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Lachlan H. Yee  
*Southern Cross University*

Carola Holmström  
*University of New South Wales*

Evi T. Fuary  
*University of New South Wales*

Nigel C. Lewin  
*University of New South Wales*

Staffan J. Kjelleberg  
*University of New South Wales*

*See next page for additional authors*

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**Authors**

Lachlan H. Yee, Carola Holmström, Evi T. Fuary, Nigel C. Lewin, Staffan J. Kjelleberg, and Peter D. Steinberg

## **Inhibition of fouling by marine bacteria immobilised in $\kappa$ -carrageenan beads**

LACHLAN H. YEE<sup>1,2,\*</sup>, CAROLA HOLMSTRÖM<sup>1,2</sup>, EVI T. FUARY<sup>1</sup>, NIGEL C. LEWIN<sup>1,2</sup>, STAFFAN KJELLEBERG<sup>1,2</sup>, PETER D. STEINBERG<sup>1,2</sup>

<sup>1</sup>*Centre for Marine Biofouling and Bio-Innovation, The University of New South Wales, Sydney 2052, Australia;*

<sup>2</sup>*Environmental Biotechnology – Cooperative Research Centre, Australian Technology Park, Eveleigh NSW 1430*

### **Abstract**

Antifouling solutions that leave little or no impact in the world's oceans are constantly being sought. This study employed the immobilisation of the antifouling bacterium *Pseudoalteromonas tunicata* in  $\kappa$ -carrageenan to demonstrate how a surface may be protected from fouling by bacteria, i.e. a “living paint”. Attempts so far to produce a “living paint” have been limited in both longevity of effectiveness and demonstration of applicability, most noticeably regarding the lack of any field data. Here we demonstrate long term survival of bacteria immobilised in  $\kappa$ -carrageenan for 12 months in the laboratory and evidence for inhibition of fouling for up to 7 weeks in the field, Sydney Harbour, NSW, Australia.

**Keywords:** Immobilised bacteria, *Pseudoalteromonas tunicata*,  $\kappa$ -carrageenan, marine antifouling coating, living paint

Correspondence: Dr Lachlan Yee, Centre for Marine Biofouling & Bio-Innovation, School of Biotechnology and Biomolecular Sciences, The University of New South Wales, 2052 Sydney, Australia. Fax: +61 (2) 93 85 25 54. Email: [L.Yee@unsw.edu.au](mailto:L.Yee@unsw.edu.au)

### **Introduction**

All surfaces exposed to the marine environment, from artificial substrates such as ships to the living surfaces of marine plants and animals, are subjected to colonisation by marine organisms, referred to as biofouling. Current attempts to prevent marine biofouling on man-made surfaces rely primarily on coatings that release organic biocides or heavy metals (Yebra et al. 2004). These additives are generally released into the environment and have a variety of

detrimental, non-target effects, leading (for example) to the recently proposed global ban on antifouling coatings containing tributyl tin (TBT) (IMO 2001). Environmentally more favourable options are under development but the challenge remains to develop a solution that is effective against a broad spectrum of fouling organisms (Chambers et al. 2006).

Many authors have suggested that natural systems may provide useful models for new commercial antifoulants. These suggestions include the use of naturally produced, deterrent chemicals from marine organisms (Rittschof 2001), surfaces based on molluscan shells which have microtopographies that are deterrent or difficult to adhere to (Adkins et al. 1996; Bers and Wahl 2004), or the use of deterrent bacteria (Dobretsov et al. 2006; Holmström et al. 1998). This last model has been captured in the notion of a “living paint” (Goupil et al. 1973), in which bacteria that release antifouling compounds are immobilised in a polymer. If the immobilised bacteria could be maintained alive in such a coating and continue to release natural inhibitory metabolites, such a coating would have an indefinite lifespan, in contrast to current antifouling coatings that fail when they exhaust their reservoir of biocides.

In principle, such technology is well within the realms of possibility. The immobilisation of bacteria in polymer matrices both natural hydrogels (Prakash and Martoni 2006) and water based latex (Flickinger et al. 2007) is very well established, although relatively few have used marine bacteria. The marine *Chromatium* sp. was immobilised in an agar polymer in order to treat organic and inorganic waste leading to hydrogen production (Matsunaga and Mitsui 1982; Mitsui et al. 1985). Continuous denitrification of artificial seawater has been achieved using a marine bacterial community immobilised within a macro-porous cellulose carrier (Catalan-Sakairi et al. 1997). Even an extremophile, *Thermotoga maritima*, has been immobilised in latex at temperatures of 80 °C (Lyngberg et al. 2005).

