more cost-efficient sampling of a wide variety of NIS as it only detected a proportion of the NIS found on control plates.

In summary, the results of field trials showed:

- More NIS were sampled by non-biocidal plates than by biocidal plates for the same unit effort.
- Compositionally different assemblages of NIS were sampled by non-biocidal plates that were oriented vertically and horizontally.
- Higher incidence of NIS, particularly ascidians, on horizontal plates with several species occurring only on horizontal plates (e.g., *S. clava, Ciona intestinalis*).
- Greater numbers of NIS were sampled in deployments over summer than in winter, although a single NIS (the bryozoan *Tricellaria inopinata*) was only found during the winter deployment.
- Compositionally different assemblages of NIS were sampled by pitted, non-biocidal plates.

Therefore, we recommend incorporating plates oriented horizontally and vertically, with all plates pitted to promote recruit survival past vulnerable life-stages. The undersides of horizontal plates deployed in this study detected a higher number of NIS than vertical surfaces. Other studies suggest that shade tolerance is an important mechanism of NIS translocation (Dafforn et al, 2015). However, the ability of vertical and horizontal surfaces to target different suites of species makes the incorporation of both orientations essential to capturing a wider range of taxonomic groups.

While settlement arrays can be used to target early life-history stages of biofouling communities, the detection of new incursions is dependent upon the size of the population, the fecundity of that population and the intensity of sampling. New incursions of NIS can be considered rare in their new environment and this poses significant challenges to the early detection of these species (Hulme, 2006). Because of this, most NIS discoveries occur once they have established and attained ecologically significant densities (Myers et al, 2000, Bax et al, 2002, Inglis et al, 2006b). In order to decrease detection thresholds to detect increasingly rare species, an increase in sampling intensity is required (Gotelli and Colwell, 2001, Hortal et al, 2006). Even high numbers of samples (100 samples) were unable to detect an introduced water flea, in an enclosed lake environment, at low population densities (Harvey et al, 2009). In this example and others (e.g., Rohr et al, 2006, Soria-Auza and Kessler, 2008), the detection probability was strongly affected by sampling intensity and organism abundance. Furthermore, Harvey et al, (2009) targeted a specific species, whereas the goal of this study was to optimise settlement arrays for diverse biofouling assemblages. While settlement arrays have the potential to sample NIS not targeted by the MHRSS, the utility of settlement arrays

for detecting early stage incursions will be limited by the sampling intensity (i.e., the number of plates deployed).

Analysis of species richness sampled by settlement plates using rarefaction curves shows that NIS richness was well sampled by non-biocidal controls after 40 samples (plates), suggesting that the NIS sampled were relatively common in the sampled environment. Doubling the sampling effort to 80 plates showed no gain in NIS sampled across all three deployments. By contrast, the rarefaction curves for IS showed that doubling the sampling effort would potentially increase the number of species sampled by 1-2 species. Sampling rare IS is likely to be a more appropriate metric for detection of NIS at low population sizes (i.e., early stage incursions), and as such, suggests that the number of samples required is potentially very high. To decrease the detection thresholds a high number of settlement plates will be required to detect early stage incursions, with extrapolation of rarefaction curves beyond 40 samples showing that rare IS are still likely to be sampled after 80 samples. Therefore, based on extrapolation of rarefaction curves and other studies focusing on the detection of species which are rare in the sampled environment, between 80-160 plates are recommended to sample low population sizes (Rohr et al, 2007, Harvey et al, 2009).

To maintain a high level of shade for attracting shade-seeking larvae (e.g., ascidian larvae; Howes et al, 2007), deployment of settlement arrays with 8-12 plates is recommended to enhance the shadowing of arrays. The spatial arrangement of arrays should consider the proximity to potential vectors of invasion, with evidence showing that a risk-based sampling design can enhance the potential for early detection (Inglis et al, 2006b, Harvey et al, 2009). In the context of marine NIS incursions, areas with high turnover of international vessels should be targeted. Furthermore, modifying the number of plates per array (i.e., 8-12 plates) would allow flexibility in the number of arrays deployed for the same processing costs. For example, deployment of 120 plates at a small marina may be best sampled by 10 arrays each with 12 plates, whereas a larger area might be better sampled by 15 arrays, each with 8 plates.

Temporal sampling of biofouling communities should consider the ability to detect species using traditional taxonomic approaches (i.e., this approach requires refinement to identify some species) and the loss of temporal resolution for detections associated with longer deployments. A deployment time no longer than three months is therefore recommended to enable sufficient development of biofouling species, while maintaining the ability to detect new incursions or range extensions at an early stage.

Although year-round deployment has the potential to provide high temporal resolution for detection of NIS, higher numbers of NIS during summer and faster growth rates of fouling organisms suggests that settlement array deployment should target the spring-summer period. However, to maintain capacity for detecting incursions of NIS, sampling the period following the boreal spring-summer could maintain capacity for detecting new to New Zealand NIS arriving from the Northern Hemisphere.

