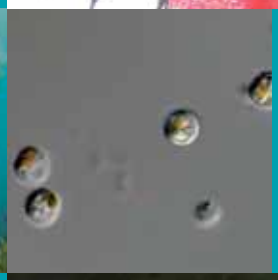



Natural Heritage Trust

*Helping Communities Helping Australia*

An Australian Government Initiative

March 2007

# Empirical validation: Small vessel translocation of key threatening species

## Stage II – Undaria pinnatifida


Prepared by:

Keith R. Hayes, Rasanthi Gunasekera, Jawahar Patil, Cath Sliwa, Sasha Migus, Felicity McEnnulty, Piers Dunstan, Mark Green, and Caroline Sutton



Final Report for the **Australian Government** Natural Heritage Trust (Project No. 46630) by **CSIRO Marine and Atmospheric Research**

CSIRO

# Empirical validation: Small vessel translocation of key threatening species

## Stage II – Undaria pinnatifida

Keith R. Hayes, Rasanthi Gunasekera, Jawahar Patil, Cath Sliwa, Sasha Migus, Felicity McEnnulty, Piers Dunstan, Mark Green, and Caroline Sutton

Australian Government Natural Heritage Trust Project 46630

Final Report, revised February 2007

Hayes, Keith R., 1968- .
Empirical validation : small vessel translocation of key threatening species. Stage ii, Undaria pinnatifida.

ISBN 9781921232534 (pdf).

1. Plant translocation - Australia. 2. Plant introduction - Australia. 3. Invasive plants - Ecology - Australia. 4. Undaria pinnatifida - Ecology - Australia. 5. Fouling organisms - Ecology - Australia. 6. Ships - Fouling - Australia. I. CSIRO. Marine and Atmospheric Research. II. Title.

579.8817062

This Report documents work carried out under Project 46630 of the Australian Government's Natural Heritage Trust.

Enquiries should be addressed to:

K.R. Hayes
CSIRO Division of Marine and Atmospheric Research
GPO Box 1538, Hobart, Tasmania, 7001
+61 3 62325260
+61 3 62325485
keith.hayes@csiro.au

## Distribution list

| | |
|---|---|
| Project Manager | |
| On-line approval to publish | |
| Client | |
| Authors | |
| Other CSIRO Staff | |
| National Library | |
| State Library | |
| CMAR Library as pdf (Meredith Hepburn) | |
| CMAR Web Manager as pdf (Diana Reale) | |

## Important Notice



## Use of this Report

# CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

# EXECUTIVE SUMMARY

This document is the final report of the second of two one-year projects designed to collect quantitative information on small vessel biofouling. Both projects support the development and implementation of the new National System for the Prevention and Management of Marine Pests in Australia, with particular emphasis on *Asterias amurensis* (Stage I) and *Undaria pinnatifida* (Stage II) – this report.

The four primary aims of Stage II of this project were: a) to develop a new genetic probe for *Undaria pinnatifida*, b) to sample the internal and external spaces and surfaces of small vessels to determine their translocation potential using the genetic probes for *Asterias amurensis* and *U. pinnatifida*; c) develop a Bayesian journey survival model to estimate the probability of target species survival on small vessels; and, d) develop quantitative risk factors for fouling biomass based on vessel activity, paint type and management practices. The Bayesian journey survival model, however, could not be developed because the project team were unable to take successive, daily, hull fouling or internal water samples from any of the sample vessels. A fifth Stage II objective – to empirically verify the results of the Infection Modes and Effects Analysis – has also been carried over from Stage I of the project.

The development of a gene probe specific to *Undaria pinnatifida* took much longer to develop than expected, primarily because the initial target – 602 nucleotides of the Large Sub Unit ribosomal DNA (LSU rDNA) – was identical (or nearly so) for at least two other native species: *Ecklonia radiata* and *Macrocystis pyrifera*. The project team therefore sought other regions of the *U. pinnatifida* genome for inter-specific variation, eventually settling on the RuBisCo spacer region and the rDNA internal transcribed spacer regions (ITS1 & ITS2).

Probes designed around two regions of significant variation in the RuBisCo spacer region proved specific to all samples of the non-native test species in the *Undaria* genus (*Undaria pinnatifida* and *Undaria.undarioides*) but also returned positive PCR results when tested against two other non-native Laminariales species: *Lessonia nigrescens* and *Alaria esculenta*. The utility of this probe was further undermined by its ambiguous signature on agarose gel. It is therefore unlikely to prove effective without further laboratory refinements. Probes designed around sequences of the ITS spacer region were also specific to all samples of non-native test species in the *Undaria* genus. This probe did give unexpectedly positive PCR results for three species outside this genus, but in all cases subsequent sequencing confirmed that these results were caused by contamination of the samples with *Undaria pinnatifida* DNA at the time of DNA extraction.

The ITS probes were subsequently applied to 430 hull fouling samples and 69 plankton samples collected during Stage II of the project. All of the plankton samples, bar one, taken during August and November returned positive for *Undaria*. These results add further weight to other independent observations of *Undaria pinnatifida* zoospores in the plankton at this time of year, but also act to mask any patterns in the high proportion of hull fouling samples that returned positive.

The project team sampled 54 vessels at 5 locations during the course of Stage I and Stage II, taking 1116 samples, and making further 365 inspections, in 63 different locations around the hull, internal spaces, deck and fishing gear. The distribution of bio-fouling is quite

different between locations. Many of the locations, on many occasions, were clean, or nearly so, returning samples that weighed less than the reliable limit of a wet weight sample (1.5 grams). The large number of samples in this category, together with inspections that resulted in no sample (i.e. the inspected area was clean and dry), gives rise to a zero-inflated dataset.

The wet weight of the more substantially fouled samples can be approximately described by a log-normal distribution, although in some cases (such as the hull quadrats) rare incidences of very heavily fouled vessels results in a distribution with greater probability mass in the right tail. For all the very heavily fouled vessels the management and use patterns of these vessels are atypical, bordering on derelict – i.e. left on a mooring for periods of a year or more without being used. These types of vessels clearly pose an unacceptable translocation risk. They, however, easily identified as a distinct sub-set of small craft and can therefore be managed separately from the vast majority of small pleasure craft and fishing vessels.

The majority of vessels that we inspected and sampled were cleaned and slipped at least once a year, and used at least once every one to two weeks. Some of these vessels returned positive results when tested with the *Undaria* (and *Asterias*) gene probes. These results suggest well maintained small craft can still act as a possible vector for the translocation of marine pests. Seawater held in the internal water systems of vessels that tested positive for *Undaria*, for example, indicates that vessels that travel with closed seacocks from an area where *Undaria* is present in the water column could potentially transfer "contaminated" water to new areas.

The wet weight of biofouling on the wet areas (below the water line) of these regularly used vessels can be approximately described by a delta distribution – i.e. a log-normal distribution with a higher than usual probability mass at zero, or in this case near zero. An Analysis of Covariance model confirms that the number of days since the vessel was last cleaned, and the median number of trips per annum, are statistically significant explanatory variable of the bio-fouling wet weight of the "non-zero" samples. The effect of the vessel activity, however, is strongly mediated by the type of antifouling paint used by the vessel. This effect is strongest when the vessel is painted with an ablative paints, is relatively weak when the vessel is painted with a self-polishing paint and totally eliminated if the vessel is painted with a hard paint. These results highlight the importance of regular maintenance, including the use of anti fouling paints that are appropriate to the vessel use patterns, in minimising the translocation potential of recreational vessels.

The quantitative samples taken during this project also confirmed that, by and large, the severity and occurrence components of the IMEA analysis provide a reasonable indication of the level of biofouling in any given part of the vessel. Many parts of the vessel, however, were systematically underrated in the analysis, such as the rudder surface, propeller, stabilisers/trim tabs, garboard plank and block spaces. A few parts of the vessel were systematically overrated in the analysis, such as the bob-stay fitting. In some instances these errors can be explained by misinterpretation of vessel components (e.g. bob-stay fitting) or by very different use patterns between vessels (e.g. propellers that are constantly used versus intermittently used). On other occasions, however, it appears as if participants at the IMEA workshops (completed three years ago in collaboration with the Victorian Department of Natural Resources and Environment) simply underestimated how the biofouling hazards of some parts of a boat relative to others (e.g. the garboard plank).

# 1. INTRODUCTION

## 1.1 Background

Introductions of non-indigenous species (NIS) are a serious threat to global biodiversity (Baltz, 1991) and have been occurring in the marine environment since humans started exploring the world's oceans. The transport and introduction of organisms throughout the world's oceans is happening at an unprecedented rate (Walford and Wicklund, 1973; Carlton, 1985; 1995). A number of anthropogenic vectors are, or have been, responsible for spreading marine organisms beyond natural bio-geographic boundaries (Carlton, 2001). The vectors responsible for the introduction of marine organisms vary with time and with geographical region. In Australia, hull fouling, accidental release or translocation associated with mariculture (predominately oysters) and the ballast water discharges of large commercial vessels are the most prominent sources of marine NIS (Thresher et al., 1999).

In addition to large commercial vessels, it is becoming increasingly clear that small craft, including yachts and fishing vessels, are capable of introducing NIS to new sites and contributing to their subsequent spread. A well known recent example is the introduction of the black-striped mussel *Mytilopsis* sp. to Darwin in 1999 (Willan et al., 2000). It is almost certain that this species arrived on the hull of an ocean-going yacht, and spread to nearby marinas by other local yachts. Adult *Mytilopsis* mussels have subsequently been found on the hull and in the seawater piping of Indonesian fishing vessels and other ocean-going yachts (*pers. comm*. A. Marshall, Northern Territory Aquatic Pests Program). Similarly, the spread of *Undaria pinnatifida* around the south-east and east coasts of Tasmania is in part attributed to the movement of the vessels of recreational and commercial fishers and divers.

This project is the second of two one-year projects designed to collect quantitative information on small vessel biofouling. Both projects support the development and implementation of the new National System for the Prevention and Management of Marine Pests in Australia, with particular emphasis on *Asterias amurensis* (Stage I) and *Undaria pinnatifida* (Stage II). The sampling strategy for both projects was based on the results of an Infection Modes and Effects Analysis (IMEA) completed three years ago in collaboration with the Victorian Department of Natural Resources and Environment (Hayes, 2002). The IMEA identified (and ranked) locations on fishing vessels and recreational vessels that might be capable of transporting marine pests. The results were used to identify potential "hot-spots" on small vessels that were subsequently sampled in the two projects.

The first project (Stage I), sampled 30 vessels, at two Tasmanian locations (Royal Hobart Yacht Club and the Domain Slipway) between the 12th August 2003 and the 11th June 2004. A total of 750 samples were taken. A further 204 inspections were made on board the vessels that resulted in no sample being taken. An additional 120 estuarine plankton samples – 4 for each vessel sampled – were also taken at the survey sites (Hayes et al., 2004). In most cases the wet weight of the samples collected in Stage I supported the results of the IMEA. Notable exceptions include the garboard plank (which had much higher levels of fouling than suggested by the analysis), the block spaces and other small niche areas on the hull (which had much

lower levels of fouling than suggested by the analysis) and the rudder surface (which had higher levels of fouling than predicted).

Most of the vessels inspected in the first project were well maintained and relatively free of biofouling. There were, however, rare occurrences of very heavily fouled vessels. This pattern gives rise to zero inflated, positively skewed data that may be well described by a delta distribution. One of the aims of the second project is to model the effect of potential explanatory co-variates (such as vessel cleaning and activity patterns) on the biomass of small vessel biofouling. A preliminary analysis of the results from the first project suggested that significant hull fouling (> 50 grams per 0.5$m^2$) is likely to develop on anti-fouled vessels after approximately 400 days.

All of the 750 vessel samples and 120 plankton samples collected in Stage I were processed and probed using a gene probe designed to detect the presence of species in the *Asterias* genus (Deagle et al., 2003). The proportion of positive plankton samples reflected the reproductive cycle of *Asterias amurensis* in the Derwent estuary: a small window between the 3rd of February and the 18th of March returned no positive plankton samples. This is consistent with previous data on the life-cycle of *A. amurensis* in the Derwent. Samples taken on the 23rd of April (boat 25) were also negative but thereafter, from the 28th of April onwards, one or more of the plankton samples tested positive. This suggests that the *A. amurensis* may spawn in the Derwent in mid-to late April, approximately two months earlier than previously reported (Sutton and Bruce, 1996).

Five Stage I vessels – 6, 18, 19, 20 and 25 - returned *Asterias* positive samples with negative plankton samples. Two of these vessels (6 and 25) do not provide strong evidence for settlement because the sample dates on both occasions (1st of October and 23rd of April) are within, or at least close to, the spawning season of *Asterias amurensis*, and hence the positive sample may simply reflect the presence of larvae or gametes in the seawater associated with the sample. The results from the three remaining vessels, however, sampled contiguously between the 3rd of February and the 25th, provided the first strong evidence of *Asterias* settlement on small recreational and commercial vessels. The plankton samples during this period were consistently negative, suggesting that the positive result is not due to contamination of the sample by larvae or gametes in the associated seawater.

## 1.2 Aims and objectives

This project (Stage II) has four main objectives:

1. develop a genetic probe for *Undaria pinnatifida*, and apply it to samples obtained from small vessels (recreational yachts, commercial fishing vessels) to determine the presence or absence of this key threatening species, with a low probability of Type II error;

2. sample the hull fouling of small vessels during the 2004 spawning season of *Undaria pinnatifida* using the results of the IMEA to target potential “hot spots”;

3. develop a Bayesian journey survival model to estimate probability of survival as a function of vector dynamics and journey duration based on (1) and (2) to the extent allowable by the data[1]; and,

4. develop quantitative risk factors for fouling biomass based on vessel activity, paint type and management practices that assist in the development and/or implementation of new national protocols designed to minimise the bio-invasion risks associated with small craft.

The objectives of the project were modified slightly subsequent to the submission of the Stage I proposal to reflect and support the development of the new National System for the Prevention and Management of Marine Pests in Australia. In particular the fourth objective was added to assist in the development and implementation of the national protocols for the management of bio-invasion risks associated with recreational vessels and small fishing vessel.

The Stage II deliverables listed in the project proposal are:

1. An optimised gene probe for *Undaria pinnatifida* that can be applied to plankton and hull fouling samples to determine the presence or absence of the species.;

2. A quantified estimate of the translocation potential of various internal and external spaces and surfaces on fishing vessels, recreational vessels and aquaculture equipment. These results will provide:

    a. empirical verification of the predictions of the Infection Modes and Effects Analysis;

    b. vessel infection estimates to support bio-invasion risk assessment and management strategy evaluation for preventing and minimising the spread of *Asterias amurensis* and *Undaria pinnatifida* in southeast Australia; and,

    c. data to support AQIS vessel inspection protocols and education campaigns for recreational boatmen, fishermen and aquaculture operators.

3. A Bayesian journey survival model for *Asterias amurensis* and/or *Undaria pinnatifida* to support bioinvasion risk assessment and management strategy evaluation.

## 1.3 Project milestones

The project milestones and delivery dates are summarised in Table 1 This report addresses milestones 1 to 6 to the extent allowable by the data.

[1] The delivery of this model is contingent on our ability to track vessels over times. The models cannot be developed without a sufficient number of positive samples, collected continuously over a period of at least two or three days.

Table 1 Empirical Validation – Stage II: Project milestones

| Milestone | Conditions to be met for payment to proceed | Date due |
|---|---|---|
| Milestone 1 | Contracts signed by all parties | 29 February 2004 |
| Milestones 2, 4 and 5 | First progress report against the milestones that:<br>1. states that the *Undaria pinnatifida* probe has been developed<br>2. provides a detailed sampling and analysis plan for the fieldwork<br>3. progress on sampling Stage 1 and II<br>4. includes a description of Stages I and II of the empirical validation project that is suitable for presentation to NIMPCG | 1 June 2004 |
| Milestones 2, 3, 4, 5 | Second progress report against the milestones that:<br>1. outlines the progress of the fieldwork to date<br>2. describes the effectiveness and any problems encountered with the species specific probe<br>3. provides a work plan for the analysis of the collected samples | 30 September 2004 |
| Milestone 4 | Notification to DEH that all field work has been completed. | 30 November 2004 |
| Milestone 5 | Notification to DEH that all analysis has been completed. | 29 April 2005 |
| Milestones 1, 2, 3 4, 5, 6 | Final report addressing all milestones and that includes<br>1. relevant components of the risk assessment of the translocation potential for *Undaria pinnatifida* posed by small craft and through the aquaculture industry;<br>2. models to assess the likelihood of transmission by vectors to the extent allowable by the data of *Undaria pinnatifida*;<br>3. data to support possible future education campaigns for small vessel operators and the aquaculture industry to reduce the risk of *Undaria pinnatifida* translocation by these means, and an indication of the implications of these data .<br>4. an assessment of the implications of the findings of the project for national control and management of *Undaria pinnatifida* and for control of established introduced marine pests generally, for presentation to the National Introduced Marine Pests Co-ordination Group. | 30 May 2005 |

# 2. UNDARIA PINNATIFIDA PROBE DEVELOPMENT AND VERIFICATION

## 2.1 Background

Stemming from the successful development of a genetic probe to detect a microalga - the toxic dinoflagellate *Gymnodinium catenatum* (Patil et al., 2005), we initially targeted both small subunit (SSU) and large subunit (LSU) ribosomal DNA (rDNA) loci to develop a gene probe to detect the macroalga *Undaria pinnatifida*. The existence of hundreds of copies of these ribosomal genes in the nuclear genome of eukaryotes makes them nearly as abundant as mitochondrial DNA (mtDNA) and therefore abundance was not expected to compromise detection levels. Limited sequence variance at this loci between the members of the Order Laminariales (kelps – large brown seaweeds), however, prompted us to examine the RuBisCo spacer region and the ribosomal internal transcribed spacer region (ITS) as potential alternative targets for gene probe development. The RuBisCO spacer region has been previously used to investigate the phylogenetic relationships between members of the Laminariales (Yoon and Boo, 1999; Yoon et al., 2001). Similarly the ribosomal ITS region has been studied extensively in the context of Laminariales phylogenetics (Yoon et al., 2001; Saunders and Druehl, 1993). This phylogenetic scrutiny has generated relatively large DNA sequence information at the target regions for several species within the Laminariales..

This chapter summarises the systematic assessment of three target loci/regions during the development of *Undaria pinnatifida* specific probes. Two of these probes may be deployed to assess the risk associated with translocation of the genus *Undaria* by ballast water and hull fouling in Australia.

## 2.2 Macro algal sample collection and DNA extraction

A total 126 samples were successfully procured or sourced (freshly collected, frozen, silica-dried or as extracted DNA samples) from specimens of *Undaria pinnatifida* (n=59) and other related species of macroalgae of the Order Laminariales. Appendix 1 summarises the specimens, collection location and sample codes. Fresh collections were made by CSIRO Marine Research (CMR) scuba divers, while the frozen, silica-dried or extracted DNA samples were obtained from researchers in other temperate locations of the world.