Arguably the major challenge for a realistic “living paint” is the design of an appropriate polymer system for immobilisation. Gatenholm et al. (1992; 1993; 1995; 1996) were the first to address some of the materials and chemical challenges of designing a polymer system for immobilising bacteria for use in anti-fouling. Immobilising the marine bacterium *Pseudoalteromonas tunicata* in hydrogels, Gatenholm et al. (1995) found that calcium alginate degraded within 24 hours of exposure to seawater but the more robust poly hydroxyethyl methacrylate (pHEMA) was toxic to the bacteria. Polyacrylamide (PAA) was then used at a monomer concentration between 3-5%. This system entrapped the bacteria with minimal escape but allowed the deterrent metabolites to diffuse out, as determined through bioassays against larvae. This polymer system (without bacteria) also withstood marine exposure for 90 days. However *P. tunicata* immobilised in PAA survived for only 6 days in the laboratory

(Gatenholm et al. 1995). Polyvinyl alcohol (10%) as an immobilisation system was employed by Holmström et al. (2000) and survival of the *P. tunicata* was reported as 14 days. Other systems which have been explored are layered acrylic latex coatings for immobilisation of terrestrial bacteria that produce amylolytic or proteolytic enzymes (Polsenski and Leavitt 2006; Powers et al. 2002).

Polymers such as pHEMA and PAA are still relatively toxic to bacteria and thus for future development of a “living paint” system, there is still a clear need to identify a durable but non-toxic polymer for a living antifouling system.  $\kappa$ -carrageenan is a food grade polysaccharide polymer derived from red algae (Rhyodphyta) and is used in pharmaceuticals, cosmetics and food products (van de Velde et al. 2002). In this study we trial the use of  $\kappa$ -carrageenan for the immobilisation of *Pseudoalteromonas tunicata* and *Phaeobacter* sp. strain 2.10 (formerly *Roseobacter gallaeciensis* strain 2.10) (Martens et al. 2006). *P. tunicata*, is a dark green pigmented, biofilm forming bacterium which produces metabolites that are inhibitory to a range of common marine fouling organisms – invertebrates, fungi, algae, diatoms, bacteria and protozoa (Holmström et al. 1998). It’s antifouling properties have been extensively studied (Egan et al. 2001; Egan et al. 2002; Franks et al. 2005; Holmström et al. 1992). *Phaeobacter* sp. (Martens et al. 2006; Ruiz-Ponte et al. 1998), is a brown pigmented, biofilm forming bacterium that has inhibitory effects against fouling organisms such as bacteria and bryozoans (Rao 2005). Both bacterial viability and activity when immobilised within the  $\kappa$ -carrageenan immobilisation system were investigated. Activity was tested both in laboratory bioassays against invertebrates and algae and, for the first time, a “living antifouling paint” containing a viable marine bacterium was trialed in the field, in Sydney Harbour, New South Wales, Australia.

## **Methods**

### *Bacteria*

The complex marine medium Vääätänen nine-salt solution (VNSS) (Maarden et al. 1985) was used to grow *P. tunicata* and *Phaeobacter* sp. All cultures were grown at 20 °C.

### *Bacterial sensitivity to heat shock*

In order to determine the tolerance of *P. tunicata* to heat and consequently define conditions to employ for immobilisation, this bacterium was subjected to temperatures suitable for immobilisation and viability was monitored. *P. tunicata* was subsampled from an established

culture on a VNSS growth medium agar plate and used to inoculate 20 mL of VNSS overnight at room temperature. The cells were centrifuged (6000 rpm for 25 min) and resuspended in 10 mL 1.2% NaCl. One mL of these resuspended cells was dispensed into 1.5 mL eppendorf® tubes and subjected to 45 and 50 °C for 30 s in duplicate. This period chosen was a maximum time the bacteria experienced elevated temperatures before being cooled back to ambient (20 °C) through exposure to the VNSS gelling solution (see Immobilisation of bacteria - methods). A dilution series was prepared in phosphate buffered saline (PBS). Ten µL drops of dilutions were plated onto VNSS agar. Resuspended cells not exposed to heat were also diluted and plated to obtain a control cell count.