In summary, the following recommendations are provided for incorporating passive sampling methods into the national Marine High Risk Site Surveillance (MHRSS) programme:

- Deployment of 10-20 arrays each consisting of 8-12 plates (80-160 plates) depending on the site-specific scale.
- Plates deployed both horizontally and vertically in equal numbers.
- All plates are pitted to increase species richness.
- Plates should be made of dark (grey or black) PVC with no paint treatment.
- Arrays should be distributed 10’s to 100’s of metres apart to sample distinct species assemblages.

- Plates should be deployed for three to four months to assist in taxonomic identification.
- Deployments should target the spring-summer period (October–January) to sample NIS spawning during warmer conditions, and the autumn-winter period (April-July) to sample NIS potentially arriving from the northern hemisphere ready to spawn (i.e., following the Northern Hemisphere spring).

Processing of 80–160 plates would take an estimated 22–43 h (16 ± 7 min per plate), 8–12 h initial array construction, 12–15 h to deploy and retrieve, and 40–60 h for species identification using the MITS service. Array materials are estimated at NZ$1 942–3 634 per annum. At a rate of NZ$150/h for construction, processing, deployment and retrieval, and NZ$180/h for MITS taxonomists (30-50 hours per annum) settlement array surveys would cost ~ NZ$19 496 for 80 plates, ~NZ$27 388 for 120 plates, and ~NZ$35 280 for 160 plates per annum for two deployments at a single location. These prices do not allow for travel to remote locations.

Comparing the effectiveness of settlement plate arrays to other survey methods, such as diving surveys, is difficult as each method targets different life-stages of biofouling communities. However, diving surveys have been responsible for the detection of incursions of *S. spallanzanii* at low population sizes in Lyttelton Harbour during March 2008 (380 individuals subsequently removed by December 2009; Read et al, 2011), whereas settlement arrays deployed at the same location were not able to detect *S. spallanzanii*, or the abundant *S. clava* (Floerl et al, 2012). While settlement arrays can contribute to the secondary objectives of the MHRSS programme, the high cost of deploying and processing large numbers of settlement plates would be better spent on diving surveys by trained taxonomists and parataxonomists.

It is noted, however, that as the issues related to the sensitivity and specificity of HTS methods are overcome, the combination of settlement arrays and metagenomic sequencing could increase the efficiency of plate processing, enabling the deployment of larger numbers of plates for the same processing cost. These gains in efficiency would need to be reassessed as continuing research is done to operationalise HTS methods for biosecurity surveillance.

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8 Appendices

Appendix A: Settlement array design and deployment protocol

This document details the protocol for the design, construction and deployment of settlement arrays for use in biosecurity surveillance, as recommended to complement the current MHRSS programme.

This document provides a guide for deployment of standardised settlement arrays optimised for capturing biofouling NIS. Equipment and materials required for assembling and deploying settlement arrays are listed in Table 1.

Table 1. Equipment list for settlement arrays. Details given for deployment of 10 arrays with 80 plates total

Array component	Breakdown	Quantity required
Tools	Crescent, scissors, lighter, gloves, pliers, snips, sandpaper (80 grit), drill (with 2, 8, and 12-mm drill bits)	1 of each
Anchor and rope	Cinder blocks	10
	Rope	5-10 m (site/depth dependent)
	Chain (galvanised grade L, 8 mm)	2 m per array (20 m total)
Frame	1 m lengths of PVC pipe (32 mm diameter)	30
	0.25 m lengths of PVC pipe (32 mm diameter)	40
	PVC elbow joints (32 mm inner diameter)	40
	PVC T joints (32 mm inner diameter)	20
Frame attachment	Stainless steel threaded rod (12-mm thick)	10 x 1 m lengths
	stainless steel L brackets	20
	stainless steel D shackles	30
	stainless steel nuts	60
	stainless steel washers	60
	stainless steel locking nuts	20
Settlement plates	4.8 mm x 300 mm black cable ties	80
	PVC plates “pitted”	80
Labels	Array warning labels[1]	10