Genomic DNA was extracted from frozen or dried samples using DNeasy plant mini kit (QIAGEN) following supplier's instructions but with some modifications. About 10-50 mg of tissue was taken from blade or midrib area. The samples were then frozen in liquid nitrogen and ground to fine powder using a mortar and pestle before DNA extraction. Due to difficulties encountered in extracting good quality DNA from most macroalgae, the amount of tissue sample used for extraction was reduced to at least half of the recommended (100mg) and buffer volumes were increased by about 2 fold. DNA templates were quantified and diluted 1:100 - 1:500 times before amplification. Samples that were received as DNA from other researchers were diluted as per the instructions of the sender or as appropriate for PCR reaction.

A PCR reaction was carried out on all samples using universal nuclear 18S ribosomal DNA primers (Table 2; NSF1179 and NSR 1642) to confirm suitability of each DNA sample for PCR. Extended annealing and extension times were used. Thermal cycling conditions for 18S universal primers were: one initial cycle with denaturation at $94^0$C for 4 min, annealing at $54^0$C for 2 mins and extension at $72^o$C for 2 mins, followed by 35 cycles with 1 min at $94^0$C, 1 min at $54^0$C and 1 min at $72^0$C and a final elongation at $72^0$C for 4 mins. Standard PCR reactions were done in 25 µl volume. The reaction cocktail contained 0.2 µM of each primer, 0.2 mM dNTPs, 2 mM MgCl2, 1X AmpliTaq Gold® buffer, and 0.625 units AmpliTaq Gold® (Applied Biosystems). Aerosol-resistant pipette tips were used with all PCR solutions and negative control reactions were performed with each PCR cocktail.

Table 2 Sequences of 18S primers used

| Name | Gene | Sequence (5'-3') | Application | Reference |
|---|---|---|---|---|
| NSF 1179 | 18S rDNA | AATTTGACTCAACACGGG | PCR –Universal positive control | Wuyts et al., 2001 |
| NSR 1642 | 18S rDNA | GCGACGGGCGGTGTGTAC | PCR –Universal positive control | Wuyts et al., 2001 |

## 2.3 Sequencing and analysis

PCR products were purified using the QIAquick PCR purification kit (QIAGEN). Sequencing reactions were carried out on both strands, using the universal amplification primers in targeted gene probe areas, with the ABI Big Dye dideoxy terminator cycle sequencing kit (Applied Biosystems). Electrophoresis was carried out on an ABI-3100 automated DNA sequencer and sequence data were edited with Sequence Navigator software (Applied Biosystems). Sequence data were aligned using CLUSTAL_X (Thompson et al., 1997). These sequences along with additional sequences from GenBank were used to assess the level of variation in targeted gene regions within *Undaria pinnatifida* and between this species and other species of the Family Alariaceae (of which *U. pinnatifida* is a member), as well as species in the Families Laminariacea and Lessoniaceae within the Order Laminariales. Note that *Undariella peterseniana* is also known *as Undaria peterseniana* in the literature.

## 2.4 Probe design and testing

Three target loci/regions were examined to develop *U. pinnatifida* specific DNA probes: the ribosomal rDNA LSU, the RuBisCo spacer region and the ribosomal rDNA internal transcribed region (rDNA ITS1 and ITS2**)**. The details of the methodology are described separately for all the three loci.

### 2.4.1 Large sub-unit ribosomal DNA

The nuclear large sub-unit ribosomal DNA (LSU rDNA) was identified as a potential target to develop *Undaria pinnatifida* specific molecular probes because this region was successfully

used in the past to develop probes for the toxic dinoflagellate *Gymnodinium catenatum* (Patil *et al*., 2005).

There is very limited sequence information (in the public web-based GenBank database) on LSU rDNA from different strains of *U. pinnatifida* and its closely related species. This loci was therefore amplified from 59 individuals of *U. pinnatifida* and from 22 individuals of closely related species of the Laminariales. Amplification and sequencing of the nuclear LSU rDNA was carried out using the universal primers C'1 and D2 (see Table 3 for sequence and references). A standard PCR reaction cocktail (Section 2.2) was used with all primer combinations. Thermal cycling conditions for C'1 and D2 universal primers were: one initial cycle with denaturation at 94°C for 4 min, annealing at 58°C for 2 mins and extension at 72°C for 2 mins, followed by 35 cycles with 1 min at 94°C, 1 min at 58°C and 1 min at 72°C and a final elongation at 72°C for 4 mins. Samples were sequenced according to the protocol given in Section 2.3.

Table 3 Sequences of primers used in LSU rDNA region

| Name | Gene | Sequence (5'-3') | Application | Reference |
|---|---|---|---|---|
| C'1 | LSU-rDNA | ACCCGCTGATTTAAGCAT | Universal-PCR & sequencing | Rousseau et al 1997 |
| D2 | LSU- rDNA | TCCGTGTTTCAAGACGG | Universal-PCR & sequencing | Rousseau et al 1997 |
| UP_LSU2F | LSU- rDNA | CGTTCTTGCCCCACGGCAGC | "PCR-specific primers" | This study |
| UP_LSU2R | LSU- rDNA | GGGTCTTCACCCTCCGAAGAG | "PCR-specific primers" | This study |
| UP_LSU3R | LSU-rDNA | CTCCGAAGAGAGGCTACCTTCCC | "PCR-specific primers" | This study |

The generated sequences were aligned with those available on the public database (Appendix 2). Two regions with significant inter-specific variation were identified as potentially suitable for developing *Undaria pinnatifida* specific gene probes. In all three primers were designed, one forward and two reverse (Table 3). The primers were then tested for their specificity on all samples of *U. pinnatifida* and the other closely related species listed in Appendix 1. PCR amplifications for the potentially specific primers (UP_LSU2F, UP_LSU2R and UP_LSU3R) were carried out under the following conditions; one initial cycle with denaturation at 94°C for 4 min, annealing at 64°C for 2 mins and extension at 72°C for 2 mins, followed by 35 cycles with 1 min at 94°C, 1 min at 64°C and 1 min at 72°C and a final elongation at 72°C for 4 mins.

### 2.4.2 RuBisCo spacer region

The RuBisCO spacer region was evaluated as another potential target for developing *Undaria pinnatifida* specific gene probes. The DNA sequences available in the public database were quickly aligned with in-house generated sequences. Amplification of the RuBisCo spacer region was carried out using the primers RS1 and RS2 (Table 4). A standard PCR reaction cocktail

(Section 2.2) was used with all primer combinations. Thermal cycling conditions for the RS1 and RS2 universal primers were: one initial cycle with denaturation at 94°C for 4 min, annealing at 45°C for 2 mins and extension at 72°C for 2 mins, followed by 35 cycles with 1 min at 94°C, 1 min at 45°C and 1 min at 72°C and a final elongation at 72°C for 4 mins. Samples were sequenced according to the protocol given in Section 2.3.

Table 4 Sequences of primers used in RuBisCo spacer region

| Name | Gene | Sequence (5'-3') | Application | Reference |
|---|---|---|---|---|
| RS1 | RuBisCo spacer | GCCAAATGCACCAACTTCTT | Universal-PCR | Yoon & Boo 1999 |
| RS2 | RuBisCo spacer | AGACCCCATAATTCCC | Universal-PCR | Yoon & Boo 1999 |
| UP_RBCsp_1F | RuBisCo spacer | ATACTGAAATTAAACTCATAAAAGATTAAG | PCR-Specific primers | This study |
| UP_RBCsp_1R | RuBisCo spacer | AGTTAAGTCTTAATTTTATAACAATATCAC | PCR-Specific primers | This study |
| UP_RBCsp_2R | RuBisCo spacer | TTTAAAGTTAAGTCTTAATTTTATAACAAT | PCR-Specific primers | This study |
| UP_RBCsp_3R | RuBisCo spacer | ATAACAATATCACTTTAAATAATAAAGTAG | PCR-Specific primers | This study |

One forward and three reverse primers were designed and synthesised to serve as *Undaria pinnatifida* specific probes (Table 4) based on the sequence alignment (Appendix 3). Preliminary amplification suggested the primer pair UP_RBCsp_1F and UP_RBCsp_1R to be more specific. The reaction conditions were first optimised adopting a gradient PCR protocol. The identified primer pairs were then tested on all the samples listed in Appendix 1. Thermal cycling conditions for the RuBisCo spacer specific primers (UP_RBCsp_1F and UP_RBCsp_1R) were: one initial cycle with denaturation at 94°C for 4 min, annealing at 61°C for 2 mins and extension at 72°C for 2 mins, followed by 35 cycles with 1 min at 94°C, 1 min at 61°C and 1 min at 72°C and a final elongation at 72°C for 4 mins.

### 2.4.3 The ribosomal rDNA internal transcribed spacer regions

The ribosomal rDNA internal transcribed spacer regions (ITS1 and ITS2) were evaluated as a third potential target for *Undaria pinnatifida* specific probes. The entire region spanning the ITS1, 5.8S rDNA and ITS2, including partial 3' end of SSU and 5' end of LSU was amplified with primers LB1 and LB2 (Table 5) from 59 individuals of *U. pinnatifida* sourced from different regions of the world. A standard PCR reaction cocktail (Section 2.2) was used with all primer combinations. Thermal cycling conditions for the LB1 and LB2 universal primers were: one initial cycle with denaturation at 94°C for 4 min, annealing at 55°C for 2 mins and

extension at 72°C for 2 mins, followed by 35 cycles with 1 min at 94°C, 1 min at 55°C and 2 min at 72°C and a final elongation at 72°C for 10 mins. Samples were sequenced according to the protocol given in Section 2.3.

Sequencing proved to be difficult because the amplified products were large and had long nucleotide repeats. We therefore had to employ additional primers (2.4; YB1 and BC2), along with the universal LB1 and LB2 primers, to achieve an accurate sequence. This results in more than 228 sequence reactions for *Undaria pinnatifida* alone. The entire c1000bp region was eventually sequenced and aligned with those available in the public database (Appendix 4). Two forward and one reverse potentially specific primers were designed and synthesised from these alignments (Table 5)

Table 5 Sequences of primers used in ribosomal Internal Transcribed Spacer region

| Name | Gene | Sequence (5'-3') | Application | Reference |
|---|---|---|---|---|
| LB1 | SSU - rDNA | CGCGAGTCATCAGCTCGCATT | Universal-PCR & sequencing | Yoon et al 2001 |
| LB2 | LSU - rDNA | AGCTTCACTCGCCGTACTGG | Universal-PCR & sequencing | Yoon et al 2001 |
| YB1 | 5.8S - rDNA | TTGCAGAATCCAGTGAATCATC | Sequencing only | Yoon et al 2001 |
| BC2 | 5.8S - rDNA | CGAGTGGTGTCAACAGACACTCC | Sequencing only | Saunders & Druehl 1993 |
| UP_ITS_1F | ITS1 - rDNA | TCCTGACACTACCGTCGTGCGCGT | PCR-Specific primers | This study |
| UP_ITS_2F | ITS1 - rDNA | TCAGGAAGGGGACACCCTCCTGACACTA | PCR-Specific primers | This study |
| UP_ITS_1R | ITS2 - rDNA | ATGAGCCGGAATGAAGCAGGCGAAT | PCR-Specific primers | This study |

Preliminary amplification suggested the primer pair UP_ITS_1F and UP_ITS_1R to be more specific. The reaction conditions were first optimised adopting a gradient PCR protocol. The identified primer pair was then tested on all the samples listed in Appendix 1. The thermal cycling conditions for the ITS spacer region specific primers (UP_ITS_1F and UP_ITS_1R) were: one initial cycle with denaturation at 94°C for 4 min, annealing at 70°C for 2 mins and extension at 72°C for 2 mins, followed by 35 cycles with 1 min at 94°C, 1 min at 70°C and 1 min at 72°C and a final elongation at 72°C for 4 mins. Annealing at a much higher temperature (70°C) was essential to exclude *Alaria esculenta.*

## 2.5 Gene probe results and discussion

### 2.5.1 Large sub-unit ribosomal DNA

The PCR results from several individuals of *Undaria pinnatifida* as well other members of the Laminariales are presented in Table 6, Table 7 and Table 8. It was possible to amplify regions of the LSU rDNA and 18S rDNA from individuals of *U. pinnatifida* using universal primers. All the DNA samples were successfully PCR amplified by the universal 18S rDNA primers except *Undariella peterseniana* and one sample of *Ecklonia radiata*. All the templates (samples) were therefore deemed suitable for PCR reaction except the two that didn't amplify.

The PCR fragments (LSU rDNA) were successfully sequenced and aligned with the corresponding sequences from the public sequence database. Clustal alignments of the partial LSU rDNA sequence (602 nucleotides) of *Undaria pinnatifida* obtained from in-house sequencing (locations = Tinderbox, Tasmania and Port Phillip Bay, Victoria) and from GenBank AF071152 were identical. *Ecklonia radiata* from Tinderbox and Port Phillip Bay also had identical sequences in this region. Furthermore, the Tinderbox sample of *Macrocystis pyrifera* had only one base pair mismatch when compared to the sequences in GenBank (Accession No. AF053116).

Contrary to expectations, these results indicate that this region of LSU rDNA exhibits minimal variation between these three species. We therefore aligned the entire LSU region sequences for those species of Laminariales for which DNA sequence information is available in the public database. This eventually identified two regions of inter-specific variation, which were used to design three potentially specific primers (Table 3). We tested these probes for specificity against DNA samples of other closely related species, and found them to be non-specific: DNA samples of *Lessonia corrugata* (n=4), *Lessonia nigrescens* (n=1), *Alaria marginata* (n=3), *Egregia menziesii* (n=4), *Alaria esculenta* (n=3), and a number of other species unexpectedly yielded PCR positive results (Table 7 and Table 8, column 5). These results indicate that the PCR probe targeting the LSU DNA loci is not specific to *Undaria pinnatifida*. Several attempts to optimise the probes, by increasing annealing temperature and altering the reaction conditions, were unsuccessful. The results are perhaps not surprising given the low inter- and low intra-specific sequence variation observed at the LSU locus of the Laminariales.

### 2.5.2 RuBisCo spacer region

We obtained (and subsequently aligned) complete sequences of the RuBisCo spacer region for 42 taxa (mainly from the Laminariales) from the public database. The size of the spacer ranged between 250 - 300 bp. Two regions of significant variation were identified and this allowed the design and synthesis of 1 forward and 3 reverse primers (Table 4) with potential to be *Undaria pinnatifida* specific.

Our initial results suggested that the primer pair "UP_RBCsp_1F and UP_RBCsp_1R" were the most promising. PCR amplifications were carried out on genomic DNA of all the 126 individuals listed in Table 6, Table 7 and Table 8 in order to further test the specificity of the primer pair. An *Undaria* specific signal was amplified from all the *Undaria pinnatifida* samples (Table 6, column 7) while virtually all of the other species tested PCR negative. *Lessonia*

*nigrescens* (n=1) and *Alaria esculenta* (n=3; very faint band), however, yielded PCR positive results (Table 7, column 7).

These results indicate that the probes are not 100% specific to the *Undaria* genus. *L nigrescens* and *A. esculenta*, however, are not native to Australia. These probes may therefore be useful for detecting the *Undaria* genus along with *L nigrescens* and *A. esculenta* in environmental and ballast water samples. It is important to note, however, that the non-specific amplification of *L. nigrescens* may have been caused by contamination with *Undaria pinnatifida* DNA. Potential contamination of this sample was discovered at a much later stage in the study (see below).

A major limitation of the RuBisCo specific gene probe is the inability to unambiguously visualise the amplified species-specific signature on an agarose gel. The amplified product was relatively small (72 bp) and the primer pair tends to exhibit primer-dimer formation. The diagnostic *Undaria* signal and the primer-dimer molecule are difficult to separate from each other on a normal 1.8- 3% agarose gel. Attempts to improve resolution using metaphor agarose were unsuccessful. This difficulty may be overcome in the future by modifying the primer ends to avoid primer dimerisation or by using polyacrylamide gel electrophoresis. This probe therefore requires further experimentation and refining.

### 2.5.3 The ribosomal rDNA internal transcribed spacer regions

ITS regions are known to exhibit significant sequence variation between species but they also exhibit relatively high within species variation. When developing species-specific probes, the within species variation may contribute to false negative results. We amplified and sequenced the entire ~1000 bp of the ITS region along with the 5.8S rDNA from all 59 *Undaria pinnatifida* samples so as to establish the within species variation at the ITS region. The entire region was also amplified and sequenced for 15 closely related Laminariales (including native species) and sequences for 49 other species were obtained from the public database. We were surprised to find that the ITS region of these kelps had very little within species variation, and were thereby encouraged to target this region for *U. pinnatifida* specific probes.

When the *Undaria pinnatifida* sequences were aligned with the corresponding sequences of the other closely related species (Appendix 4), two between-species hyper-variable regions (sufficient for species-specific probes) were identified. This allowed us to design and synthesise two forward and one reverse primer for a potentially specific probe. The most suitable primer pair (UP_ITS_1F and UP_ITS_1R) was then tested on all the 126 samples (Table 6, Table 7 and Table 8, column 9). The primers pair appears to be specific to the genus *Undaria*. Nonetheless one sample of each of the species: *Lessoniopsis littoralis, Lessonia nigrescens* and *Egregia menziesii* unexpectedly yielded positive results. These results are puzzling on two counts: a) these species exhibit relatively higher sequence variation at the primer binding site and were therefore expected to yield PCR negative results; and, b) in the case of *L. littoralis* and *E. menziesii* only one of the four samples tested was positive (Note: only one sample of *L. nigrescens* was available for testing).

Table 6 Undaria pinnatifida probe test results

| Species | Sample code/s | 18S | LSU rDNA universal | LSU rDNA specific | RuBisCo universal | RuBisCo specific | ITS rDNA universal | ITS rDNA specific |
|---|---|---|---|---|---|---|---|---|
| *Undaria pinnatifida* | UP 7-9 | + | + | + | + | + | + | + |
| *U. pinnatifida* | 1-GWS000967 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 10-12 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP13-15 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 16-18 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 22-24 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 25-27 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 28-30 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 1-6 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 31-33 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 34-36 | + | + | + | + | + | + | + |
| *U. pinnatifida* | 2-UF01ME | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 37-39 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 40-42 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 43-45 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 46-48 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 49-51 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 52 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 53 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 54 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 55-57 | + | + | + | + | + | + | + |
| *U. pinnatifida* | UP 58-60 | + | + | + | + | + | + | + |
| *U. undarioides* | Uu 1 | + | + | + | + | + | + | + |
| *Undariella peterseniana* | Upet1 | - | - | - | - | - | - | - |

+ indicates PCR positive, – indicates PCR negative, ** indicates that the samples were contaminated, blank cell indicates that the test was not carried out.