#### *Immobilisation of bacteria in κ-carrageenan*

*P. tunicata* was grown overnight with 2.5% (w/v) cellulose (AVICEL-101) in VNSS (total 40 mL centrifuged and resuspended in 5 mL 1.2% (w/v) NaCl). Cellulose is an attachment substrate for *P. tunicata* that promotes cell viability (Dalisay-Saludes et al. 2006). The immobilisation of *P. tunicata* was achieved by mixing the 2 mL *P. tunicata* pellet with 8 mL of 45 °C κ-carrageenan (Fluka, 1% w/v) in 1.2% (w/v) NaCl and syringed dropwise into 200 mL sterile VNSS (stirred) with 3 mL paraffin oil. The temperature chosen for immobilisation was 45 °C as this was the minimum temperature required for immobilisation with κ-carrageenan used in this study. The various salts found in VNSS aid the gelation of the κ-carrageenan hence immobilising the bacteria. Fifty mL of polymer was added to the VNSS, stirred for 1 hr, filtered through a stainless steel autoclaved sieve and rinsed 3 times with sterile VNSS. Removal of the paraffin oil was achieved by adding the newly formed κ-carrageenan beads to a glass container. The container was then filled with filtered seawater (Millipore Opticap Filter, 0.2 µm Milligard) and the less dense paraffin rose to the surface and was decanted. The resulting κ-carrageenan beads now containing immobilised bacteria, ranged from approximately 1 – 2 mm in diameter. The choice of κ-carrageenan beads over sheets was to enable uniform polymer structure and to emphasise the bacteria to surface ratio of the immobilisation system as a whole. *Phaeobacter* sp. was also immobilised according to these procedures. After approximately 24 hours the beads were used in the various assays and field experiments described below.

### *Survival of bacteria*

The survival of *P. tunicata* and *Phaeobacter* sp. within the carrageenan beads was measured for periods of up to a year. A range of temperatures (20 °C, 4 °C and - 80 °C) and storage solutions (filtered sea water, VNSS, and 4% CaCl<sub>2</sub>) were examined. Beads were prepared using the immobilisation method outlined above, stored in petri dishes and sealed with paraffin film containing the solution of interest. Bacterial survival was determined by breaking open the beads and plating out onto VNSS agar plates. Multiple colonies of pigmented bacteria indicated viable bacteria were recovered from within the beads. Bacterial agar overlays were performed on newly made κ-carrageenan beads, beads kept for storage in the laboratory and beads that had been recovered from the field trial. The overlay bacteria used were the marine strains *P. undina* and *P. nigrifaciens* previously shown to be sensitive against *P. tunicata* (Holmström et al. 2002). Finally, an additional viability test for *P. tunicata* was included based on PCR detection of the *P. tunicata alpP* gene. PCR amplification of the gene for the antibacterial protein, AlpP, followed the protocol of Skovhus et al. (2006). This determination of bacterial survival and gene detection was also applied to the field trial samples.

### *Invertebrate laboratory bioassay*

*P. tunicata* both in immobilised form and as a biofilm were examined for antilarval properties in a settlement assay using the bryozoan, *Bugula neritina* following the procedure of (Gribben et al. 2006). Each treatment had replicate dishes (N = 7) and the effect of immobilisation on settlement was examined using one-way ANOVA and Tukey's pairwise comparison of means. Data were square root transformed to ensure homogeneity of variance. In order to check the bacteria were totally immobilised within the κ-carrageenan beads, the supernatant of the bioassay was used to inoculate a solution of VNSS post assay. No detectable *P. tunicata* grew in this solution. Beads containing bacteria that were used in this bioassay were confirmed to have living bacteria at the time of the bioassay by plating out an aliquot of the crushed beads onto VNSS agar and observing pigmented bacterial colony formation.

### *Algal laboratory bioassay*

The efficacy of immobilised *P. tunicata* against algae was tested using spores of the red alga *Polysiphonia* sp. following Egan et al. (2001). In addition to this protocol, we also placed into each dish a circular piece of autoclaved nylon (mesh 25 µm). The mesh prevented the spores mixing in among the beads, which made them hard to detect under the microscope, and

inaccurate counting would have resulted. It was hypothesised that the 3 - 10 kDa anti-algal compound (Egan et al. 2001) would pass freely through this mesh but prevent spores (~ 50  $\mu\text{m}$ ) from travelling beneath this barrier. Approximately  $30 \pm 5$  spores were added to each dish and spore settlement was assessed after 24 hr. Each treatment had replicate dishes ( $N = 9$ ) and the effect of immobilisation on settlement was analysed using 1-way ANOVA and Tukey's pairwise comparison of means as per the bryozoan assay above. The supernatant from this bioassay was also used to inoculate a solution of VNSS post assay. No detectable *P. tunicata* grew in this solution. Beads containing bacteria that were used in this bioassay were confirmed to have living bacteria at the time of the bioassay by plating out crushed beads onto VNSS agar and observing pigmented bacterial colony formation.