[1] See Figure 1, example warning card below

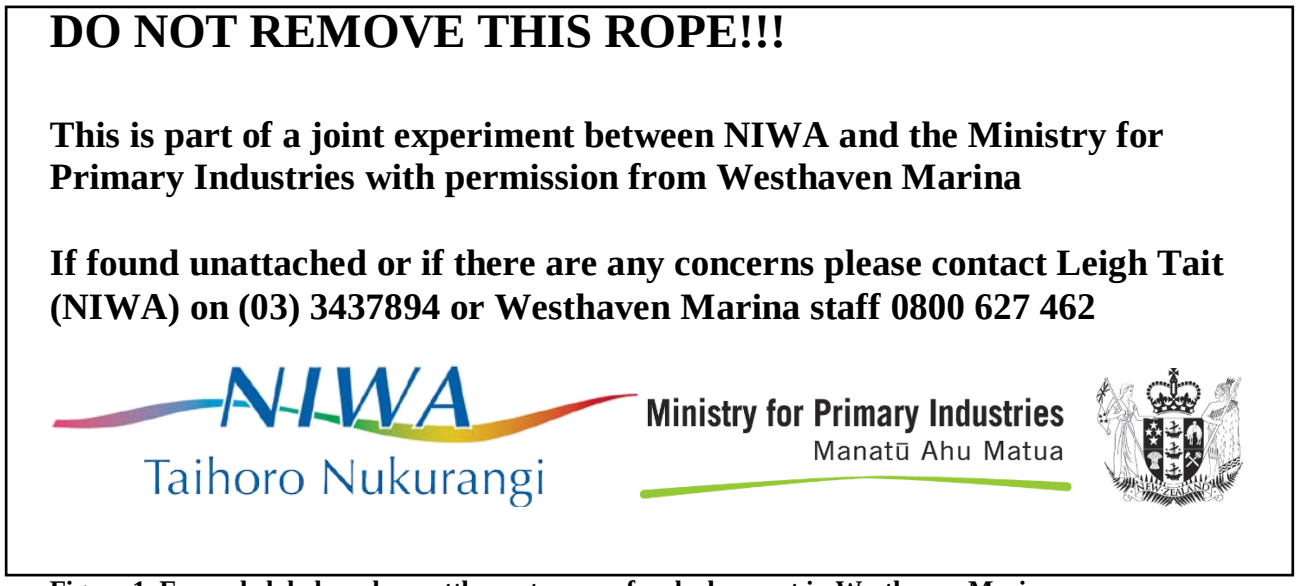

Figure 1. Example label used on settlement arrays for deployment in Westhaven Marina.

Plate treatment

Using equipment itemised in Table 1 prepare the settlement plates as follows:

1. *See equipment required for settlement plates (Table 1).*
    a. Pre-cut 80 plates to 14.5 x 14.5 cm (this is typically done when ordering the PVC sheet from suppliers such as Mulford Plastics Ltd).
    b. All plates will need two holes for attachment to the PVC frames (using zip-ties). Positioning of holes is shown in Figure 2. A template with pilot holes (2 mm wide holes) should be used to drill holes in settlement plates and frames (to ensure all pilot holes and subsequent 8mm holes are 4 cm apart).
    c. Surface abrasion treatment should be applied to each plate. This can be done using a hand-held router (Dremel) device set to take out a circle 6 mm wide and 2 mm deep (depth should be set with an adjustable shroud surrounding the router bit). In total 22 “pits” should be routed from the PVC. These pits should be evenly spaced in rows (Figure 2).

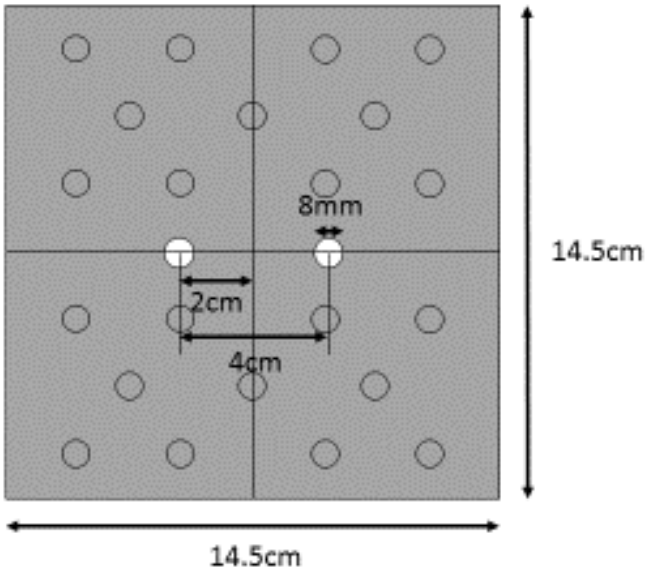

Figure 2. Settlement plate dimensions and positioning of attachment holes and pattern of "pits".

Frame assembly

To accommodate high numbers of plates to enhance rates of detection, up to 12 plates per array are required (2 surface orientations). Plates are attached to frames made of PVC pipe (Figure? 3). Settlement arrays are deployed at two metres depth and secured at the surface to pontoons and at the seafloor with a cinder-block (Figure 4).

Assembly of the frame should be completed in advance of site deployment to allow glue to properly cure. However, complete assembly of arrays for deployment should be completed at or near the deployment site. On site assembly will require several hand tools, including; crescent or spanner, pliers, and snips (for cutting zip-ties). Using equipment itemised in Table 1 assemble frames as such:

To be completed in advance (frame assembly). You will need tools and frame materials (Table 1):

1. Using three 1 m lengths of PVC pipe, four smaller 25 cm sections of PVC pipe, two PVC "t-connectors" and four PVC "elbow joints" connect all units together, and re-assemble while gluing joints in place with PVC cement.
2. Once the PVC cement has cured, paired holes for plate attachment will need to be drilled into the PVC frame. Holes should be 4 cm apart and must alternate in horizontal and vertical orientation (Figure 3). A pilot hole (2-3 mm) should be drilled before the final (8 mm) hole is drilled.
3. For the anchor, a 12 mm hole should be drilled in the centre of the frame (Figure 3, "Rope position"). Again a pilot hole will be necessary before using the 12 mm drill bit.