Table 7 Undaria pinnatifida probe test results cont…

| Species | Sample code/s | 18S | LSU rDNA universal | LSU rDNA specific | RuBisCo universal | RuBisCo specific | ITS rDNA universal | ITS rDNA specific |
|---|---|---|---|---|---|---|---|---|
| *Ecklonia radiata* | Er 4-6 | + | + | - | | - | - | - |
| *E. radiata* | Er1-3 | + | + | - | | - | - | - |
| *E. radiata* | Er 7-9 | + | + | - | | - | - | - |
| *E. radiata* | 3-ECO1WR | - | - | - | | - | - | - |
| *Lessonia corrugata* | 4-GWS001454 | + | | + | | - | + | - |
| *L. corrugata* | Lc 1-3 | + | + | + | | - | + | - |
| *L. nigrescens* | 29-LESS | + | | + | | + ** | + | + ** |
| *L. flavicans* | 30-SF1597 | + | | - | | - | + | - |
| *Macrocystis pyrifera* | Mp 1-3 | + | + | - | | - | + | - |
| *M. angustifolia* | Ma 1-3 | + | + | - | | - | + | - |
| *M. integrifolia* | 21-MI03SC | + | | + | | - | + | - |
| | 22-MI04SC | | | | | | | |
| *M. integrifolia* | 39-MI02ST | + | | - | | - | + | - |
| | 40-MI05ST | | | | | | | |
| *Agarum clathratum* | 5-CL000602 | + | | - | | - | + | - |
| *Alaria esculenta* | Ae 1-3 | + | + | + | | + | + | - |
| *A. marginata* | 31-AM01SR | + | | + | | - | + | - |
| | 32-AM02SR | | | | | | | |
| *A. marginata* | 6-AM01KP | + | | + | | - | + | - |
| *A. nana* | 33-AN06GB | + | | + | | - | + | - |
| *Costaria costata* | 7 – COST | + | | - | | - | + | - |
| *Cymathere triplicata* | 8 – CYMA | - | | - | | - | - | - |
| *Eisenia arborea* | 10-EA01KP | + | | - | | - | + | - |
| | 11-EA02KP | | | | | | | |
| *Hedophyllum sessile* | 12-CL001501 | + | | - | | - | + | - |
| *Kjellmaniella gyrata* | 13-Kgcul | + | | - | | - | + | - |
| *Laminaria digitata* | 14-CL030103 | + | | - | | - | + | - |
| *L. saccharina* | 15-Lscul | + | | - | | - | + | - |

+ indicates PCR positive, – indicates PCR negative, ** indicates that the samples were contaminated, blank cell indicates that the test was not carried out.

Table 8 Undaria pinnatifida probe test results cont…

| Species | Sample code/s | 18S | LSU rDNA universal | LSU rDNA specific | RuBisCo universal | RuBisCo specific | ITS rDNA universal | ITS rDNA specific |
|---|---|---|---|---|---|---|---|---|
| *Lessoniopsis littoralis* | 18-LL04AP | + | | + | | - | + | - |
| *L. littoralis* | 19-LL01KP | + | | + | | - | + | + ** |
| *L. littoralis* | 20-LL02FI | + | | + | | - | + | - |
| *L. littoralis* | 38-LL06GB | + | | + | | - | + | - |
| *Pleurophycus gardneri* | 23-CL000903 | + | | + | | - | + | - |
| *Postelsia palmaeformis* | 24-PP01BA<br>25-PP02BA | + | | + | | - | + | - |
| | 26-PP04BA | | | | | | | |
| *P. palmaeformis* | 46-PP06GB | + | | + | | - | + | - |
| *Pterygophora californica* | 27-PC01CB | + | | + | | - | + | - |
| | 28-PC02CB | | | | | | | |
| *Dictyoneurum californicum* | 34- DR01AB | + | | - | | - | + | - |
| *Egregia menziesii* | 9-EM02BA | + | | + | | - | + | - |
| *E. menziesii* | 35-EM03BB | + | | + | | - | + | + ** |
| *E. menziesii* | 36-EM04BB | + | | + | | - | + | - |
| | 37-EM05BB | | | | | | | |
| *Nereocystis leutkeana* | 41-NL02GP | + | | - | | - | + | - |
| | 42-NL04GP | | | | | | | |
| *N. leutkeana* | 43-NL03TR | + | | + | | - | + | - |
| | 44-NL05TR | | | | | | | |
| *Pelagophycus porra* | 45-PELA | + | | - | | - | + | - |

+ indicates PCR positive, – indicates PCR negative, ** indicates that the samples were contaminated, blank cell indicates that the test was not carried out

We suspect that these unexpected positive results were caused by contamination with *Undaria pinnatifida* DNA. An alternative explanation is the chance amplification of another region entirely. We subsequently sequenced the 550 bp of the "*Undaria* specific" fragment amplified from the suspect samples. The sequences obtained from all the three suspect samples were very similar to those obtained from the *Undaria pinnatifida* samples. We then subjected the primary amplicon (column purified) of ~1000bp of the ITS region from all the three suspect samples to a second round of PCR using the *Undaria* specific probes and found them to be PCR negative. These two results combined suggest that the three spurious results are indeed due to contamination. Furthermore, the latter result in combination with the strict PCR protocols followed in the laboratory suggest that the three samples were most likely contaminated with genomic DNA of *Undaria pinnatifida* at the time of DNA extraction.

# 3. HULL FOULING SAMPLES

## 3.1 Field methods

Fishing vessels and recreational yachts were sampled at four locations over the course of the first and second stages of the empirical validation project. The first project sampled 32 vessels between the 12th August 2003 and the 19th July 2004 at the Royal Hobart Yacht Club and the Domain Slipway (Hayes *et al*., 2004). A further 22 vessels were sampled during two one-week field trips on the 28th August 2004 and the 29th November 2004, at the Sandringham Yacht Club, Hobson's Bay Yacht Club and the Royal Yacht Club of Victoria for the second stage. Vessels were haphazardly selected based on the slipping schedules of the yacht club. Vessel selection, access and permission from boat owners was organised by liaising with the Bosun and slipway managers at each of the sites.

Five field staff were necessary for quick and efficient sample collection and to prevent delays on the slip. On average, sample collection took between 45 to 60 minutes depending on the size of the vessel. Upon introduction the vessel owners were given a brief explanation of the project and methodology, provided with a project summary sheet (Appendix 5) and asked to close all sea-cocks prior to slipping so that internal water samples could be collected. Sampling commenced as soon as the boat was safely secured on the slipway.

Samples were collected from external surfaces and accessible internal surfaces (see list of sample locations in Appendix 6) using plastic putty knifes, metal putty knives (for excessive growth of barnacles etc.) and metal vegetable peelers (for water inlets and outlets). Samples were rinsed from the utensils with 0.2µm filtered seawater into sample jars. Hull surface samples were taken by scraping six 0.5$m^2$ quadrats placed haphazardly within the 3 zones of fore, mid-ships and aft somewhere along the hull and keel (three port and three starboard). Two samples of the rudder surface were taken (one port, one starboard) using 0.5$m^2$ quadrats.

Garboard planks, keel cooling pipes, stabilisers and boot-tops were sampled by scraping the entire width of the structure along a one metre long section, usually from the top and bottom surfaces (wherever appropriate). All other biofouled external features, such as paddle wheels, water inlet/outlet cover plates, echo sounders were sampled as completely as possible. The interior surfaces of water inlets and outlets were sampled to maximum extent allowable by the diameter of the inlet and the width and length of the vegetable peelers.

All vessels were boarded to inspect the deck, fishing gear (if applicable) and internal spaces, such as anchor wells, cockpit bins, rudder control rooms and bilges, etc. Samples of water (and sediment) were collected when present wherever possible. Water samples were collected using a 60 ml syringe, electric mono pump (depending on volume) or by simply opening the sea-cocks and collecting the water that drained from the vessel into a sample jar or bucket.

All vessel owners were interviewed to obtain information on the dimensions of the vessel, hull material, voyage history, cleaning activities, name of antifouling paint applied to the vessel, and the date last applied. The degree of fouling on the boot-tops, vertical bottom and flat bottom surfaces of the hull (port and starboard) was scored using industry standard hull fouling indices.

All information was recorded on survey sheet specifically designed for this project (Appendix 6).

Six additional sample locations were added to the survey sheet (originally designed around the results of the IMEA analysis – Hayes, 2002) during the course of the fieldwork: the boot-top (HAH), transom (HAI), radio earth plate (HAF), auto-pilot sensor (HAE), rudder pintel (PT) and rudder cavity (PU). The boot-top (HAH) was specifically isolated for the second stage of the project to investigate the potential for preferential fouling in this location by macroalgae such as *Undaria pinnatifida*. One further change was made to the sample locations upon analysis of the data: the transducer – HM and depth sounder – HH were merged into a single category

Plankton samples were also collected at the sampling site in order to confirm the presence or absence of *Asterias* (Stage I) and *Undaria* (Stage II) in the surrounding waters. In the first project, four (one for visual inspection), five minute samples were collected with an electric mono pump (CP 25) and sieved through a 100µm mesh plankton net. The mono pump was calibrated at 1-2m head, delivering 31 litres per minute (range of 30 – 31.6 litres per minute). The total volume of water sampled for each plankton sample was approximately 155 litres.

In the second project three, ten (later reduced to five) minute, samples were collected with a 'Shurflow' deck wash pump (working pressure of 3.1 bar) and filtered through glass fibre (GF6 Schleicher and Schuell) filter paper with a pore size of 0.5 - 1.5µm. The filter paper was supported by a fine stainless steel mesh, bracketed in place, during the pumping. The pump was calibrated at less than 1m head, delivering approximately 2 litres per minute. The total volume of water sampled for each plankton sample was approximately 20 (later reduced to 10) litres. The filter paper was preserved after each sample in 25X SET buffered ethanol fixative.

## 3.2 Laboratory methods

### 3.2.1 Pre-extraction and sample processing

Upon returning to the laboratory all field equipment was thoroughly washed in hot water and detergent and air dried. All samples were washed with 0.2µm filtered seawater and sieved through a 106µm (*Asterias amurensis* samples) or 45µm (*Undaria pinnatifida* samples) sieve within a few hours of being collected. Sieved samples were then weighed and if the wet weight exceeded 5g were split by weight into approximately two halves. Samples were weighed on Sartorius BL3100 scales (readability = 0.1g, linearity = 0.2g). Samples that weighed less than 1.5 grams were allocated a nominal weight of 0.5 grams biomass because of the unreliability of very low wet-weight measurements – i.e. the mass of the water associated with the sample contributes a significant (but un-quantified) proportion of the overall mass of the sample.

After weighing, all samples were preserved in SET buffered ethanol fixative and placed into a fridge prior to DNA extraction or pre-extraction processing where necessary (see below). The SET buffered ethanol fixative comprises 25ml of 90% reagent grade ethanol, 2ml Milli-Q-water and 3ml of 25X - SET buffer (Table 9).

All samples with large biomasses or with bulky organic matter (e.g. barnacles) underwent a second processing stage prior to DNA extraction and amplification. The average wet weight of samples from all locations was approximately 63g, whereas the maximum biomass the genetic probe can process without sub-sampling is approximately 1g. It proved necessary therefore to remove as much of the biomass as possible to eliminate the need to sub-sample during DNA extraction and thereby preserve the sensitively of the probe. This process was undertaken as a second step, after initial preservation due to concerns that live organisms may not be readily washed off their substrates.

Table 9 Preparation of 25X SET buffer

| Reagent | Stock in 400ml | To make 400ml of 25X SET | Final molarity |
|---|---|---|---|
| 3.75M NaCl | | 87.66g | 3.75M |
| 0.5M Na2 EDTA (ph8) | 74.4g | 20ml | 25mM |
| 0.8M Tris HCl (ph 7.8) | 50.42g | 200ml | 0.4M |

During the second stage processing, bulky samples were rinsed with un-buffered reagent grade ethanol; this ethanol was used for one rinse only to avoid contamination between samples. Samples were rinsed through a 2mm mesh sieve in plastic dish full of ethanol and agitated for approximately one minute to encourage the finer particles to move through the sieve. The contents of the dish were then sieved through a 106µm or 45µm sieve. The contents of this sieve were then washed with SET buffered ethanol fixative into a sample jar and placed in the fridge ready for DNA-extraction.

### 3.2.2 DNA extraction and amplification

All pre-processed samples were concentrated by vacuum filtration through a 5 µm pore-sized hydrophilic Durapore Filter (Millipore). The residue was briefly air-dried, weight measured, transferred to a 2 ml tube and DNA extracted using the DNeasy Plant Kit (QIAGEN) following suppliers instructions. DNA was retrieved in 200 µl elution buffer quantified and stored at 4°C. All the samples were diluted to get <15ng DNA before PCR amplification.

A two-step nested PCR was used to enhance the sensitivity of the test. Primary enrichment PCR was conducted using the universal primer pair LB1 and LB2 (Table 5). PCR conditions were the same as the standard PCR described previously. The secondary *Undaria* specific PCR was carried out using the primer pair UP_ITS_1F and UP_ITS_1R (Table 5) with 1/25 the volume of the primary reaction as template. PCR conditions were the same as the ITS spacer region specific PCR described previously. Randomly selected *Undaria* positive samples were sequenced for further confirmation.

## 3.3 Hull fouling data analysis

The data analysis was performed in several discrete steps:

1. An initial exploration of the data to identify broad patterns and statistical characteristics;

2. A comparison of the environmental suitability and occurrence predictions of the Infection Modes and Effects Analysis with the actual biomass (measured as wet weight) collected at the various sample sites;

3. An analysis of the probe results, focussing in particular on the incidence of *Undaria pinnatifida* positive samples in the plankton and the vessel samples[2]; and,

4. The development of quantitative biofouling risk factors describing:

    a. the relationship between the proportion of zero weight (clean) samples and potential explanatory variables such as vessel activity, cleaning schedules and use patterns; and,

    b. the relationship between the wet weight of fouling biomass in non-zero weight samples and potential explanatory variables such as vessel activity, cleaning schedules and use patterns

The Stage I and Stage II projects were unable develop journey survival model for *Asterias amurensis* or *Undaria pinnatifida* because the project team were unable to collect the data necessary to develop such a model– i.e. we were unable to gain access, follow and sample individual (positive) vessels.

All data summary and analysis was performed using the programming language R (R Development Core Team, 2004). R is an open source code (http://www.r-project.org/) similar to S.

## 3.4 Hull fouling results

### 3.4.1 Vessels

The project team sampled 54 vessels at 5 locations during the course of both projects. Most vessels were sampled in Hobart at the Domain Slipway (16) and the Royal Hobart Yacht Club (18). The remaining vessels were sampled in Melbourne at the Sandringham Yacht Club (14), the Hobson's Bay Yacht Club (4) and the Royal Yacht Club of Victoria (2). Most of the sample vessels were yachts (32), followed by fishing vessels[3] (10) and motor cruisers (10). The project team also sampled a ferry and a tug.

[2] The incidence of *Asterias amurensis* positive samples are summarised in Section 1 of this report
[3] Three types of fishing vessels were sampled: an abalone "mother ship", 8 lobster/scallop vessels and a long line fishing vessel.

Figure 1 and Table 10 summarise the dimensions of the vessels that were sampled, with particular reference to the wetted surface area (Appendix 7). The yachts and motor cruisers are roughly equal in length, width and draught, with an average wetted surface area of approximately 37m$^2$. The fishing vessels and ferry are slightly larger with an average wetted surface area between 54 and 86m$^2$.

The tug is an outlier in this context with an approximate wetted surface area of 140m$^2$, however the largest vessel sampled was a lobster/scallop vessel with an approximate wetted surface area of 239m$^2$. These two vessels are clearly evident as outliers in the kernel density estimate of wetted surface area (Figure 1d). With the exception of these outliers the distribution of wetted surface area is approximately normal with a mean of 38.4m$^2$ and standard deviation of 16.8m$^2$.

Table 10 Sampled vessel dimensions

| Vessel type | n[a] | Mean L (m) | Mean W (m) | Mean D (m) | Mean WSA (m$^2$) |
|---|---|---|---|---|---|
| Ferry | 1 | 18.0 | 5.0 | 1.5 | 54.0 |
| Fishing vessel (Abalone mother ship) | 1 | 19.8 | 6.1 | 1.4 | 55.0 |
| Fishing vessel (Lobster/scallop) | 8 | 15.3 | 4.5 | 2.7 | 84.8 |
| Fishing vessel (Long line) | 1 | 16.5 | 6.1 | 2.6 | 86.0 |
| Motor cruiser | 10 | 12.8 | 4.1 | 1.4 | 37.3 |
| Tug | 1 | 25.5 | 9.0 | 2.7 | 140.0 |
| Yacht | 32 | 10.8 | 3.5 | 1.8 | 36.8 |

[a]n = number of vessels sampled, L = Length, W = Width, D = Draught, WSA = Wetted Surface Area (approximate)

### 3.4.2 Vessel activity

Figure 2 summarises the activity and cleaning practises of the vessels sampled during the two projects. Most of the owners and contractors questioned (94%) were able to report/recount their voyage histories and cleaning activity during the last year or at least since the last time the vessel was antifouled. Over half of the owners and contractors (53%) reported that they last used their vessel in the two weeks prior to the sample date, whilst 69% reported that they had last used their vessel less than 50 days before the sample date (Figure 2a) Approximately one third (37%) of the owners/contractors reported that they had cleaned and/or antifouled their vessel within the last 6 months, whereas 69% reported they had cleaned and/or antifouled their vessel within the last year (Figure 2b). A histogram of the median number of trips per annum suggests that most of the vessels that were sampled were used less than 50 times in the previous year (Figure 2c). Indeed only 10 vessels (19%) were used at a frequency equal to, or greater than, once a week.