To be completed on site (frame rigging and settlement plate attachment). You will need tools, assembled frames, anchor and rope and frame attachment materials (Table 1):

1. Frame rigging
    a. Wind a nut and washer 10 cm down the threaded rod, and push rod through the centre hole of the frame. Secure washer and nut on the other side of the PVC frame and tighten with care as not to overly compress the PVC frame.

b. At each end of the threaded rod wind a nut and washer approximately 3 cm down, place the steel 'L' bracket over the threaded rod and secure the 'L' bracket on the other side with a locking nut (Figure 4).
c. Secure rope to 'D' shackles at each end of the threaded rod and secure shackles to the 'L' brackets.
d. Using another 'D' shackle, secure the chain through a hole in the cinder block and attach rope to the other end of the chain. The chain length is dictated by the tidal range at the deployment site, so that slack rope is taken up at low tide. In most cases two metres of chain will be sufficient.

2. Plate attachment
    a. Secure settlement plates to the frame using zip-ties. Up to twelve plates are attached per frame with six deployed horizontally and six deployed vertically.
3. Array deployment
    a. Fully assembled frames should be deployed by first lowering the cinder block to the seafloor, then lowering the frame, and securing the rope to the surface pontoon or structure.

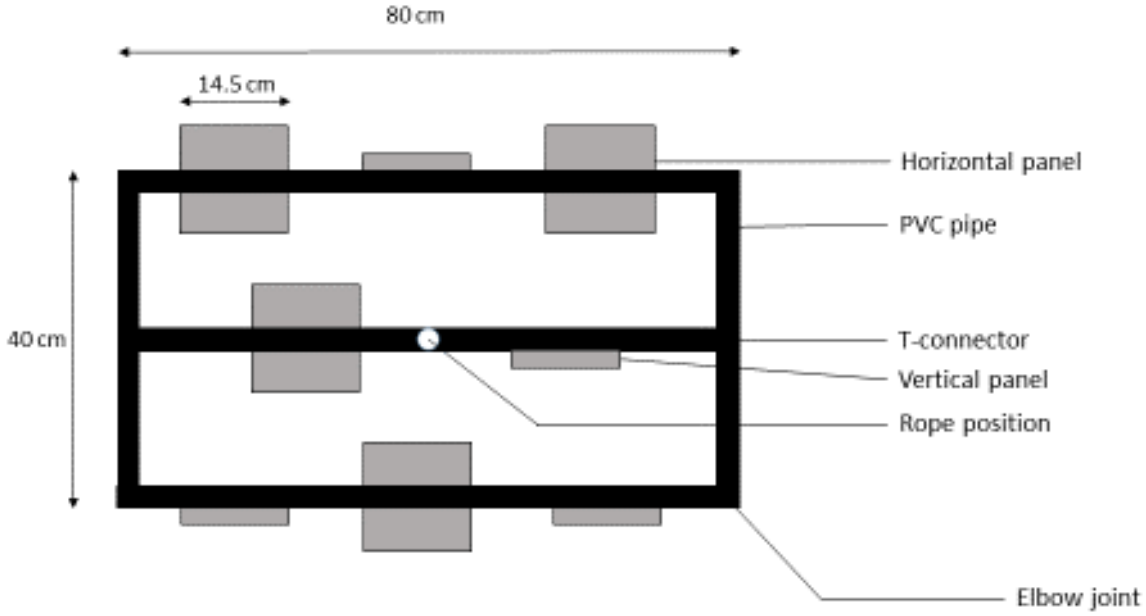

Figure 3. Settlement array design and plate layout. Diagram shows birds-eye-view of the frame with horizontal plates facing downwards.

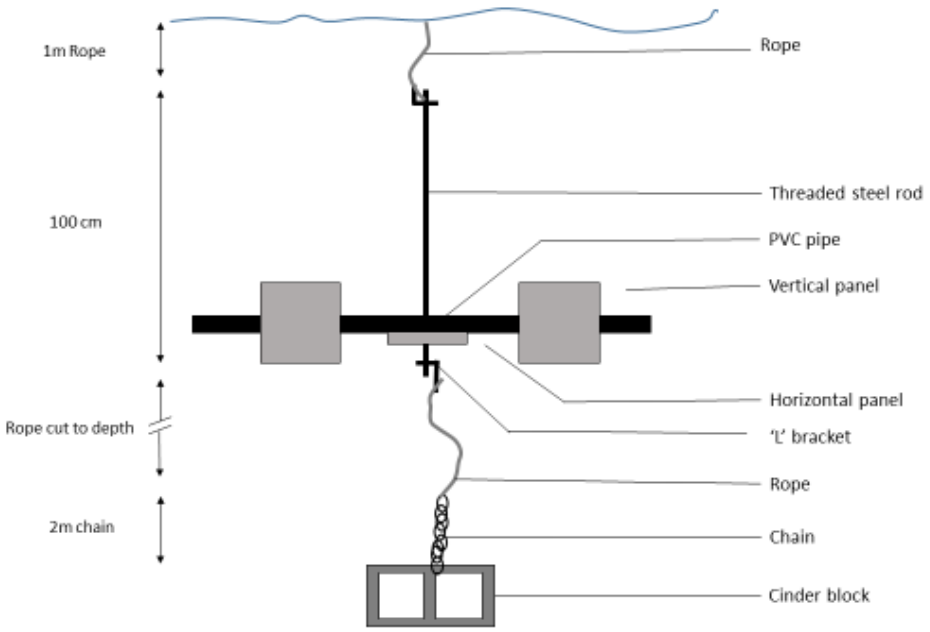

Figure 4. Side view of settlement array including the anchor and rope setup.

Appendix B: Sample sorting and labelling of marine collections for submission to the Marine Invasives Taxonomic Service

1. Introduction

This document outlines the procedures for processing marine algae and invertebrates collected for various MPI projects. Please follow these procedures to ensure that all specimens can be reliably tracked and correctly identified. Incorrect handling can easily render specimens unidentifiable, even by experienced taxonomists. Collections made are intended to provide early warning of any new non-indigenous species arrivals as well as baseline distributional data of existing species at selected localities (for some projects). Therefore, all collections must be made carefully, and specimens sorted, labelled, fixed and preserved in the best possible condition for identification.

2. Processing facilities

All collections of marine algae and invertebrates should be kept shaded, cool and wet, and processed within 1–2 hours of collection according to the following procedures. A field lab should be set up to provide working space that is sheltered from wind, sun and rain, but well ventilated. Good bench space is vital and there should be ready supplies of fresh seawater on hand.

The field lab should be equipped with various containers, ranging from buckets and tubs, through to shallow plastic trays for sorting collections. A good supply of appropriately-sized plastic vials and jars are essential for storing sorted collections. These containers should be non-rigid plastic as far as practical, clear and have **water-tight** screw caps. A supply of waterproof paper and pencils should be available.

3. Fixatives and preservatives

DO NOT use isopropyl alcohol (IPA) for fixing or preserving any specimens.

Formalin should be diluted to 5 % and 10 % using seawater, NOT freshwater.

Ethanol must be diluted using freshwater, NOT seawater.

3.1 Narcotising/relaxing

Some marine invertebrates will disintegrate, contract violently or otherwise be rendered unidentifiable unless relaxed gradually and completely before fixing (Table 3). For example, anemones will contract into hard balls if placed straight into formalin (even worse in ethanol) – leaving it difficult to identify them using morphological characteristics. Ascidians also must be relaxed or they will contract on fixing, making them difficult or impossible to identify. For the few groups that require relaxing, add fresh seawater to their container (jar or bowl) and sprinkle a few menthol crystals onto the surface. Leave for a few hours if possible. Check the response of specimens periodically by gently touching part of the body (e.g., with forceps). When there is no response to a moderate touch, it is safe to fix the sorted collection by adding fixative (= preservative in most cases). Start by adding fixative at about 10–20 % of the seawater volume just to kill the specimens. Leave to fix for 30 mins, then pour off the seawater-fixative mix and re-fill with the appropriate preservative (Table 3).

4. Labelling

Accurate labelling of collections is essential to the success of the entire project. Unlabelled collections or collections with illegible (unreadable or faded printing) labels cannot be used because the information cannot be salvaged. It's vital to get field labelling right and to be very disciplined in labelling practice.

4.1 Label materials and preparation

Label material

Labels should be made from high quality, water-resistant parchment paper, light card, or archival quality paper that will remain intact for at least 5 years in 100 % ethanol (e.g., Laundry Tag Manilla; Byron & Weston's Resistall paper).

Label writing

Write in pencil or preferably permanent ink using a pigment pen. Pens must be water- and alcohol-proof (e.g., Staedtler Pigment Liner or Uni Pin pigment pens). If using laser printed labels, post-printing treatment must be performed to maximize permanency of the print (i.e., heating in a small baking oven for approx. 30 seconds at 150°C or passing them through the photocopier/printer twice). Avoid ink-jet printed labels because most inks will run in water or alcohol. Remember, inadequate or poor quality labelling at this stage can render the collections useless.

Label placement

Labels for every Sorted Collection must go **inside** the container and preferably so they **can be read easily from outside**. Permanent marker pen labels on the outside of containers may increase convenience, but are often dissolved by leaking ethanol, may be abraded by friction during transit or may be forgotten when a container is changed.