Figure 1 Histograms (a, c) and kernel density estimates (b, d) of length and wetted surface area of sampled vessels

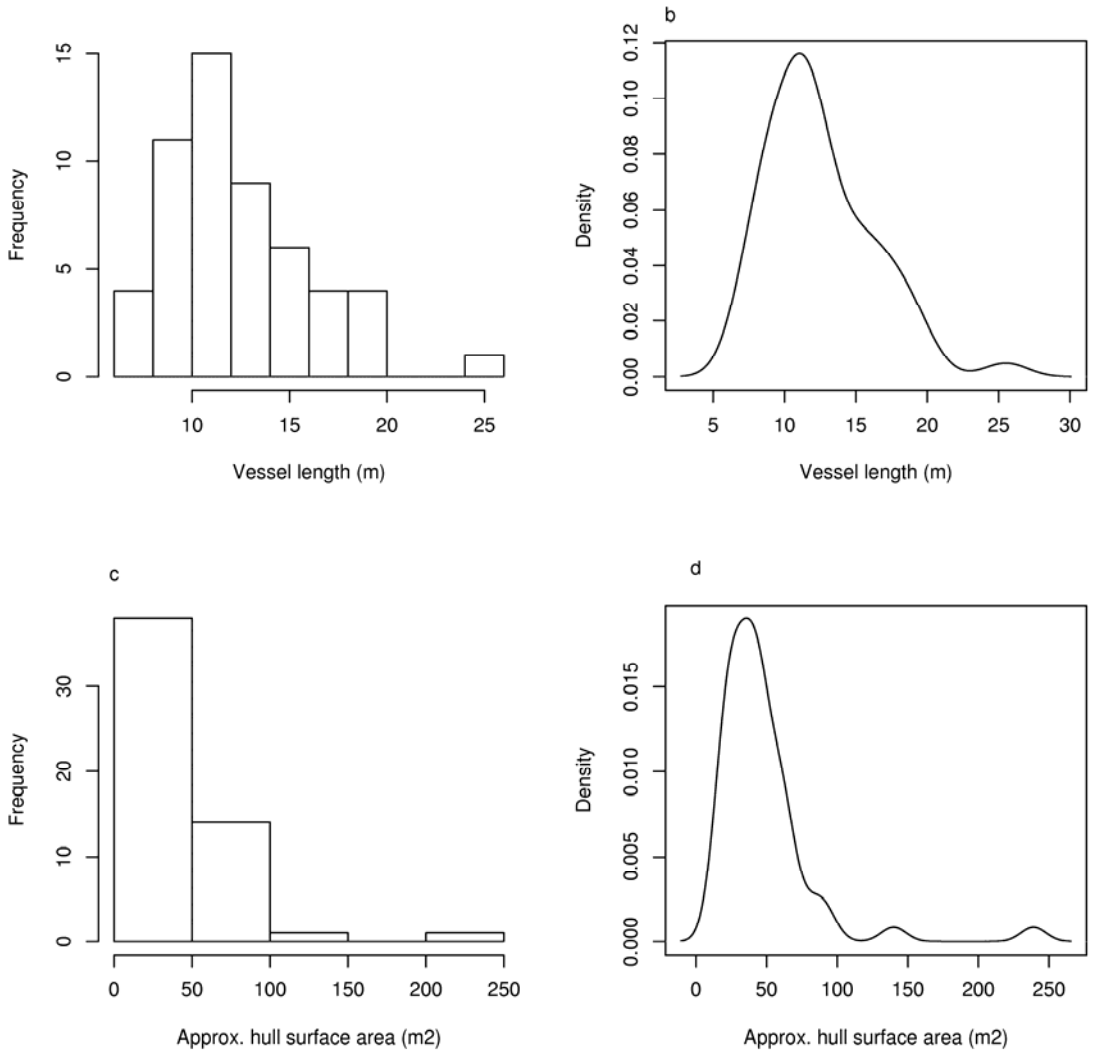
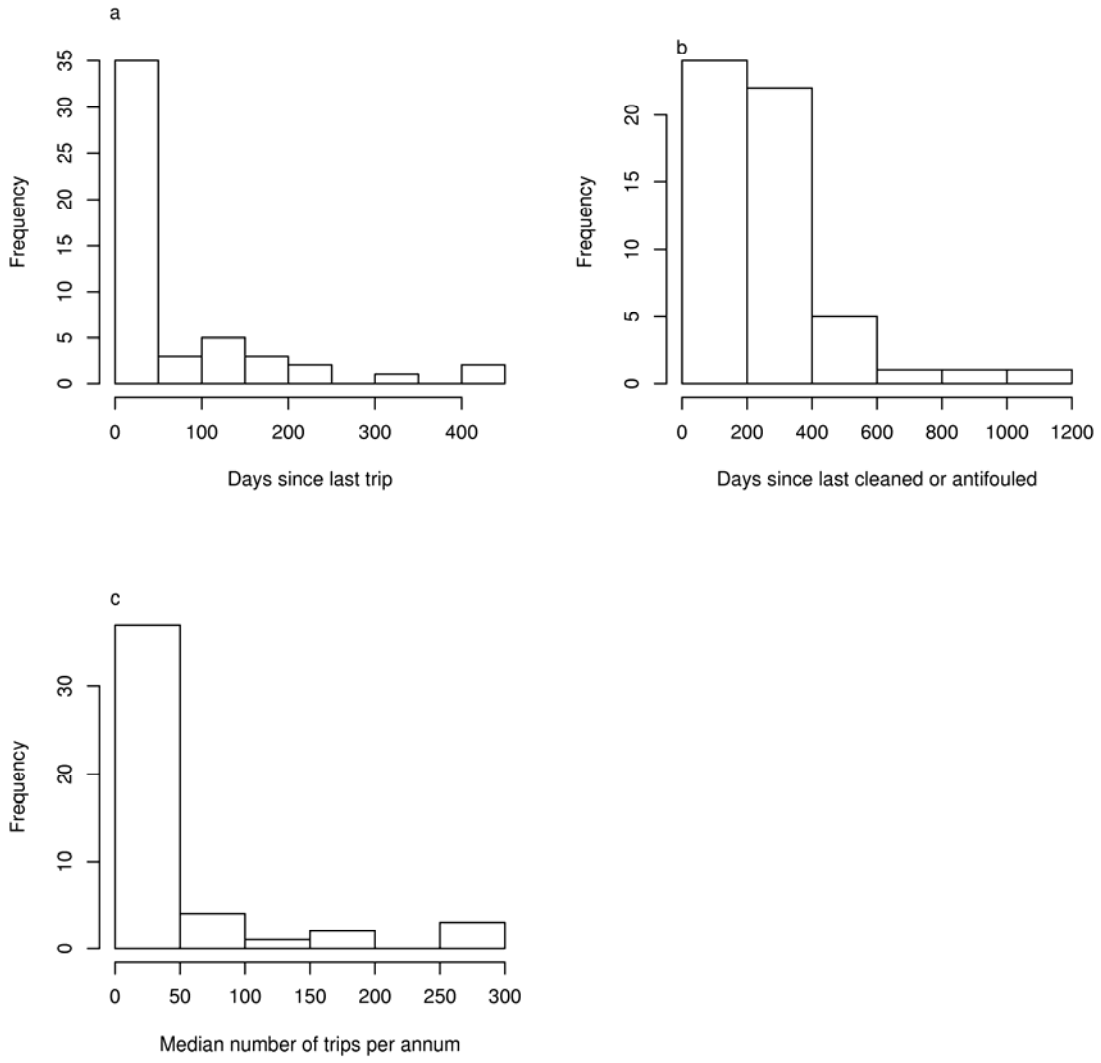


Figure 2 Histograms summarising the number of days since the vessel's last trip (a), the number of days since it was last cleaned or antifouled (b) and the median number of trips per annum (c)



### 3.4.3 Antifouling paints

At least 20 separate anti-fouling products were used across the 54 vessels that were sampled. The most popular products were Jotun Seaguardian (6), International Copper Coat (4), Wattyl Sigmaplane Ecol IV (4), Wattyl Seapro (3) and International Long Life (3) (Table 11). The split between ablative (27) and non-ablative (24) products is approximately equal. We were unable to obtain details of the anti-fouling paint used for the three of the vessels.

### 3.4.4 Biofouling samples

The project team sampled or inspected 63 different locations in and around the hull, propeller, rudder and anchor, internal spaces, fishing gear and deck of the sample vessels. A total of 1116 samples were taken, mainly from the hull. A further 365 inspections were made on board the vessels that resulted in no sample being taken. An additional 162 plankton samples – 3 for each vessel sampled – were also taken from the ocean on each occasion. No samples were collected from the deck or fishing gear of any of the vessels taken – in all cases these locations were clean and dry when inspected by the project team.

Figure 3 plots histograms of the wet weight (grams) of the samples taken from wet areas of the hull[4], internal spaces, propeller and rudder. The data are zero-inflated and censored. The lower limit on the reliability of the wet weight of the samples introduces (left) censorship because samples weighing $\leq 1.5$ grams were assumed to contain 0.5 grams of biomass. The large number of samples in this category, together with inspections that resulted in no sample (i.e. the inspected area was clean and dry), gives rise to zero-inflation. The term "zero inflated" is used to describe datasets that contain an excessive number of zeros, and in this case near zeros. The word inflation is used to emphasise that the probability mass at the point zero exceeds that allowed under any standard parametric family of distributions (Tu, 2002). For example, the mean and standard deviation of the wet weight of the hull quadrats was 75 and 412 grams respectively, but 56 of the 257 hull quadrats weighed less than 1.5 grams.

Figure 4 plots the histogram of the log-transformed data $x = f(x)$ where $x$ is the wet-weight of the sample in grams, and $f(x)$ is given by:

$$f(x) = \ln(x + a) \ . \qquad [1]$$

The parameter $a$ in equation [1] is a "shift-parameter" which is commonly used if the data cannot be smaller than a certain bound different from zero (Limpert *et al*., 2001). In this instance the lower limit on the reliability of wet weights suggested an upper bound for small samples of 1.5 grams. All such samples were assumed to contain 0.5 grams biomass, hence in this analysis $a$ was arbitrarily set to 1 to reflect this bound. The transformed data are still zero-inflated, but thereafter appear more normally distributed. It is important to recognise, however, that these results group different sample areas, particularly in the case of the hull, and thereby mask patterns within each sample location. All further analysis is therefore conditioned upon sample location.

[4] Defined as areas of the hull at or below the water line – i.e. the submerged portion of the hull.

Table 11 Anti-fouling paints used on the hulls and propellers of sampled vessels

| Survey name[a] | Commercial name | Active agent(s) | Type | n |
|---|---|---|---|---|
| ABC 3 | Ameron ABC #3 Antifouling (Altex ABC-3) | Cuprous oxide, Zinc oxide, Thiram | Ablative | 1 |
| Ablative International | Unknown | Unknown | Ablative | 1 |
| Awlcraft | Awlcraft Marine Paint Awlcraft Antifouling | Cuprous oxide, Diurion | Ablative | 1 |
| Awlgrip | Altex Yacht & Boat Paint No. 5 | Cuprous oxide, Zinc oxide, Thiram | Ablative | 5 |
| Bottom coat | International Epiglass Bottomkote Eroding Antifouling | Cuprous oxide | Ablative | 1 |
| Ecol IV | Wattyl Marine Coatings Ecol IV Antifouling | Cuprous oxide, Diurion | Ablative | 4 |
| Hempel | Hempels Antifouling Olympic 7154 | Cuprous oxide | Self-polishing | 1 |
| International (soft) | | | Ablative | 1 |
| International Copper coat | International Epiglass Copper coat Ablative Antifouling | Cuprous oxide, Diurion | Ablative | 7 |
| Interspeed 2000 | International Epiglass Interspeed 2000 Hard Antifouling for Aluminium | Cuprous thiocyanate, Diurion | Hard | 4 |
| Jotun Seaguardian | Jotun Antifouling Seaguardian | Cuprous oxide, Zinc oxide | Self-polishing | 6 |
| Long-life | International Epiglass Longlife High Strength Hard Antifouling | Cuprous oxide, Diurion | Hard | 4 |
| Micron | International Epiglass Micron Optima Antifouling | Cuprous oxide, Zinc pyrithione | Self-polishing | 2 |
| Micron CSC | International Epiglass Micron CSC High Strength Self Polishing Antifouling | Cuprous oxide, Diurion | Self-polishing | 2 |
| Olympic Black | Hempels Antifouling Olympic 7154 | Cuprous oxide | Self-polishing | 1 |
| Seapro | Wattyl Marine Coatings Seapro Antifouling | Cuprous thiocyanate | Hard | 3 |
| Seasafe | Jotun Antifouling Seasafe | Cuprous thiocyanate, Zinc oxide, Zineb | Ablative | 1 |
| Sigmaplane Ecol HA120 | Wattyl Marine Coatings Sigmaplane Ecol HA120 Antifouling | Cuprous oxide, Diurion | Self-polishing | 3 |
| Taspaint T782 | Unknown | Unknown | Unknown | 1 |
| VC Offshore | International Epiglass VC Offshore Racing Antifouling | Cuprous oxide, Diurion | Hard | 1 |
| Wattyl Marine Coating | Unknown | Unknown | Unknown | 1 |

[a]Survey name = name used by questionnaire respondent

Figure 3 Histograms of the sample wet weight (grams) taken from the wet hull, internal spaces, propeller and rudder

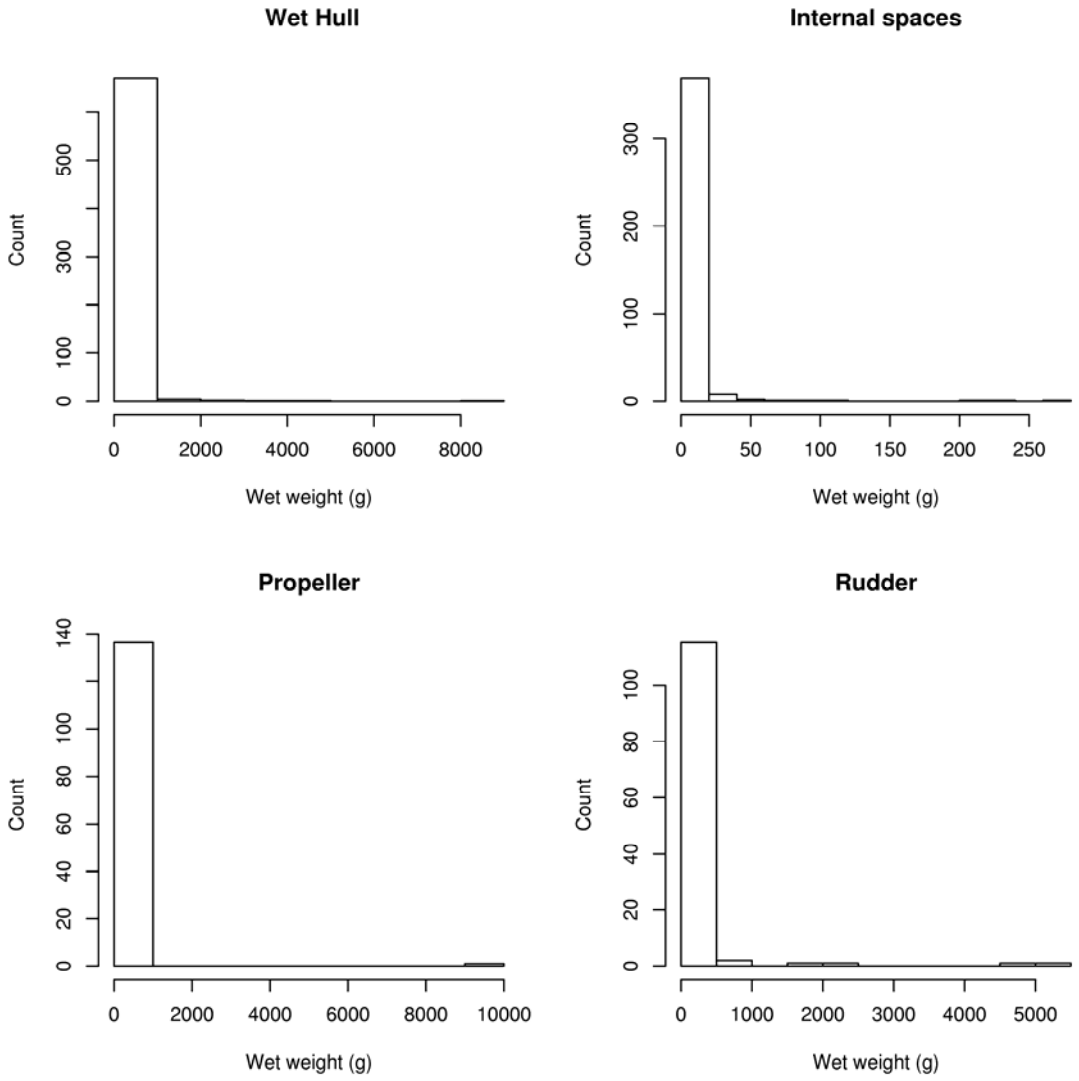
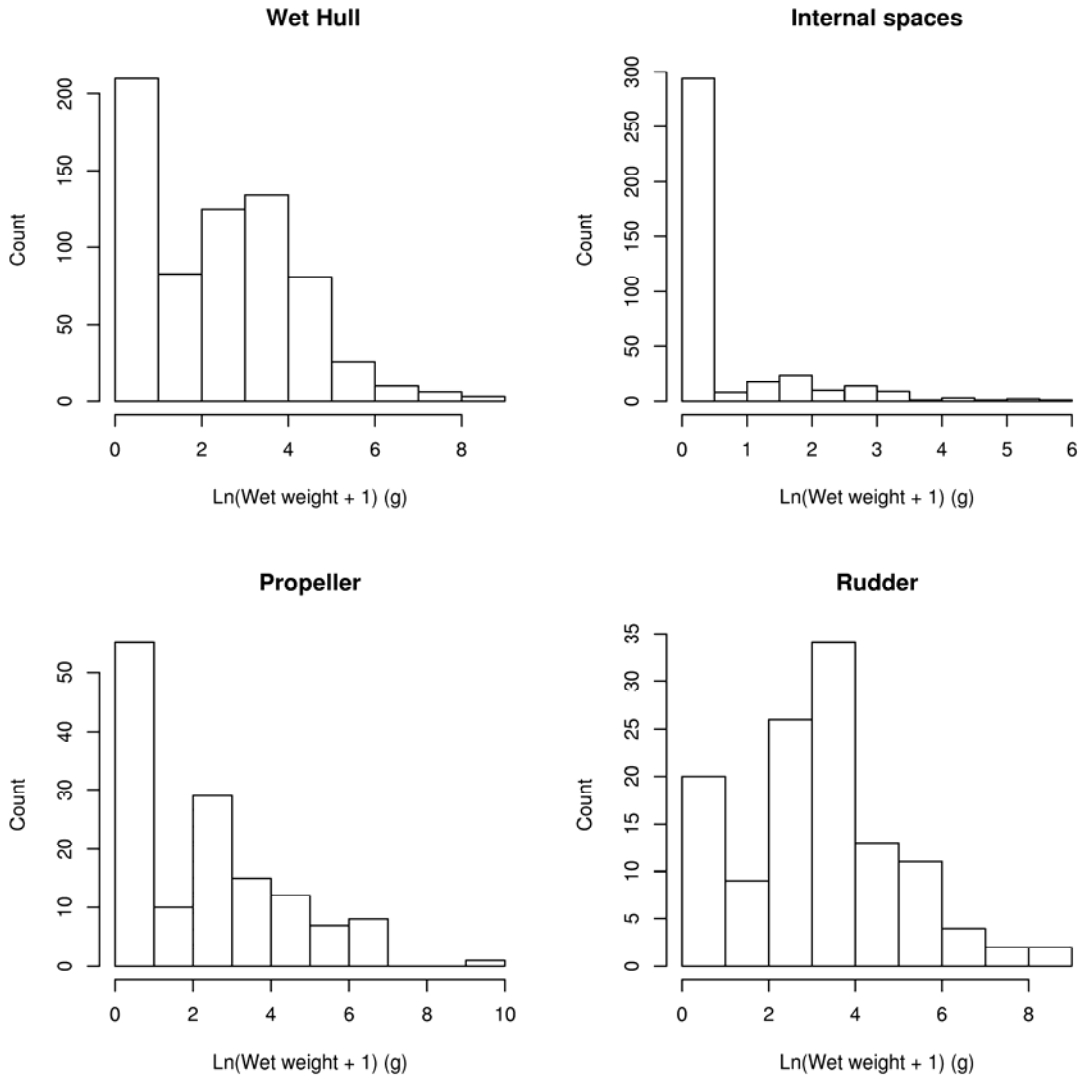


Figure 4 Histograms of the log-transformed sample wet weight (grams) taken from the hull, internal spaces, propeller and rudder



Histograms of the log-transformed data $x = f(x)$ by sample location are shown in Appendix 8. The pattern of zero-inflated, log normally distributed data are evident in a number of locations, notably: the hull quadrats (HA); boot-top (HAH); radio earth plates (HAF), external transducer surfaces (HM); fixed keel (HP); garboard plank (HX) stern tubes cover/gland (PG), rudder surface (PJ), propeller shaft (PA) and perhaps to a lesser extent in the seawater/grey water inlets/outlets (IB); propeller surface (PB), water inlet/outlet cover plates (HD), the paddle wheel and booth (HK), external surfaces of keel cooling pipes (HF) and depth sounder booth (HH).

Zero-inflated, log normally distributed data may be adequately described by a delta distribution (Tu, 2002). A delta distribution with parameters $p$, $\mu$ and $\sigma$, denoted $Y \sim \Delta(p, \mu, \sigma)$, is defined as:

$$Y \sim \begin{cases} 0, & \text{with probability } p \\ \text{lognormal}\left(\mu, \sigma^2\right), & \text{with probability } (1-p) \end{cases} \quad [2]$$

To explore this possibility the sample wet weights are divided into zero (≤ 0.5 grams) and non-zero (> 0.5 grams) categories. Histograms of the non-zero, log-transformed sample wet weights by sample location together with Q-Q plots (Appendix 8) and the Shapiro-Wilks test (Table 12 and Table 13) confirm the proposition that the non-zero samples are log-normally distributed ($n_1 > 25$ and $p > 0.05$ in the Shapiro-Wilks test) on the fixed keel (HP), the boot-top (HAH), depth sounder booth (HH) and the water inlet/outlet cover plates (HD).

The wet weight of hull fouling biomass is approximately log-normally distributed ($n_1 > 25$ and $p > 0.01$ in the Shapiro-Wilks test) on the propeller surface (PB). The wet weight has a notably longer right tail than the log-normal distribution on the rudder surface (PJ) and hull quadrats (HA), whilst the fouling in the seawater/grey water inlets/outlets (IB) appears to be asymmetrically skewed in both tails. In these cases, however, the effect of non-normality on statistical models may be mitigated by the large sample size, particularly for the hull quadrats (Faraway, 2002).

In the interests of expediency only the eight locations above, together with the paddle wheel and booth (HK) ($n < 30$ but $p > 0.05$), will be taken forward for more detailed statistical modelling. The hull quadrats (HA)will provide the basis for much of this modelling because the sample area ($0.5m^2$) in each case is held constant and therefore comparable between vessels. In most other instances the sample area is determined by the size of the relevant location (e.g. water inlet/outlets, transducer) and therefore varies between vessels.

Table 12 Shapiro-Wilks test results for non-zero log transformed data where the sample size n > 3 by sample location

| Location name | LocID | n | n1 | n0 | w | p |
|---|---|---|---|---|---|---|
| Hull surface: External | HA | 257 | 197 | 60 | 0.97 | 0.0001 |
| Seawater/grey-water inlet/outlets: Internal | IB | 149 | 81 | 68 | 0.92 | 0.0001 |
| Rudder surface: External | PJ | 98 | 79 | 19 | 0.93 | 0.0006 |
| Keel - fixed: External | HP | 75 | 53 | 22 | 0.97 | 0.1868 |
| Water inlet/outlet cover plates: External | HD | 67 | 39 | 28 | 0.95 | 0.0567 |
| Zinc blocks: Front | HAB | 53 | 25 | 28 | 0.92 | 0.0440 |
| Propeller surface: External | PB | 53 | 34 | 19 | 0.92 | 0.0127 |
| Boot-top (1m scrape) | HAH | 47 | 40 | 7 | 0.97 | 0.2860 |
| Seawater/grey-water inlet/outlets: Water | IA | 42 | 2 | 40 | NA | NA |
| Anchor well: Water | IH | 39 | NA | 39 | NA | NA |
| Depth sounder booth: External | HH | 37 | 27 | 10 | 0.97 | 0.7359 |
| Anchor surface: External | PM | 36 | NA | 36 | NA | NA |
| Paddle wheel and booth: External | HK | 35 | 17 | 18 | 0.96 | 0.5522 |
| Bilge - closed: Water | ID | 35 | 1 | 34 | NA | NA |
| Chain: External | PN | 35 | NA | 35 | NA | NA |
| Propeller shaft: External | PA | 33 | 18 | 15 | 0.92 | 0.1184 |
| Stern tubes cover/stern gland: External | PG | 29 | 18 | 11 | 0.93 | 0.1728 |
| Anchor well: Sediment | II | 27 | NA | 27 | NA | NA |
| Cracks in deck/between plates: Water | DA | 22 | NA | 22 | NA | NA |
| Engine cooling water filter: Water | IF | 21 | NA | 21 | NA | NA |
| Cockpit/Wheelhouse: Water | IV | 21 | NA | 21 | NA | NA |
| Keel extension/Skeg: External | PH | 21 | 20 | 1 | 0.96 | 0.6348 |
| Exhaust outlet: External | HAC | 20 | NA | 20 | NA | NA |
| Garboard plank: External | HX | 20 | 17 | 3 | 0.96 | 0.6723 |
| Skin fittings: External | HC | 17 | 12 | 5 | 0.86 | 0.0435 |
| Radio earth plate: External | HAF | 16 | 12 | 4 | 0.95 | 0.6590 |
| Cockpit/Wheelhouse: Sediment | IW | 16 | 1 | 15 | NA | NA |
| Stabilisers/Trim tabs - folding: Top | HR | 14 | 14 | NA | 0.93 | 0.2908 |
| Keel cooling pipes: External | HF | 13 | 10 | 3 | 0.99 | 0.9986 |
| Outboard sail drive legs: External | PE | 12 | 7 | 5 | 0.74 | 0.0096 |
| Stabilisers/Trim tabs - folding: Bottom | HS | 9 | 6 | 3 | 0.83 | 0.1064 |

Table 13 Shapiro-Wilks test results for non-zero log transformed data where the sample size n > 3 by sample location cont.

| Location name | LocID | n | n1 | n0 | w | p |
|---|---|---|---|---|---|---|
| Surface: Water | DG | 8 | NA | 8 | NA | NA |
| Cockpit bins/open storage: Water | DE | 7 | NA | 7 | NA | NA |
| Zinc blocks: Behind | HAA | 7 | 1 | 6 | NA | NA |
| Bob-stay fitting: External | HY | 7 | NA | 7 | NA | NA |
| Marlin board: External | HZ | 7 | 1 | 6 | NA | NA |
| Rudder control room: Water | IP | 7 | NA | 7 | NA | NA |
| Live catch wet well: Water | IL | 6 | 1 | 5 | NA | NA |
| Rudder control room: Internal | IQ | 6 | NA | 6 | NA | NA |
| Hatches: Water | DD | 5 | NA | 5 | NA | NA |
| Live catch circulation tank: Internal | IN | 5 | 2 | 3 | NA | NA |
| Gunwale (toe rail): Sediment | DC | 4 | NA | 4 | NA | NA |
| Block space: External | HL | 4 | 4 | NA | 0.77 | 0.0550 |
| Storage rooms: Water | IT | 4 | NA | 4 | NA | NA |
| Propeller cowling: external | PS | 4 | 4 | NA | 0.90 | 0.4230 |

### 3.4.5 Infection Modes and Effects Analysis

The Infection Modes and Effects Analysis (IMEA) ranks vessel sub-components according to: a) their environmental suitability; b) the occurrence of marine organisms; and, c) the likelihood of detection (Hayes, 2002). The results of this project verify the occurrence of marine organisms as foulers on delineated areas of a vessel, through their abundance measured via wet biomass, and to a lesser extent environmental suitability in so far as it is related to the presence/absence of marine organisms in certain locations of a boat. In this context we assume that the mean wet weight of biofouling taken from each location is a reasonable proxy for the bio-invasion hazard associated with that location

Table 14 and Table 15 summarise the mean, variance and sample size of the log-transformed sample wet weights *f(x)*, together with the average environmental suitability score (AvSevRat), the average occurrence score (AvOccRat) and the multiple of the latter scores (SOR). Figure 5 plots the mean wet weight of the log-transformed data *f(x)*, for each sample location, against the severity score multiplied by the occurrence score allocated to that location in the IMEA analysis. Points that lie close to, or parallel to the line $y = x$ reflect accurate predictions, points which deviate from this line represent predictions that were either too high or too low. In this context it is important to note that the IMEA analysis does not provide an absolute measure of biofouling wet-weight, but rather a relative measure of biofouling hazard – hence points that lie along any single line, parallel to the line $y = x$ represent good agreement between the two data sets.

Table 14 Mean, variance, sample size and IMEA score of transformed sample wet weights by location

| Location name | LocID | mu | var | n | AvSevRat | AvOccRat | SOR |
|---|---|---|---|---|---|---|---|
| Seawater/grey-water inlet/outlets: Water | IA | 0.44 | 0.04 | 42 | 8.83 | 8.5 | 75.08 |
| Keel extension/Skeg: External | PH | 3.53 | 2.71 | 21 | 8.5 | 8.5 | 72.25 |
| Paddle wheel and booth: External | HK | 1.36 | 1.54 | 35 | 8.33 | 8.67 | 72.22 |
| Seawater/grey-water inlet/outlets: Internal | IB | 1.39 | 1.45 | 149 | 8.71 | 8.25 | 71.89 |
| Skin fittings: External | HC | 1.97 | 2.73 | 17 | 9 | 7.88 | 70.88 |
| Keel cooling pipes: External | HF | 2.33 | 1.75 | 13 | 9.12 | 7.75 | 70.72 |
| Block space: External | HL | 4.33 | 0.65 | 4 | 8.12 | 8.5 | 69.06 |
| Bob-stay fitting: External | HY | 0 | 0 | 7 | 9 | 7.5 | 67.5 |
| Keel - fixed: External | HP | 2.37 | 2.83 | 75 | 8.5 | 7.88 | 66.94 |
| Depth sounder booth: External | HH | 2.54 | 3.56 | 37 | 8.38 | 7.88 | 65.95 |
| Hull Surface: External | HA | 2.7 | 3.09 | 257 | 8.25 | 7.75 | 63.94 |
| Stern tubes cover/stern gland: External | PG | 2.18 | 3.64 | 29 | 8.8 | 7.17 | 63.07 |
| Garboard plank: External | HX | 4.35 | 4.95 | 20 | 7.38 | 7.88 | 58.08 |
| Outboard sail drive legs: External | PE | 1.48 | 3.67 | 12 | 7.8 | 6.83 | 53.3 |
| Live catch circulation tank: Internal | IN | 1.07 | 2.21 | 5 | 6.5 | 7.5 | 48.75 |
| Stabilisers/trim tabs - folding: Top | HR | 3.76 | 1.18 | 14 | 7 | 6.88 | 48.12 |
| Stabilisers/trim tabs - folding: Bottom | HS | 2 | 2.39 | 9 | 7 | 6.88 | 48.12 |
| Marlin board: External | HZ | 0.64 | 2.83 | 7 | 6.17 | 6.67 | 41.11 |
| Engine cooling water filter: Water | IF | 0.37 | 0.01 | 21 | 5.5 | 5.62 | 30.94 |
| Live catch wet well: Water | IL | 0.5 | 0.06 | 6 | 6.5 | 4.5 | 29.25 |
| Traps - Cray/king crab: Water | FT | 0 | NA | 1 | 5 | 5.5 | 27.5 |
| Traps - Cray/king crab: External | FU | 0 | 0 | 2 | 5 | 5.5 | 27.5 |
| Zinc blocks: Behind | HAA | 0.6 | 1.14 | 7 | 5.88 | 4.62 | 27.17 |
| Floats - pots: Water | FN | 0 | NA | 1 | 4 | 5.5 | 22 |
| Floats - pots: External | FO | 0 | NA | 1 | 4 | 5.5 | 22 |
| Bilge - closed: Water | ID | 0.35 | 0.18 | 35 | 4.33 | 4.83 | 20.94 |
| Propeller Shaft: External | PA | 1.89 | 3.82 | 33 | 4.43 | 3.75 | 16.61 |
| Propeller Surface: External | PB | 2.5 | 5.5 | 53 | 4.43 | 3.75 | 16.61 |
| Anchor well: Water | IH | 0.04 | 0.02 | 39 | 3.83 | 3.5 | 13.42 |
| Anchor well: Sediment | II | 0 | 0 | 27 | 3.83 | 3.5 | 13.42 |
| Stern tubes cover/stern gland: Internal | PF | 0 | 0 | 2 | 4.29 | 3 | 12.86 |
| Exhaust outlet: External | HAC | 0 | 0 | 20 | 3.5 | 3.5 | 12.25 |

Table 15 Mean, variance, sample size and IMEA score of transformed sample wet weights by location cont.

| Location name | LocID | mu | var | n | AvSevRat | AvOccRat | SOR |
|---|---|---|---|---|---|---|---|
| Zinc blocks: Front | HAB | 1.54 | 2.5 | 53 | 3.5 | 2.5 | 8.75 |
| Rudder surface: External | PJ | 3.08 | 3.67 | 98 | 4.25 | 1.75 | 7.44 |
| Marker buoys: Water | FAC | 0 | NA | 1 | 2.75 | 2.5 | 6.88 |
| Propeller nozzle: External | PC | 0 | 0 | 2 | 2.6 | 2.5 | 6.5 |
| Cracks in deck/between plates: Water | DA | 0 | 0 | 22 | 2.75 | 2.25 | 6.19 |
| Chain: External | PN | 0 | 0 | 35 | 2.33 | 2.5 | 5.83 |
| Rudder control room: Water | IP | 0.12 | 0.04 | 7 | 2.83 | 1.83 | 5.19 |
| Rudder control room: Internal | IQ | 0 | 0 | 6 | 2.83 | 1.83 | 5.19 |
| Anchor Surface: External | PM | 0 | 0 | 36 | 2.33 | 1.75 | 4.08 |
| Cockpit bins/open storage: Water | DE | 0 | 0 | 7 | 2.5 | 1.5 | 3.75 |
| Winch box: Water | DF | 0 | 0 | 2 | 2.5 | 1.5 | 3.75 |
| Cockpit/Wheelhouse: Water | IV | 0 | 0 | 21 | 1.5 | 2.5 | 3.75 |
| Cockpit/Wheelhouse: Sediment | IW | 0.25 | 1.04 | 16 | 1.5 | 2.5 | 3.75 |
| Gunwale (toe rail): Sediment | DC | 0 | 0 | 4 | 2 | 1.83 | 3.67 |
| Hatches: Water | DD | 0 | 0 | 5 | 1.67 | 2 | 3.33 |
| Surface: Water | DG | 0 | 0 | 8 | 1.5 | 1.83 | 2.75 |
| Storage rooms: Water | IT | 0 | 0 | 4 | 1.83 | 1.33 | 2.44 |
| Storage boxes: Water | IU | 0 | 0 | 3 | 1.83 | 1.33 | 2.44 |
| Dead catch storage – spray room: Water | IX | 0 | 0 | 2 | 1.5 | 1.5 | 2.25 |
| Dead catch storage – insulated: Water | IY | 0 | 0 | 2 | 1.5 | 1.5 | 2.25 |
| Bullwarks: Sediment | DI | 0 | 0 | 3 | 1.5 | 1.25 | 1.88 |
| Canvas screens: Water | DH | 0 | NA | 1 | 1.5 | 1 | 1.5 |
| Sea anchors/parachutes: External | PO | 0 | 0 | 2 | 1.5 | 1 | 1.5 |
| Sea anchors/parachutes: Water | PP | 0 | NA | 1 | 1.5 | 1 | 1.5 |

Figure 5 Mean transformed wet weight by sample location plotted against IMEA results