4.2 Field labels and collection codes

Collection codes

The specific sampling locations, number of stations and replicates (if necessary) are determined prior to field collecting. Therefore, each sample to be collected is pre-assigned a Collection Code that uniquely distinguishes each sample collected per port (e.g., **2LYT037**). The Collection Code is composed of three elements.

1. The first element indicates the repeat number for each survey of the port. In the example, '**2**' indicates this is the second time this port has been surveyed. (No number was assigned for the first round of surveys).

2. A three-letter port code (e.g., **LYT** for the Port of Lyttelton). These are the standard three-letter United Nations codes for Trade and Transport Locations (UN-LOCODES) for each port (http: //www.unece.org/cefact/locode/service/main.htm). Note that some locations presently do not have a UN-LOCODE in which case MITS can be contacted for a suggested code.

3. The three-digit *Collection Number* (e.g., **037**) (previously called a *sample lot code*) provides a unique identifier for each collection that is made for the particular round of surveys for the port in question. Because the number and location of samples to be collected is known, each sample can be allocated a number before commencement of fieldwork.

Thus, from the outset, each sample receives a permanent, unique Collection Code.

Additional 'unplanned' collections

If further field samples are taken in addition to those already planned, a new Collection Code should be created for each sample with numbering following from the last pre-assigned Collection Code.

Field labels

Each collection or sample receives a **Field Label** at the point of collection specifying:

- port name,
- station/site location,
- station number,

- collection code,
- sample type or method,
- replicate number/identifier, and
- date.

Sample type and methods are coded as follows:

Anchor box dredge	ANCH	Pile scraping	PSC
Beach seine net	SEINE	Plankton tow: phyto-	PHYT
Beach wrack	WRACK	Plankton tow: zoo-	ZOOP
Benthic core	BCOR	Poison stations	POIS
Benthic sled	BSLD	Sediment	SEDIMENT
Dinoflagellate cyst	CYST	Trap: crab	CRBTP
Dive: visual search	VISD	Trap: shrimp	SHRTP
Dive: visual transect	TRAND	Settlement plate	SPLATE

Table 1. Sample type and method codes

For example, below are Field Labels for two pile-scrape collections (replicates 3 and 4) made at Station 018 beside Gladstone Quay in Lyttelton (bold shows pre-printed parts of the label):

Port	**Lyttelton**	**Location** Gladstone South	
Station L018		**Collection code**	2LYT037
Method PSC	**Replicate no.**	3	
Date:	16 May 2005		

Port	**Lyttelton**	**Location** Gladstone South	
Station L018		**Collection code**	2LYT038
Method PSC	**Replicate no.**	4	
Date:	16 May 2005		

Note that station number is not a unique identifier because replicates and collections using different methods are usually taken at each station. These labels include a small amount of redundancy (station, method, and replicate, but the Collection Code captures all of this information).

4.3 Collections record and labels (field lab)

In the field laboratory, each collection should be registered prior to sorting. This involves making a **Collections Record** by recording data from each Field Label on an electronic (e.g., Excel) or hardcopy registration sheet. This is an important step. Although the data (locality, date, sampling method, replicate, etc) should already have been determined when assigning Collection Codes prior to fieldwork, the Collections Record allows the field team to verify that the projected number of samples has actually been collected, and to record any variation from the original sampling plan.

4.4 Taxon group codes

Once a collection has been registered with its Collection Code, it is sorted into broad taxonomic groups (Table 3). Each taxonomic group extracted from the sample (**Sorted Collection (SC))**, is placed in a separate container with a separate label. These labels comprise the Collection Code, plus a 2-letter code for the taxonomic group (Table 3; see

Section 5: Processing), so that the unique identifier becomes the **Sorted Collection Number**. For example, algae are given the code **AG**. In the case of a port survey the SC Number on the label for algae collected in sample **2LYT037** would read: **2LYT037AG**. In the case of a hull fouling project the SC Number on the label for algae collected in sample **NIW-145-AM-s-3** would read **NIW-145-AM-s-3-AG**

Each preserved SC is recorded in a hand-written or electronic *Collection Register* that is later transferred to the *Sorted Collection Inventory* (an Excel template provided by the NIWA Marine Invasives Taxonomic Service). This provides a record of all SCs that leave the field laboratory, allowing easy tracking of SCs from field laboratory to MITS.

5. Processing

1. **Initial.** Process samples within 1–2 hours of collection. Process one sample at a time (or one per person) to minimize the possibility of mixing specimens from different samples.

2. **Check for label.** If there is no legible label in the sample, the collection cannot be used and should be discarded). Replacement collections may be necessary.

3. **Sieve.** Empty collection into a sieve (1 mm mesh) and drain all water.

4. **Weigh if necessary.** If wet weights of samples are required (e.g., for hull fouling projects), blot excess free water with paper towel, weigh and record wet weight of whole sample.