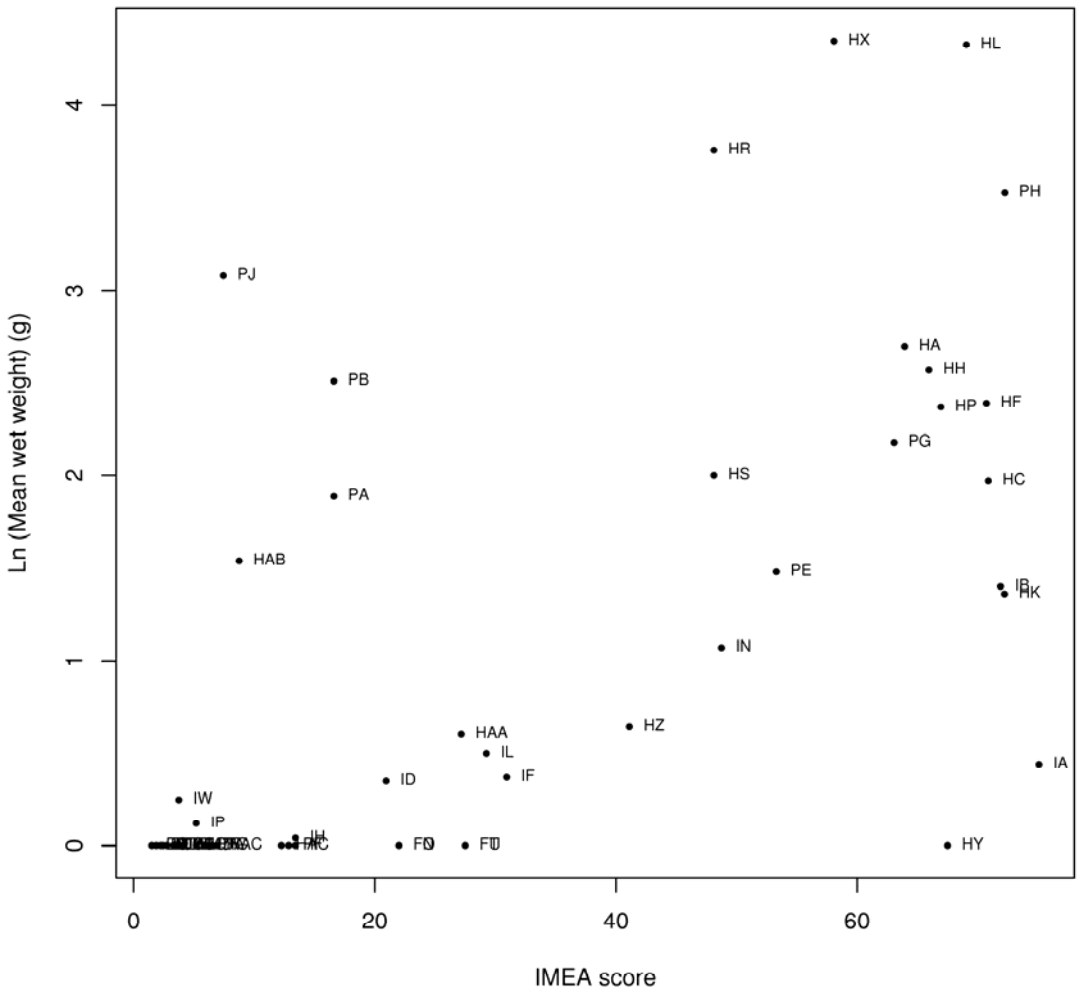


Figure 6 plots normal linear regression diagnostics for the model

$$Y = \alpha + \beta_1 x_1 \qquad [3]$$

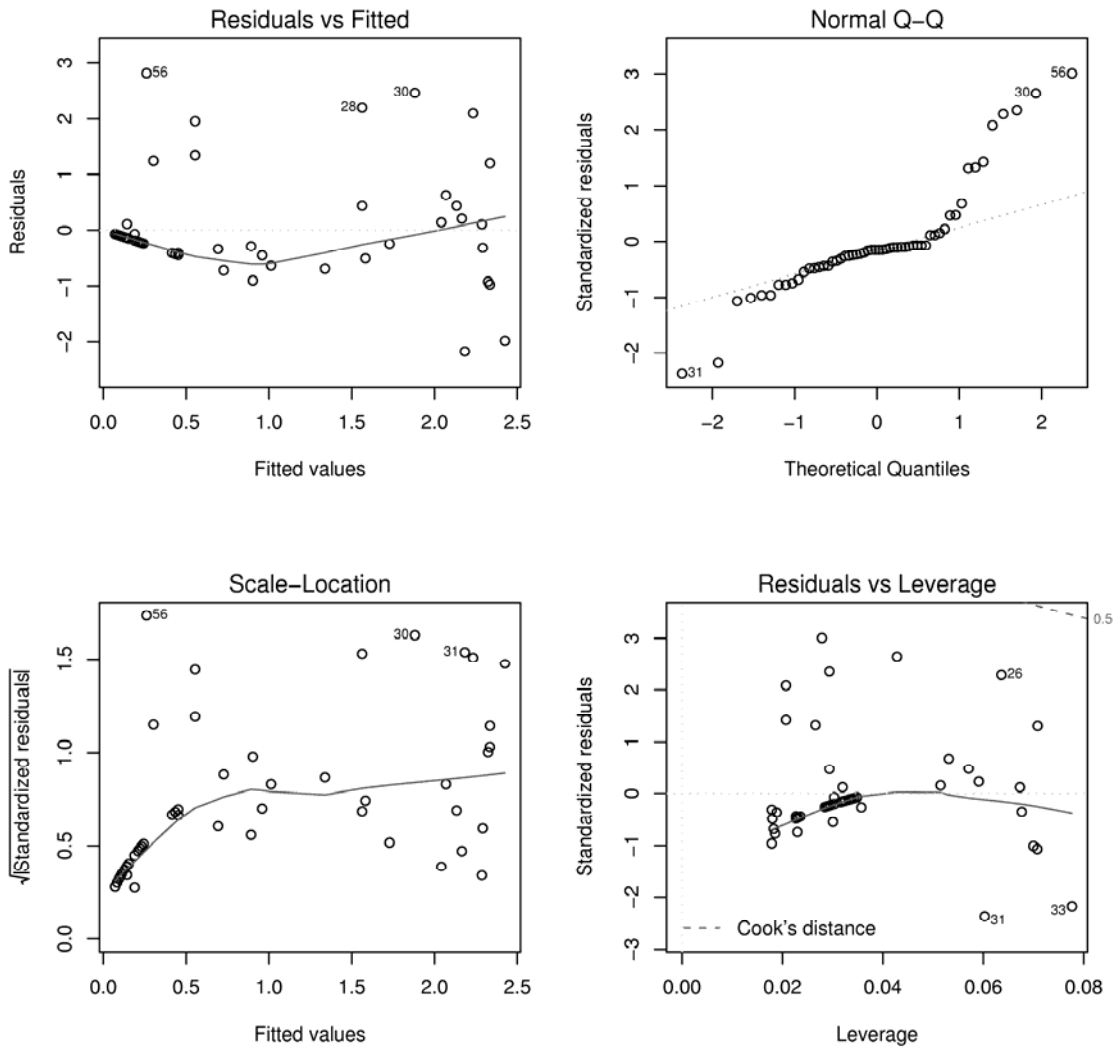
where $Y$ is the mean log-transformed sample weight $f(x)$, $x_1$ is the IMEA score (SOR) and $\alpha$ and $\beta_1$ are coefficients determined by the data (Faraway, 2002). This simple model tests the predictive ability of the IMEA analysis.

Six sample locations were not identified in the IMEA analysis: the boot-top (HAH), transom (HAI), radio earth plate (HAF), auto-pilot sensor (HAE), rudder pintel (PT) and rudder cavity (PU). The boot-top was identified and sampled as a separate location for this analysis to test the incidence of *Undaria pinnatifida* fouling in this location as compared to other locations on the hull. The radio earth plate and auto-pilot sensor are indistinguishable (morphologically) from water inlet/outlet cover plates and transducers respectively, and do not therefore represent genuine site omissions. The two additional rudder locations, however, were encountered during the vessel sampling regime and represent genuine omissions from the IMEA hazard analysis.

It is clear from Figure 5 and Figure 6 that the severity and occurrence components of the IMEA analysis provide a reasonable indication of the hull fouling bio-invasion hazard for most of the 56 IMEA locations that matched the sampled or inspected locations. In the simple linear model [3] the coefficient $\beta_1$ is a significantly different from 0 ($p < 0.001$, on 54 degrees of freedom, $R^2 = 0.44$) suggesting that the IMEA score has some predictive power in this context. There are, however, two important outlier groups. The first group consists of locations that were fouled to a much greater degree than predicted by the IMEA score. This group includes the rudder surface (PJ, data index = 56), the garboard plank (HX, data index = 30), the propeller surface (PB), folding stabilisers/trim tabs (HR, data index = 28) and block spaces (HL, data index = 26). The second group consists of locations that were much less heavily fouled than predicted by the IMEA score. This group includes the bob-stay fitting (HY, data index = 31) and the water from seawater/grey water inlets/outlets (IA).

The rudder surface (PJ) and propeller surface (PB) scored lower than the hull surface during the IMEA analysis because some of the participants believed that the high flow velocity associated with the propeller and rudder made these areas less suitable for fouling organisms. The variance of the IMEA score for the propeller surface, however, was relatively high (see Tables 3 and 4, Hayes, 2002) because of disagreement among the workshop participants about how stationary the propeller (which is not anti-fouled) may be, and therefore how much fouling might colonise it. The results of this analysis suggests that the rudder surface and propeller surface are no less amenable to fouling than the hull surface.

Figure 6 Wet weight ~ IMEA score regression diagnostics



The low IMEA score for the stabilisers/trim tabs can also be attributed to high variance and disagreement amongst the workshop participants over the environmental suitability and occurrence of fouling in this location (see Tables 3 and 4, Hayes, 2002). The disagreement in this context centres around the extent to which the stabilisers are folding (and therefore periodically dry) or fixed (and therefore in an ambient marine environment). Some of the stabilisers/trim tabs sampled in this project were folding but a number were also fixed. The fixed structures appear to have been misclassified (i.e. should have been recorded as fixed rolling chocks), leading to the anomalous score.

The anomalously low IMEA scores for the garboard plank and block spaces are more difficult to explain. Participants at the IMEA workshop recognised that the antifouling paint was abraded (garboard plank) or absent (block spaces) and scored both of these areas with a score similar to (garboard plank) or slightly higher than (block spaces) the hull surface. It appears as if the participants simply underestimated the much higher levels of fouling that accumulate in these areas.

With the exception of the inlet/outlet water, the level of biofouling found in the internal spaces is reasonably well reflected by the IMEA predictions. The wet-weight of biofouling is not a reasonable reflection of the bio-invasion hazard associated with any of the water samples taken from vessels because this metric does not capture the bio-invasion hazards associated with microscopic organisms such as larvae, diatoms and dinoflagellates. The participants at the IMEA workshop, however, recognised this hazard and gave a high score to inlet/outlet water.

The reasons for the anomalously high score attributed to the bob-stay fitting is less clear. In all the vessel inspections the bob-stay fitting was clean and dry but the IMEA participant gave this location a very high score relative to other fouling locations. In hindsight this may reflect linguistic uncertainty. The environmental suitability scores for the bob-stay fitting range from 8 to 10 suggesting an ambient environment (i.e. below the water line), whereas in the all vessels inspected the bob-stay fitting was above the water line.

No biomass samples were collected from either the deck or the fishing gear of the vessels sampled during the first year of the project. For the deck these results accord well with the predictions of the IMEA which allocated low scores to all of the locations within this category. For fishing gear, however, there is some departure from the predictions of the IMEA, particularly for the external surfaces and water collected in traps and floats and pots. The survey team found all traps, floats and pots to be clean and dry upon inspection. As noted above, however, virtually all of the fishing gear inspected by the project team had been cleaned by the crew prior to the vessel being slipped.

### 3.4.6 Undaria probe results for vessel and plankton samples

The *Undaria* specific ITS rDNA probes were initially applied to 36 hull fouling samples, from two vessels, in a preliminary attempt to confirm the specificity and sensitivity of the probe. Both of these vessels were heavily fouled by a well developed fouling community including adult specimens of *Undaria pinnatifida* (Appendix 9). A total of three PCR positive results were obtained all of which were sequenced and confirmed to be similar to those of *U. pinnatifida*. The DNA from 69 plankton and 430 hull samples was subsequently extracted, quantified and analysed using these probes.

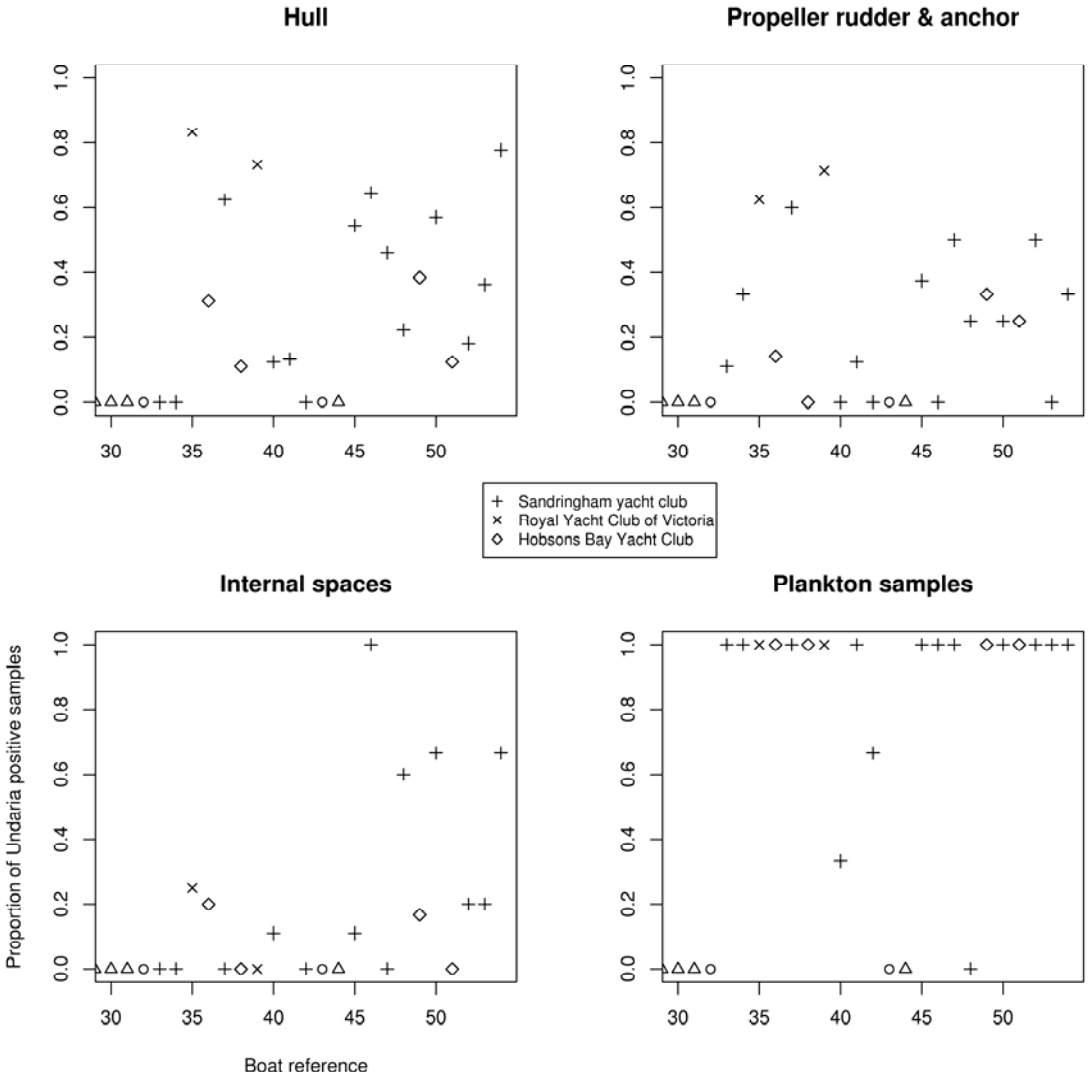
Figure 7 plots the proportion of samples from the three main vessel locations (hull, propeller rudder and anchor, and internal spaces) and the plankton, that tested positive for *Undaria.* Unlike *Asterias amurensis* relatively little was known about the seasonality of *Undaria pinnatifida's* life-cycle at the time of this project's field work. Vessel and plankton samples for Stage II of this project were taken in late August and late November (see section 3.1) – the corresponding boat references are 33 to 42 inclusive and 45 to 54[5]. All of the plankton samples (and in most cases all of the replicate samples) taken at these times, except one, tested positive for *Undaria*. The one exception are the plankton replicates taken on the 30th November (boat reference 48) that all returned negative responses. These results agree with Schaffelke *et. al.* (2005) who recently recorded high numbers of *U. pinnatifida* zoospores in the plankton between October and January at the Tinderbox marine reserve in Tasmania. Taken together these results confirm that *U. pinnatifida* zoospores are likely to be present in the water column from July through to January and possibly beyond – depending on ambient water temperatures.

The high proportion of plankton positive samples mask possible vessel-related fouling because we are unable to distinguish whether vessel positives are due to settled zoospores and/or gametophytes on the vessel or from zoospores from the seawater that may have been deposited on the vessel as it was pulled from the water onto the slip. Vessel positive samples were returned from the rudder, hull and internal space of boat 48 (the only vessel associated with negative plankton samples). This provides some evidence for *Undaria* settlement on the vessel but the sample size is clearly too small to warrant further analysis. The project results confirm that the *Undaria* probes can be applied to (properly treated) hull fouling samples. The project, however, was unable to detect any signals or patterns in the settlement of *Undaria* zoospores on small vessels due to the high proportion of plankton positive samples.

The second-stage of the project collected seven internal water samples, of which two (head inlet water of vessel 40 and water from the rudder cavity of vessel 49) tested positive for *Undaria*. These results suggest that the internal water systems of recreational vessels can potentially serve as a translocation vector of *Undaria pinnatifida*.

[5] Vessels 43 and 44 were sampled in Hobart and probed for *Asterias* only.

Figure 7 Proportion of *Undaria* positive samples from the three main location categories and the plankton



### 3.4.7 Quantitative risk factors

Zero (or near zero) hull-fouling biomass locations can be divided into two categories: a) those locations which are permanently or periodically dry (such as the wheelhouse, anchor well, cockpit bins, etc) and cannot therefore be colonised by marine organisms; and, b) those locations that are permanently or periodically immersed in seawater but are clean due to the management practises of the vessel owner. It is this latter group which is primarily of interest.

Appendix 10 summarises the relationship between the proportion of zero samples ($n_0/n$: see Table 12 and Table 13) and three potential explanatory variates: a) the number of days since the vessel was last used (days1); b) the number of days since the vessel was last slipped and cleaned[6] (days2); and, c) the median number of vessel trips per annum (midTrips), for nine locations. These plots suggest that days1 and midTrips have very little predictive power in this context. On initial inspection the predictive power of days2 appeared to be more promising, but only for the seawater/grey water inlets/outlets (IB), and the seawater/grey water inlet/outlet cover plates (HD), and perhaps to a lesser extent in the paddle wheel and booth (HK) and the depth sounder booth (HH). Interestingly, these are all areas whose function is likely to be threatened or at least impaired by fouling organisms.

Quasi-likelihood analysis (a less-restrictive form of Generalized Linear Model) was used to test the predictive power of days2. In this context the most appropriate GLM is the binomial logistic model wherein the response variable $y$ is the proportion of zero samples ($n_0/n$)and the explanatory variable $x$ is the number of days since the vessel was last cleaned.

In the logistic model the number of zero samples is given by

$$y = \frac{\exp(\alpha + \beta x)}{1 + \exp(\alpha + \beta x)} \qquad 0 \le y \le 1 \quad , \qquad [4]$$

where α and β are parameters to be estimated from the data. Equation [4] is linearised by the logit link function

$$\log \frac{y}{1 - y} = \alpha + \beta x \quad . \qquad [5]$$

Under this logistic model the number of zero samples is assumed to follow a binomial model with mean $E(Y) = p = n_0/n$ and variance $V(Y) = p(1 - p)/n$. The quasi-likelihood approach, however, makes no assumption about the distribution form of the response variable (which in turn determines the mean-variance relationship) but rather simply specifies a mean-variance function of the form

$$\upsilon(p) = \phi p(1 - p)/n \qquad [6]$$

[6] In a rare number of cases this represents the number of days since the vessel was last clean whilst still in the water.

The parameter $\Phi$ is a dispersion parameter which allows the variance of the model to be inflated (or deflated) around the mid-values of $y$ (Agressi, 2002). The maximum likelihood equations of a quasi-likelihood model are usually non-linear and are fitted to data using a variety of techniques, including the Newton-Raphson method and iterative re-weighted least squares. Model fits for this analysis were performed using the programming language R.

The results of the quasi-likelihood analysis indicate that the coefficient β (days2) in the logistic model [4] is significantly different from zero ($p < 0.05$) for the seawater/grey water inlets/outlets (IB) ($p \sim 0.001$), the paddle wheel and booth (HK) ($p \sim 0.192$) and the cover plates (HD) ($p \sim 0.0412$). Figure 8 shows the predicted proportion of zero samples for each model with 95% confidence limits. The model results suggest that the proportion of clean samples diminishes very quickly after the vessel has been cleaned. The wide confidence limits, caused in part by the low number of intermediate (between 0 and 1) samples, are indicative of a poorly fitting model with poor predictive power. Further quantitative analysis of this relationship, including outlier removal, is unlikely to provide additional predictive power and is not therefore warranted.

The wet weight fouling biomass of non-zero samples is approximately log-distributed in at least four sample locations (section 3.4.4). The distribution of the wet weight of the non-zero hull quadrat samples has a notably longer right tail than the log-normal distribution, but may nonetheless be amenable to statistical tests that assume normality because of the large sample size (Faraway, 2002). An initial analysis of the data (Table 16) suggests that there is a negative relationship between the log-transformed mean[7] wet-weight of the biofouling collected from the hull quadrats and the median number of vessel trips per annum, whereas there appears to be a positive relationship between the biofouling biomass and the number of days since the vessel was last cleaned or antifouled (Figure 9). These results suggest a linear model of the form:

$$\log(Y) = \alpha + \beta X + \varepsilon \quad , \qquad [7]$$

where $\boldsymbol{Y} = (y_1, y_2, \ldots . y_i)$ is the mean wet weight of fouling biomass on each vessel , $\boldsymbol{X} = (x_1, x_2, \ldots . x_n)$ are explanatory covariates or "predictors", α and $\boldsymbol{\beta} = (\beta_1, \beta_2 \ldots \beta_n)$ are coefficients to be determined from the data, and $\varepsilon \sim N(0, \sigma^2 I)$ are normal random measurement errors in $\mathbf{Y}$ (Faraway, 2002). A more detailed exploratory analysis of the relationship between biofouling wet weight and potential predictors suggests an analysis of covariance model of the form:

$$\log(Y) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_2 \cdot X_3 + \varepsilon \qquad [8]$$

where $\mathbf{X}_1$ is days since the vessel was last cleaned or antifouled (days2), $\mathbf{X}_2$ is median number of vessel trips per annum (midTrips), and $\mathbf{X}_3$ is a qualitative predictor (paint type) with three levels (ablative, hard and self-polishing) and $\varepsilon \sim N(0, \sigma^2 I)$ (Faraway, 2002).

[7] Averaged across the 4 quadrats taken from the hull of each vessel

Figure 8 Actual and predicted proportion of zero samples (with 95% confidence limits) based on a quasi-likelihood analysis

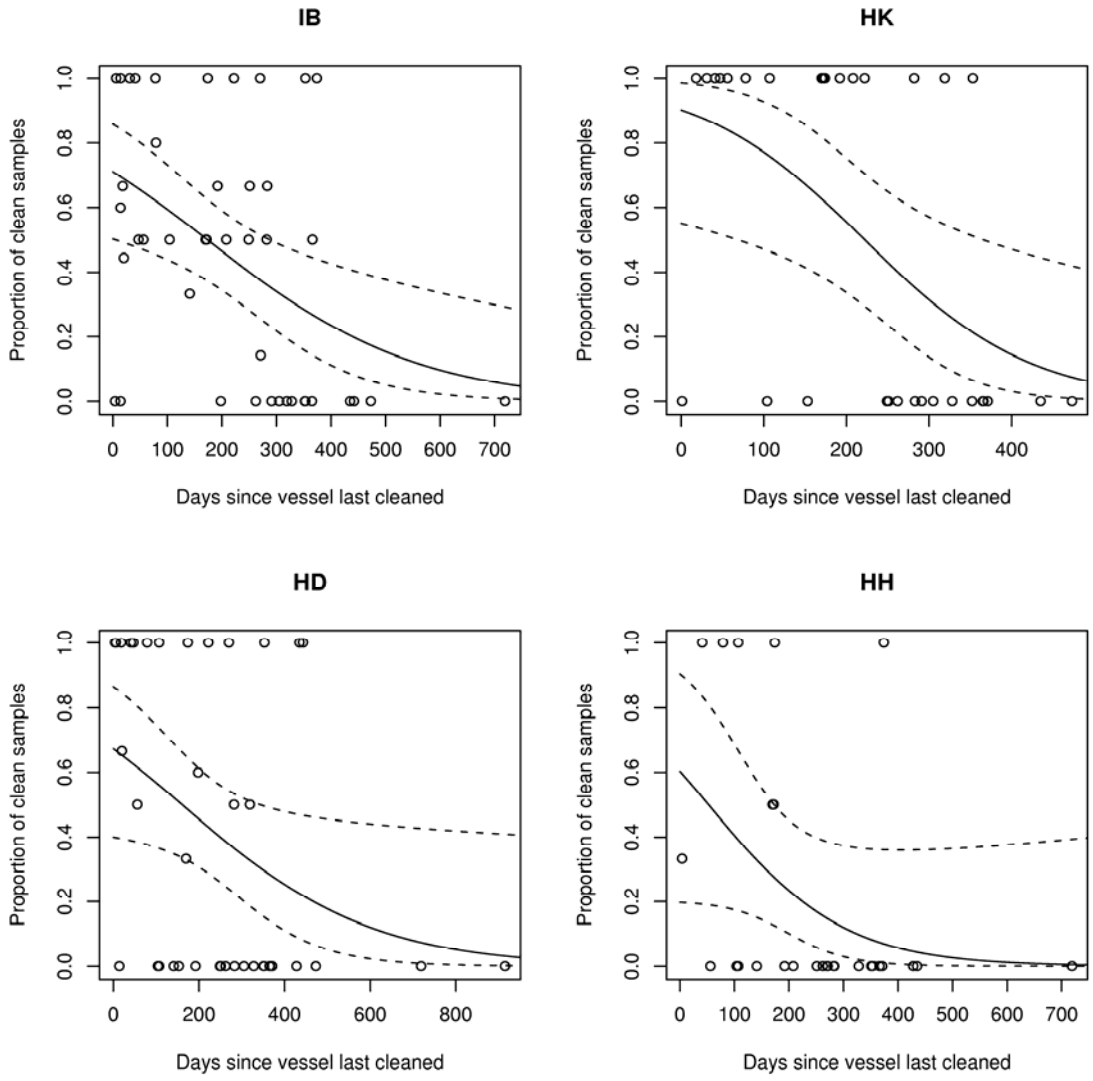
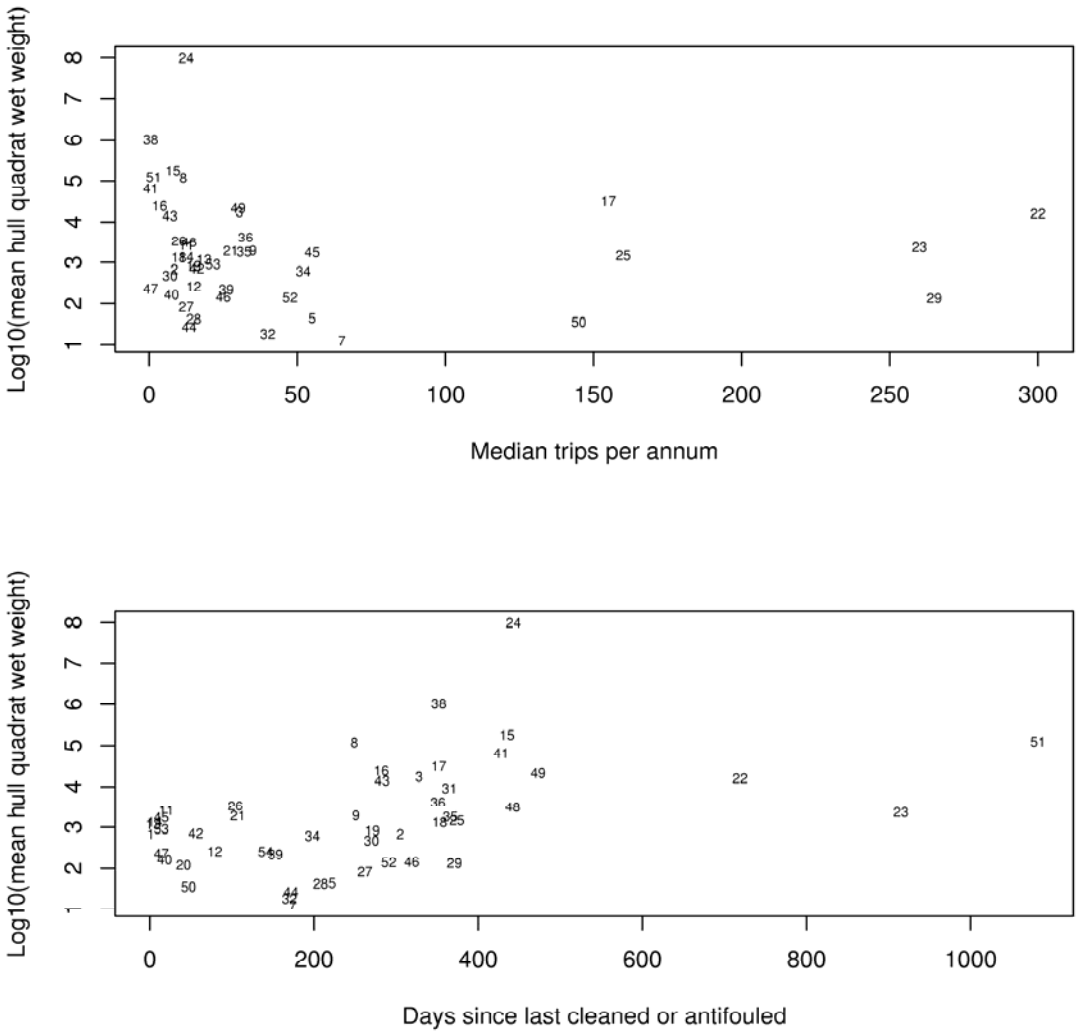


Table 16 Analysis of covariance data: Vessel characteristics and wet weight of hull quadrat biomass by levels of antifouling paint type

| ID[a] | days2[b] | midTrips[c] | paintType | Y[d] | ID[a] | days2[b] | midTrips[c] | paintType | Y[d] |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 305 | 8.5 | Ablative | 2.83 | 30 | 270 | 7 | Ablative | 2.67 |
| 5 | 222 | 55 | Ablative | 1.64 | 32 | 170 | 40 | Ablative | 1.26 |
| 7 | 174 | 65 | Self-polishing | 1.10 | 34 | 198 | 52 | Hard | 2.78 |
| 9 | 251 | 35 | Self-polishing | 3.28 | 35 | 366 | 32 | Ablative | 3.25 |
| 11 | 20 | 12.5 | Hard | 3.41 | 36 | 351 | 32.5 | Ablative | 3.62 |
| 12 | 79 | 15 | Ablative | 2.41 | 38 | 352 | 0.5 | Ablative | 6.01 |
| 13 | 4 | 18.5 | Self-polishing | 3.06 | 39 | 153 | 26 | Hard | 2.34 |
| 14 | 6 | 12.5 | Ablative | 3.13 | 40 | 18 | 7.5 | Ablative | 2.22 |
| 15 | 435 | 8 | Ablative | 5.26 | 41 | 428 | 0.5 | Self-polishing | 4.81 |
| 16 | 282 | 3.5 | Ablative | 4.38 | 42 | 56 | 16 | Hard | 2.85 |
| 17 | 352 | 155 | Hard | 4.50 | 43 | 283 | 7 | Ablative | 4.14 |
| 18 | 353 | 10 | Self-polishing | 3.11 | 44 | 172 | 13.5 | Self-polishing | 1.44 |
| 19 | 271 | 15 | Ablative | 2.92 | 45 | 14 | 55 | Self-polishing | 3.24 |
| 21 | 107 | 27.5 | Ablative | 3.28 | 46 | 319 | 25 | Ablative | 2.16 |
| 23 | 915 | 260 | Self-polishing | 3.36 | 48 | 442 | 13.5 | Self-polishing | 3.50 |
| 24 | 443 | 12.5 | Ablative | 8.00 | 49 | 473 | 30 | Ablative | 4.33 |
| 25 | 374 | 160 | Self-polishing | 3.16 | 50 | 47 | 145 | Hard | 1.55 |
| 26 | 104 | 10 | Self-polishing | 3.53 | 51 | 1082 | 1.5 | Ablative | 5.09 |
| 27 | 262 | 12.5 | Self-polishing | 1.92 | 52 | 291 | 47.5 | Ablative | 2.14 |
| 28 | 208 | 15 | Hard | 1.62 | 53 | 14 | 21.5 | Hard | 2.95 |
| 29 | 371 | 265 | Self-polishing | 2.13 | | | | | |

[a]ID = Unique vessel identification code
[b]Days2 = Days since the vessel was last cleaned or antifouled
[c]MidTrips = Median number of vessel trips per annum
[d]Y = Log-transformed wet-weight of hull quadrat biofouling samples

Figure 9 Relationship between mean wet weight of hull quadrat biofouling and two explanatory variables: vessel activity (median trips per annum) and days since the vessel was cleaned or antifouled (Numbers in each plot are the vessel references (boatID))



A diagnostic analysis of the model residuals (Table 17 and Figure 10) does not point to any higher order terms or variance problems (heteroscedascity). Several of the vessels appear to have high leverage but this was not confirmed by Cook's distance. The residual and Q-Q plots, however, clearly point to two outliers: vessel 24 (regression index 21) and vessel 51 (regression index 46). Vessel 51 was sampled at Hobson's Bay Yacht club on the 1st December 2004. It was last slipped approximately 3 years earlier and was only used up to 3 times per annum. 16 adult *Undaria pinnatifida* plants were removed from this vessel (Appendix 9) but the level of hull fouling was much lower than expected. The vessel's owner reported that he had scrubbed the waterline 6 months prior to the sample date, but it is unclear why the fouling biomass is so low. Vessel 24 was sampled at the Royal Hobart Yacht Club on the 2nd of April 2004. It was last slipped and cleaned in January 2004 (assumed to be the 15th), and was used up to 24 times per annum. The vessel was very heavily fouled with (on average) over 3.5 kgs of biomass taken from each of the hull quadrats (surface area 0.5 $m^2$ ). Again it is unclear why the level of fouling on this vessel was so extreme.

Table 18 shows the parameter estimates for the Analysis of Covariance model [8] when all vessels are included in the model[8] (Table 16). These results show that the log-transformed wet weight of a quadrat of hull biofouling increases by 0.003 grams for each day that the vessel is not cleaned or antifouled given an ablative antifouling paint and average levels of vessel activity. This result if highly significant but note that the vessel's cleaning activity only explains about a third of the variance in this dataset (Table 19).

The log-transformed wet-weight of biofouling decreases by about 0.05 grams for each day of increased median vessel activity given an average cleaning cycle and ablative antifouling paint. Again this result is significant but only explains an additional 16% of the variance in the data-set. Paint type of itself is not a significant explanatory variable. Paint type does, however, influence the effect of vessel activity on the wet weight of biofouling. For example, if the anti-fouling paint is hard, then this totally offsets the effect of vessel activity compared to a vessel with ablative paint – i.e. vessel activity has almost no net effect on hull fouling biomass if the vessel is painted with a hard paint compared to an ablative paint, on an average cleaning cycle. These results are consistent with the mode of action of the different paint types – hard paints are imbued with toxic chemicals to defer fouling whereas ablative paints are soft and shed layers in response to water movement across them.

The mediating effect of paint type on vessel activity's influence on biofouling is more significant if the two outliers are omitted from the Analysis of Covariance model (Table 20 and Table 21). Again the hard paints totally offset the biofouling reduction gained by using boats anti-fouled with ablative paints. This effect is less marked for vessels painted with self-polishing paints. These results suggest that, for an average cleaning cycle, the biofouling reduction gained by vessel use diminishes by almost 90% when a vessel is painted with a self-polishing paint as opposed to an ablative paint, and is totally offset when the vessel is painted with a hard paint. These results are statistically significant but it is important to note that the combined effect of the days since the vessel was last used, and its activity patterns mediated by paint type, only explain just over half of the variance in this data set. This remains true with and without the outliers included in the model.

[8] Ablative anti-fouling paint is the default reference case.

Table 17 Regression diagnostics for biofouling analysis of covariance. Figures in bold indicate potential outliers

| | Residuals | | | Leverage | Cooks | | Residuals | | | Leverage | Cooks |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ID[a] | $r_i$ | $s_i$ | $t_i$ | $h_{ii}$ | Distance | ID | $r_i$ | $s_i$ | $t_i$ | $h_{ii}$ | Distance |
| 2 | -1.2 | -1.11 | -1.11 | 0.076 | 0.0143 | 30 | -1.33 | -1.23 | -1.24 | 0.086 | 0.02037 |
| 5 | -0.02 | -0.03 | -0.03 | **0.315** | 0.00005 | 32 | -0.95 | -0.91 | -0.9 | 0.143 | 0.01953 |
| 7 | -1.49 | -1.37 | -1.39 | 0.086 | 0.02548 | 34 | -0.15 | -0.14 | -0.14 | 0.129 | 0.00044 |
| 9 | 0.32 | 0.3 | 0.3 | 0.089 | 0.00126 | 35 | 0.13 | 0.12 | 0.12 | 0.09 | 0.00020 |
| 11 | 1.01 | 1 | 1 | 0.202 | 0.03617 | 36 | 0.56 | 0.52 | 0.51 | 0.091 | 0.00382 |
| 12 | -0.7 | -0.65 | -0.64 | 0.094 | 0.00619 | 38 | 1.48 | 1.4 | 1.42 | 0.125 | 0.03968 |
| 13 | 0.7 | 0.67 | 0.66 | 0.134 | 0.00982 | 39 | -0.45 | -0.43 | -0.43 | 0.161 | 0.0051 |
| 14 | 0.1 | 0.1 | 0.1 | 0.131 | 0.00020 | 40 | -1.07 | -1.03 | -1.03 | 0.152 | 0.02703 |
| 15 | 0.84 | 0.77 | 0.77 | 0.083 | 0.00781 | 41 | 1.18 | 1.14 | 1.14 | 0.161 | 0.03538 |
| 16 | 0.18 | 0.17 | 0.17 | 0.106 | 0.00051 | 42 | 0.34 | 0.34 | 0.33 | 0.188 | 0.00377 |
| 17 | 1.04 | 1.35 | 1.37 | **0.534** | 0.2981 | 43 | 0.1 | 0.09 | 0.09 | 0.085 | 0.00012 |
| 18 | -0.26 | -0.25 | -0.24 | 0.126 | 0.00124 | 44 | -1.42 | -1.33 | -1.34 | 0.108 | 0.0306 |
| 19 | -0.73 | -0.66 | -0.65 | 0.056 | 0.00367 | 45 | 1.04 | 0.99 | 0.99 | 0.126 | 0.02005 |
| 21 | 0.67 | 0.62 | 0.61 | 0.085 | 0.00503 | 46 | -1.16 | -1.05 | -1.06 | 0.057 | 0.00963 |
| 23 | -0.23 | -0.28 | -0.28 | **0.488** | 0.01073 | 48 | -0.1 | -0.1 | -0.1 | 0.144 | 0.00024 |
| 24 | 3.76 | **3.44** | **4.21** | 0.069 | 0.12639 | 49 | 0.82 | 0.76 | 0.76 | 0.101 | 0.00929 |
| 25 | 0.53 | 0.51 | 0.5 | 0.153 | 0.00671 | 50 | -1.05 | -1.27 | -1.28 | **0.465** | 0.20032 |
| 26 | 0.84 | 0.79 | 0.79 | 0.118 | 0.01196 | 51 | -1.42 | -1.74 | -1.79 | **0.481** | 0.39803 |
| 27 | -1.19 | -1.11 | -1.12 | 0.11 | 0.0219 | 52 | -0.06 | -0.06 | -0.06 | 0.218 | 0.00015 |
| 28 | -1.3 | -1.28 | -1.3 | 0.199 | 0.0586 | 53 | 0.56 | 0.54 | 0.54 | 0.176 | 0.00895 |
| 29 | 0.07 | 0.08 | 0.08 | **0.458** | 0.00081 | | | | | | |

[a]ID = Vessel reference

Figure 10 Regression diagnostics for biofouling analysis of covariance. Numbers in plot refer to the regression index (not the vessel reference).