5. **Sort collection.** Empty the whole collection into a shallow tray of fresh seawater. SORT THE ENTIRE COLLECTION into the taxonomic groups listed in Table 3, placing specimens from each taxon into appropriately-sized containers (don't need massive jars for tiny organisms but see Step 9 below). Keep all specimens of each group except where there are several individuals of large species. In such cases, some specimens may be discarded but use considerable care to avoid discarding similar but different species. Decisions on discarding should be made only by an experienced invertebrate zoologist. If in doubt, keep all specimens.

6. **Images of collections/specimens.** If images are required, each should include a scale bar and the SC Number within the image. Be sure to create a record of the image file number associating it with the SC Number, in case the label is not legible within the image.

7. **Label each group.** Make a new label for each taxonomic group and place it in the container. Ensure that each sorted collection (i.e., set of specimens from one collection at one station that has been sorted into one taxonomic group) is appropriately labelled using the codes described above and listed in Table 3.

8. **Relaxing/narcotising** (some groups only, Table 3). For groups that must be relaxed before fixing, keep specimens in a shallow container of fresh seawater, preferably shaded and cool. Keep them alive with as little stress as possible, so that they relax and begin to behave fairly normally. Start the relaxing process once you separate the first specimen to minimize delays in fully narcotising all individuals. Even during relaxing, ensure that a label is placed IN each container to minimize possible confusion over their origin.

9. **Check container size and add preservative.** After sorting the entire collection, re-assess the container size required for each Sorted Collection. Each Sorted Collection should be placed in at least 5 times its own volume of preservative so

that water in the specimens' tissues does not dilute the preservative sufficiently as to allow tissue degradation. Change the container as necessary. Check that the label is in the container, then fill with the appropriate preservative (Table 3). Cap firmly and store up-right.

10. **Record in Sorted Collections Inventory.** After all samples are processed, record all sorted collections in the Sorted Collections Inventory. Direct entry into the Excel Sorted Collections Inventory may be the best approach. This inventory allows us to track: (a) the numbers of collections, origins and shipping dates to MITS (if a shipment goes astray, we will know exactly what was lost); (b) collection statistics and all aspects of the collection details (i.e., key data for MPI reports, and for managing the whole process); and (c) the approximate time required to identify collections from each project (for planning and reporting).

11. **Prepare samples for shipping.** For each port or field survey, group samples by major taxon (e.g., molluscs, crustaceans, polychaetes, etc). Pack containers in plastic bags (including absorbent packaging to minimize damage caused by any leakage), then into larger, tougher plastic bags, buckets or plastic bins. Seal properly.

12. **Ship to MITS.** Address to: NIWA Marine Invasives Taxonomic Service, Greta Point, 301 Evans Bay Parade, Kilbirnie, Wellington; attention Serena Wilkens/Sarah Allen. Be responsible and use an appropriate freighting company (e.g., Chem Couriers) to ship these hazardous collections.

13. **Email completed Sorted Collections Inventory to MITS** (Sarah Allen: s.allen@niwa.co.nz), advising of the full details of the shipment sent and its expected arrival date.

Note: It is the shipper's responsibility to ensure delivery of each shipment to MITS. The MITS team will endeavour to share this responsibility, but you should follow up to ensure that shipments have arrived. If they do not arrive by the predicted time, contact the courier requesting urgent location and delivery of the collections, and advise MITS of your attempts and responses at each step. If a shipment is declared lost or severely damaged, this must be reported to MITS and MPI, to facilitate contingency plans and/or adjustments to reporting requirements.

Taxon	Sorting groups	Taxon Code	Relax before fixing	Fixative and/or preservative	Conc.
Algae	Algae	AG	NA	Formalin **	5%
Ascidians	Colonial ascidians	AN	Yes	Formalin	10%
	Solitary ascidians	AN	Yes	Ethanol	70%
Bryozoa	Bryozoa	BR	No	Ethanol	70%
Crustacea	Amphipods	AM	No	Ethanol	70%
	Barnacles	BN	No	Ethanol	70%
	Crabs	CB	No	Ethanol	70%
	Other decapods	DP	No	Ethanol	70%
	Isopods	IS	No	Ethanol	70%
	Ostracods	OS	No	Ethanol	70%
	Tanaids	TN	No	Ethanol	70%
Cnidaria	Ctenophores	CN	No	Formalin	10%
	Hydroids	HY	No	Formalin	10%
	Hard corals	HC	No	Ethanol	70%
	Sea anemones	SN	Yes	Formalin	10%
	Soft corals	SF	Yes	Formalin*	10%
	Jellyfish	JF	Yes	Formalin	10%
Dinoflagellate cysts	Dinoflagellates	CY	No	Refrigerate	NA
Echinoderms	Brittle stars	BS	No	Ethanol	70%
	Echinoids	EC	No	Ethanol	70%
	Holothurians	HT	Yes	Ethanol	70%
	Sea stars	SS	No	Ethanol	70%
Fishes	Fishes	FH	No	Formalin	10%
Molluscs	Bivalves	BV	No	Ethanol	70%
	Gastropods	GP	No	Ethanol	70%
	Other molluscs (shell)	MU	No	Ethanol	70%
	Other molluscs (no shell)	MU	Yes	Formalin*	10%
	Polyplacophorans/chitons	PO	No	Ethanol	70%
	Opisthobranchs (no shell)	OB	Yes	Formalin*	10%
Pycongonids	Pycongonids	PY	No	Ethanol	70%
Sponges	Sponges	SP	No	Ethanol	70%
Flatworms	Flatworms	FW	Yes	Formalin	10%
Annelid worms	Annelid worms	WM	No	Formalin	10%
Nemerteans	Nemertean worms	NT	Yes	Formalin	10%
Sipunculans	Sipunculan worms	SI	Yes	Formalin	10%
Phytoplankton		PP	No	Seawater	Seawater
Zooplankton		ZP	No	Formalin	5%
Washings		WH	No	Formalin	10%

Taxon	Sorting groups	Taxon Code	Relax before fixing	Fixative and/or preservative	Conc.
Sediment		ST	No	Freeze	NA
Unknown	Unknown	UK	No	Formalin	10%

Table 3. Taxonomic groups into which all collections should be sorted, their taxon code for labelling, and fixation requirements. *, transfer into 70% ethanol within 1–4 days; **, except *Grateloupia* – press in nappy liner and place in silica gel to dehydrate. NA = not applicable.

6. Delivery Address for Samples

Sarah Allen
NIWA
301 Evans Bay Parade
Greta Point
Wellington
Phone (04) 386 0300 ext 7364

Appendix C Table of full range of biofouling species identified for the three deployments.

Phylum	Genus/species	Biosecurity status
Porifera	*Amphilectus fucorum*	**Non-Indigenous**
	Leucosolenia echinata	Indigenous
	Sycon pedicellatum	Indigenous
	Sycon ornatum	Indigenous
Annelida	*Neanthes kerguelensis*	Cryptogenic
	Nicolea maxima	Indigenous
	Harmothoe macrolepidota	Indigenous
	Galeolaria hystrix	Indigenous
	Spirobranchus cariniferus	Indigenous
	Hydroides ezoensis	Cryptogenic
	Hydroides elegans	Cryptogenic
	Filograna implexa	Indigenous
	Sabella spallanzanii	**Non-Indigenous**
Bryozoa	*Amathia verticillata*	**Non-Indigenous**
	Bugula neritina	**Non-Indigenous**
	Celleporaria umbonatoidea	**Non-Indigenous**
	Celleporaria nodulosa	**Non-Indigenous**
	Bugulina flabellata	**Non-Indigenous**
	Watersipora subatra	**Non-Indigenous**
	Tricallaria inopinata	**Non-Indigenous**
	Hastingsia whitteni	Indigenous
	Disporella novaeholloandiae	Indigenous
	Favosipora candida	Indigenous
	Gregarinidra sp.	Indigenous
	Schizoperlla japonica	**Non-Indigenous**
Crustacea	*Balanus trigonus*	Indigenous
	Austrominius modestus	Indigenous
Mollusca	*Pluerobranchaea maculata*	Indigenous
	Crassostrea gigas	**Non-Indigenous**
	Mytilus galloprovincialis	Indigenous
	Perna canaliculus	Indigenous
	Monia zelandica	Indigenous
Heterokontophyta	*Hormosira banksii*	Indigenous
	Colpomenia sinuosa	Indigenous
	Bryopsis sp	Indigenous
Urochordata	*Styela plicata*	Cryptogenic
	Asterocarpa humilis	Cryptogenic
	Botryllus schlosseri	**Non-Indigenous**
	Molgula mortenseni	Indigenous
	Botrylloides leachii	**Non-Indigenous**

Phylum	Genus/species	Biosecurity status
	Styela clava	**Non-Indigenous**
	Corella eumyota	Indigenous
	Ascidiella aspersa	**Non-Indigenous**
	Didemnum incanum	Indigenous
	Ciona intestinalis	**Non-Indigenous**
	Molgula mortenseni	Indigenous
	Molgula manhattensis	**Non-Indigenous**
	Cnemidocarpa nisiotus	Indigenous
	Botrylloides gigantium	**Non-Indigenous**
	Lissoclinum notti	Indigenous
	Symplegma brakenhielmi	**Non-Indigenous**
	Diplosoma listerianum	Cryptogenic
	Cnemidocarpa sp.	*Indeterminate*
	Aplidium sp.	*Indeterminate*
Cnidaria	*Anthothoe albocinta*	Indigenous
	Ectopleura sp	Cryptogenic
Chlorophyta	*Ulva sp*	Indigenous
	Cladophora sp.	Indigenous
	Chaetomorpha sp.	Indigenous
Indeterminate	Filamentous brown	*Indeterminate*
	Ciliophora	*Indeterminate*
	Filamentous red	*Indeterminate*