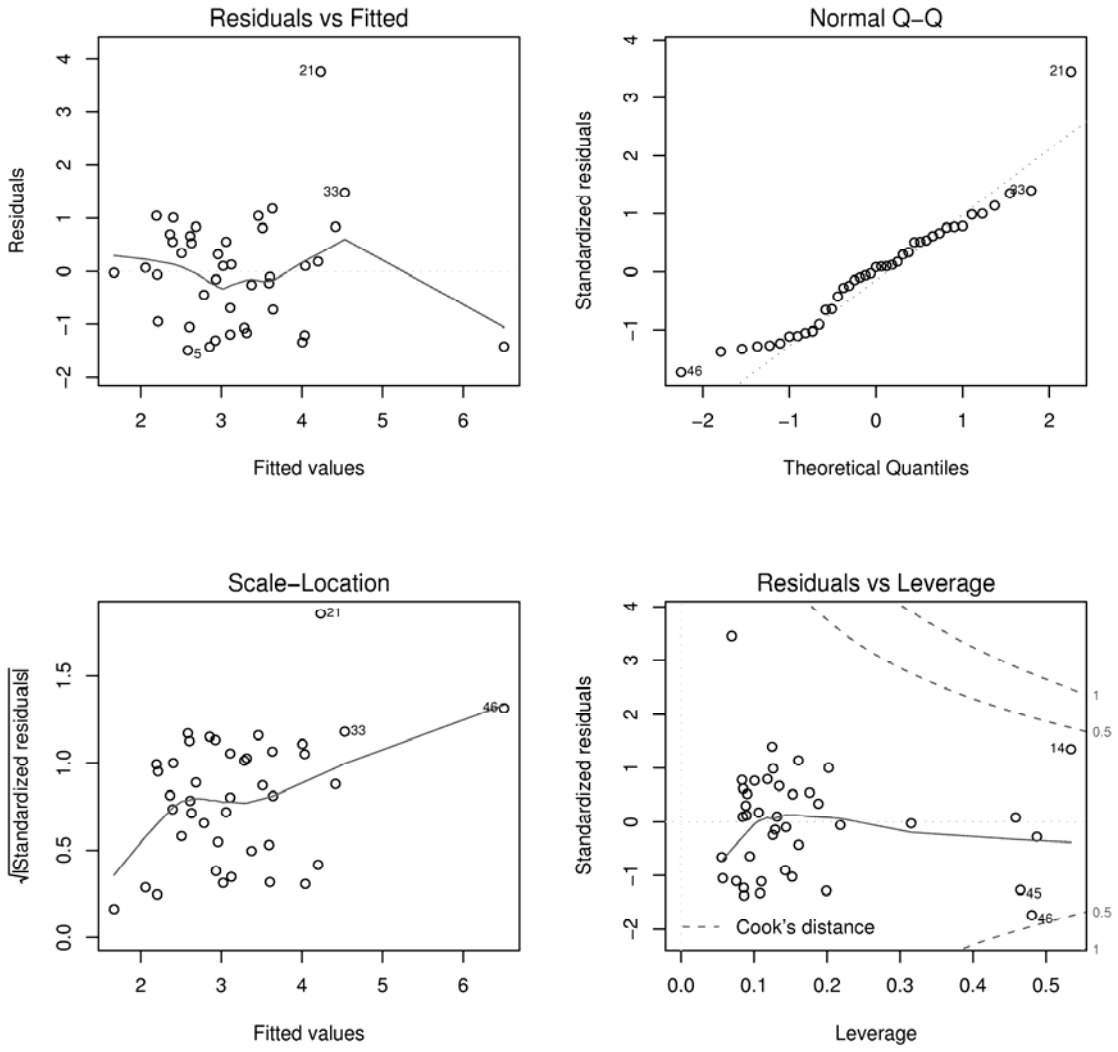


Table 18 Parameter estimates for Analysis of Covariance model (all vessels) of hull biofouling wet weight by vessel activity and antifouling paint type

Model: $\log(Y) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_2 \cdot X_3 + \varepsilon$

| Coefficients | Estimate | Std. error | t value | Pr (> \| t \|) |
|---|---|---|---|---|
| Intercept (α) | 3.580 | 0.532 | 6.735 | 0.0000*** |
| Days2 ($\beta_1$) | 0.003 | 0.001 | 2.932 | 0.0060** |
| midTrips ($\beta_2$) | -0.046 | 0.017 | -2.765 | 0.0091** |
| paintType - hard ($\beta_{32}$) | -1.243 | 0.744 | -1.672 | 0.1037 |
| paintType - self-polishing ($\beta_{33}$) | -1.129 | 0.590 | -1.911 | 0.0645 |
| midTrips:paintType – hard ($\beta_{42}$) | 0.047 | 0.018 | 2.571 | 0.0147* |
| midTrips:paintType – self polishing ($\beta_{43}$) | 0.041 | 0.017 | 2.352 | 0.0246* |

Table 19 Hierarchical ABOVA for Analysis of Covariance model (all vessels) of hull biofouling wet weight by vessel activity and antifouling paint type

| | dF | Sum of Squares | Mean square | F value | Pr (> F) |
|---|---|---|---|---|---|
| Days2 | 1 | 14.301 | 14.301 | 11.154 | 0.0020** |
| midTrips | 1 | 7.022 | 7.022 | 5.476 | 0.0253* |
| paintType | 2 | 0.708 | 0.354 | 0.276 | 0.7603 |
| midTrips: paintType | 2 | 8.481 | 4.241 | 3.307 | 0.0487* |
| Residuals | 34 | 43.594 | 1.282 | | |

*significant at an alpha level of 0.05
** significant at an alpha level of 0.01
***significant at an alpha level of 0.001

Table 20 Parameter estimates for Analysis of Covariance model (outliers omitted) of hull biofouling wet weight by vessel activity and antifouling paint type

Model: $\log(Y) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_2 \cdot X_3 + \varepsilon$

| Coefficients | Estimate | Std. error | t value | Pr (> \| t \|) |
|---|---|---|---|---|
| Intercept (α) | 3.291 | 0.438 | 7.515 | 0.0000*** |
| Days2 ($\beta_1$) | 0.003 | 0.001 | 3.284 | 0.0025** |
| midTrips ($\beta_2$) | -0.045 | 0.014 | -3.271 | 0.0026** |
| paintType - hard ($\beta_{32}$) | -1.001 | 0.604 | -1.657 | 0.1072 |
| paintType - self-polishing ($\beta_{33}$) | -0.949 | 0.485 | -1.956 | 0.0592 |
| midTrips:paintType – hard ($\beta_{42}$) | 0.046 | 0.015 | 3.047 | 0.0046** |
| midTrips:paintType – self polishing ($\beta_{43}$) | 0.039 | 0.014 | 2.762 | 0.0094** |

Table 21 Hierarchical ANOVA for Analysis of Covariance model (outliers omitted) of hull biofouling wet weight by vessel activity and antifouling paint type

| | dF | Sum of Squares | Mean square | F value | Pr (> F) |
|---|---|---|---|---|---|
| Days2 | 1 | 5.978 | 5.978 | 7.122 | 0.0119* |
| midTrips | 1 | 5.016 | 5.016 | 5.976 | 0.0202* |
| paintType | 2 | 0.475 | 0.238 | 0.283 | 0.7553 |
| midTrips:paintType | 2 | 7.795 | 3.897 | 4.644 | 0.0170* |
| Residuals | 32 | 26.857 | 0.839 | | |

*significant at an alpha level of 0.05
** significant at an alpha level of 0.01
***significant at an alpha level of 0.001

# 4. CONCLUSIONS AND DISCUSSION

The four primary aims of Stage II of this project were: a) to develop a new genetic probe for *Undaria pinnatifida*, b) sample the internal and external spaces and surfaces of small vessels to determine their translocation potential (with specific reference to *U. pinnatifida*); c) develop a Bayesian journey survival model to estimate the probability of target species survival on small vessels; and, d) develop quantitative risk factors for fouling biomass based on vessel activity, paint type and management practices. The Bayesian journey survival model could not be developed because the project team were unable to take successive (e.g. daily) hull fouling or internal water samples from any of the sample vessels. In all cases the characteristics (e.g. self draining wet wells) or activity patterns (e.g. periodic short trips of less than a day) of the vessel precluded such a sampling regime.

The development of the *Undaria pinnatifida* specific gene probe took much longer to develop than expected, primarily because the initial target – 602 nucleotides of the Large Sub Unit ribosomal DNA (LSU rDNA) – was identical (or nearly so) for at least two other native species: *Ecklonia radiata* and *Macrocystis pyrifera*. Further development of other regions of the LSU proved fruitless, despite our initially high expectations. The project team therefore sought other regions of the *U. pinnatifida* genome for inter-specific variation, eventually settling on the RuBisCo spacer region and the rDNA internal transcribed spacer regions (ITS1 & ITS2).

Probes designed around two regions of significant variation in the RuBisCo spacer region proved specific against all test species except (possibly) two non-native Laminariales: *Lessonia nigrescens* and *Alaria esculenta*. This utility of this probe was further undermined by its ambiguous signature on agarose gel. It is unlikely to prove effective without further laboratory refinements.

ITS regions are notoriously variable both within and between species. The project was therefore surprised to find very little within-species variation in the 15 species of Laminariales that were sequenced and compared. The relatively small intra-specific variation, coupled with the (as expected) high inter-specific variation allowed us to eventually develop what we believe to be an *Undaria* specific probe. This probe did give unexpectedly positive PCR results for three other species, but in all cases we are confident that these results were caused by contamination of the samples with *Undaria pinnatifida* DNA at the time of DNA extraction – i.e. before we received them.

The ITS probes were tested and the subsequently applied to 430 hull fouling samples and 69 plankton samples. The project therefore successfully developed a new gene probe for *Undaria* and confirmed that this probe can be used to test for the presence or absence of microscopic *Undaria* plants in hull fouling samples. All of the plankton samples, bar one, taken during August and November returned positive for *Undaria*. These results add further weight to other independent observations of *Undaria pinnatifida* zoospores in the plankton at this time of year, but also act to mask any patterns in the high proportion of hull fouling samples that returned positive.

The project team sampled 54 vessels at 5 locations during the course of Stage I and Stage II, taking 1116 samples, and making further 365 inspections, in 63 different locations around the hull, internal spaces, deck and fishing gear. The distribution of biofouling is quite different

between locations. Many of the locations, on many occasions, were clean, or nearly so, returning samples that weighed less than 1.5 grams – i.e. lower than the reliable limit of a wet weight sample.

The wet weight of the more substantially fouled samples can be approximately described by a log-normal distribution, although in some cases (such as the hull quadrats) rare incidences of very heavily fouled vessels results in a distribution with greater probability mass in the right tail. For all the very heavily fouled vessels the management and use patterns of these vessels are atypical, bordering on derelict – i.e. left on a mooring for periods of a year or more without being used. These types of vessels clearly pose an unacceptable translocation risk. They, however, easily identified as a distinct sub-set of small craft and can therefore be managed separately from the vast majority of small pleasure craft and fishing vessels.

The majority of vessels that we inspected and sampled were cleaned and slipped at least once a year, and used at least once every one to two weeks. Some of these vessels returned positive results when tested with the *Undaria* (and Asterias) gene probes. These results suggest well maintained small craft can still act as a possible vector for the translocation of marine pests. Seawater held in the internal water systems of vessels that tested positive for *Undaria*, for example, indicates that vessels that travel with closed seacocks from an area where *Undaria* is present in the water column could potentially transfer "contaminated" water to new areas.

The wet weight of biofouling on the wet areas (below the water line) of these regularly used vessels can be approximately described by a delta distribution – i.e. a log-normal distribution with a higher than usual probability mass at zero, or in this case near zero. An Analysis of Covariance model confirms that the number of days since the vessel was last cleaned, and the median number of trips per annum, are statistically significant explanatory variable of the bio-fouling wet weight of the "non-zero" samples. The effect of the vessel activity, however, is strongly mediated by the type of antifouling paint used by the vessel. This effect is strongest when the vessel is painted with an ablative paints, is relatively weak when the vessel is painted with a self-polishing paint and totally eliminated if the vessel is painted with a hard paint. These results highlight the importance of regular maintenance, including the use of anti fouling paints that are appropriate to the vessel use patterns, in minimising the translocation potential of recreational vessels.

The quantitative samples taken during this project also confirmed that, by and large, the severity and occurrence components of the IMEA analysis provide a reasonable indication of the level of biofouling in any given part of the vessel. Many parts of the vessel, however, were systematically underrated in the analysis, such as the rudder surface, propeller, stabilisers/trim tabs, garboard plank and block spaces. A few parts of the vessel were systematically overrated in the analysis, such as the bob-stay fitting. In some instances these errors can be explained by misinterpretation of vessel components (e.g. bob-stay fitting) or by very different use patterns between vessels (e.g. propellers that are constantly used versus intermittently used). On other occasions, however, it appears as if participants at the IMEA workshops simply underestimated how the biofouling hazards of some parts of a boat relative to others (e.g. the garboard plank).

# ACKNOWLEDGEMENTS

We would like to acknowledge the assistance and co-operation of Greg Mannering (Royal Hobart Yacht Club), Wayne Barrens (Domain slip yard), John (Hobson's Bay Yacht Club), Paul Gasgoigne (Sandringham Yacht Club) and Steve (Royal Melbourne Yacht Club) for their logistical support in sampling vessels. We would also like to thank John Lewis (Defence Science & Technology Organisation, Victoria)for providing information on the types and active compounds of antifouling paints.

We also thank the following vessel owners for allowing us to sample their vessels: Geoff Copson, Trevor Shawman, H.C. Peacock, Robert De Jonge, Ben Latham, Bruce Palmer, Jason Evans, Kim Newstead, Brian Butler, Ron Wells, Mick Daft, David Gough, Rob Rattray, Dave Weir, Peter Bristow, Ian Davenport, Tim Nolisher, Chris Parker, Steve Glover, Des Clark, Rob Giblin, Bob Drysdale, Northwest Shipping Tugs, Fred Binns, Russell Potter, David Bevan, BF Kaye-Hall, Nigel Mayes, Alan Rust, Bob White, Michael Roche, Brett Cleary, John Willett, Neil Searle, Peter Shell, John Erikson, Jeff O'Her, Ross Macaw, Richard Oliver, David Gillmore, Rod Lewis, John Parker, David Newton, Janis Zakis, Trevor Chanber, Steven Wallace, Ken Dobrich, George Shaw, Craig Begbie, Jeff Woolhouse, Geoff Roberts and Brian Patterson.

We are grateful to the following researchers for providing specimens of *Undaria* or related species for the development and ground-truthing of the genetic probe: John Lewis (Defence Science & Technology Organisation), Alecia Bellgorve (Deakin University), Britta Schaffelke (CMAR/AIMS), Ashley Coutts (Cawthron Institute, NZ), Antonella Petrocelli (IAMC-CNR, Italy), Herre Stegenga (National Herbarium of the Netherlands), Paul Farrell (Institute of Marine Sciences, UK), Stephen Kraan (National University of Ireland), Sung Min Boo (Chung Nam National University, Korea) and Graciela Casas (Centro Nacional Patagonico – CONICET, Argentina). We would especially like to thank Gary Saunders (University of New Brunswick, Canada) for his advice and providing many samples of extracted DNA.

This study was partially funded by the Australian Government's Natural Heritage Trust under the Empirical Validation project – Stage II (Natural Heritage Trust Project 46630, CSIRO reference ht21a).

# REFERENCES

Agressi A. (2002), Categorical Data Analysis. John Wiley & Sons, Hoboken, New Jersey, USA, 710 pp.

Baltz DM (1991) Introduced fishes in marine systems and inland seas. Biological Conservation, 56: 151-177.

Carlton JT (1985) Transoceanic and interoceanic dispersal of coastal marine organisms: the biology of ballast water. Oceanography and Marine Biology: An Annual Review, 23: 313-371.

Carlton JT (1995) Biological invasions and cryptogenic species. Ecology, 77(6): 1653-1655.

Carlton JT (2001) Introduced species in US coastal waters: environmental impacts and management priorities. Report prepared for the Pew Ocean Commission, Pew Ocean Commission, Arlington, USA, ?? pp.

Deagle, B.E., Bax, N., Hewitt, C.L., Patil, J.G. (2003). Development and evaluation of a PCR-based test for detection of Asterias (Echinodermata: Asteroidea) larvae in Australian plankton samples from ballast water. Marine and Freshwater Research, 54: 709-719.

Faraway JJ (2002) Practical regression and Anova using R. http://www.stat.lsa.umich.edu/~faraway/book/

Hayes KR, Sutton C, Gunasekera R, Sliwa C, Migus S, McEnnulty F, Dunstan P, Green M. and Patil J. (2004), Empirical validation: Small vessel translocation of key threatening species: Stage I – Asterias amurensis. Final report for the Australian Government Department of Environment and Heritage, CSIRO Division of Marine Research, Hobart, Australia, 60 pp.

Hayes KR (2002). Identifying hazards in complex ecological systems. Part 2: Infections modes and effects analysis for biological invasions. Biological Invasions 4(3): 251-261.

Limpert E, Stahel WA and Abbt M (2001) Log-normal Distributions across the Sciences: Keys and Clues. BioScience, 51(5): 341-352.

Patil JG, Gunasekera RM, Deagle BE, Bax NJ and Blackburn SI (2005). Development and evaluation of a PCR based assay for detection of the toxic dinoflagellate, Gymnodinium catenatum in ballast water and environmental samples. Biological Invasions (In Press).

R Development Core Team (2004), R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Rousseal, F., Leclerc, M. C. and de Reviers B. (1997). Molecular phylogeny of European Fucales (Phaeophyceae) based on partial large-subunit rDNA sequence comparisons. Phycologia 36: 438-446.

Saunders GW and Druehl LD (1993). Nucleotide sequences of the internal transcribed spacers and 5.8S rRNA genes from Alaria marginata and Postelsia palmaeformis (Phaeophyta: Laminariales). Marine Biology 115: 347-352.

Schaffelke B, Campbell ML and Hewitt CL (2005). Reproductive phenology of the introduced kelp *Undaria pinnatifida* (Phaeophycaea, Laminariales) in Tasmania, Australia. Phycologia, 44: 84-94.

Sutton CA and Bruce BD (1996), Temperature and salinity tolerances of the Northern Pacific Sea star Asterias amurensis. CRIMP Technical Report No. 6, CSIRO Marine Research, Hobart, Australia, 26 pp.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882.

Thresher RE, Hewitt CL and Campbell ML (1999) Synthesis: Introduced and cryptogenic species. In: Hewitt CL, Campbell ML, Thresher RE and Martin RB (Eds) Marine Biological Invasions of Port Phillip Bay, Victoria. CRIMP Technical Report 20, CSIRO Division of Marine Research, Hobart, Australia, pp. 283-295.

Tu W (2002) Zero-inflated data. In: El-Shaarawi AH and Piegorsch WW (Eds), Encyclopaedia of Environmetrics. John Wiley & Sons, Chichester, England, pp. 2387-2391.

Walford L and Wicklund R (1973) Contribution to a World Wide Inventory of Exotic Marine and Anadromous Organisms. Fisheries Technical Paper No. 121, Food and Agricultural Organisation, Rome, Italy, 49 pp.

Willan RC, Russel BC, Murfet NB, Moore KL, McEnnulty FR, Horner SK, Hewitt CL, Dally GM, Campbell ML and Bourke ST (2000) Outbreak of Mytilopsis sallei (Recluz, 1849) (Bivalvia: Dreissenidae) in Australia. Molluscan Research, 20(2): 25-30

Wuyts, J., De Rijk, P., Van de Peer, Y., Winkelmans, T., and De Wachter, R. (2001). The European Large Subunit Ribosomal RNA database. Nucleic Acids Research 29: 175-177.

Yoon HS and Boo SM (1999). Phylogeny of Alariaceae (Phaeophyta) with special reference to Undaria based on sequences of the RuBisCo spacer region. Hydrobiologia 398/399: 47-55.

Yoon HS, Lee JY, Boo SM and Bhattacharya D (2001). Phylogeny of Alariaceae, Laminaraceae and Lessoniaceae (Phaeophyceae) based on Plastid-Encoded RuBisCo spacer and Nuclear-Encoded ITS sequence comparisons. Molecular Phylogenetics and Evolution 21: 231-243.