#### *Field bioassay*

Antifouling effects of immobilised *P. tunicata* and *Phaeobacter* sp. were tested in the field at the Royal Sydney Yacht Squadron location, Kirribilli, Sydney Harbour, NSW, Australia ( $33^{\circ} 50'51''$  S,  $151^{\circ} 13'10''$  E). Four types of treatments were used in the experiments:  $\kappa$ -carrageenan beads with immobilised *P. tunicata* or *Phaeobacter* sp. (separately), blank beads and empty wells as controls. The field rig consisted of an acrylic frame covered with 85  $\mu\text{m}$  nylon mesh and sealed from the back side with a 30 mm diameter petri dish and Parafilm (American National Can) which created a cage-like chamber into which beads were placed (Figure 1). Every combination of bead type and control was run in triplicate and randomly arranged. Rigs were deployed vertically underwater and attached to a pontoon. Fouling on the nylon mesh overlaying the beads was recorded weekly using a digital camera (Canon S45) at the highest resolution (2272 x 1704). The images obtained were processed as 8-bit images using ImageJ® to determine the area covered by fouling. Analysis of fouling at selected weeks was done using 1-way repeated measures ANOVA followed by Tukey's pairwise comparisons.

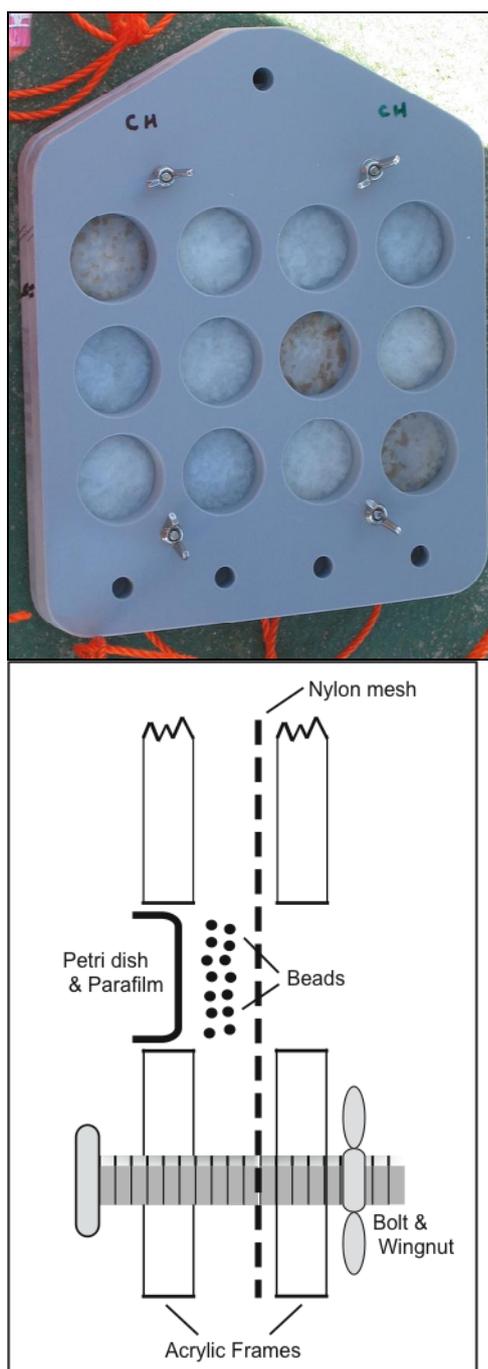


Figure 1. Photographic view (A) and side view schematic (B) of deployed field rig illustrating how the “sandwich” design exposes the  $\kappa$ -carrageenan beads containing *Pseudoalteromonas tunicata* and *Phaeobacter* sp. to fouling in Sydney Harbour, Australia. Controls included sterile carrageenan beads and empty wells. The beads were prevented from leaving the rig through use of a nylon mesh where the rear side is capped with petri dishes and Parafilm to prevent bead escape. The rig is made of heavy grade acrylic frames and bolted together with stainless steel bolts and wingnuts.

### *Detection of P. tunicata in beads using PCR amplification*

The identity of *P. tunicata* immobilised in samples after deployment in both the field and in laboratory storage was investigated using PCR amplification. After triple rinsing of beads in sterilised sea water to remove any externally adhered bacteria, DNA was extracted from beads containing immobilised bacteria cells using the FastDNA SPIN Kit (Q-BIOgene). The chromosomal DNA was used in PCR for detection of the gene for the antibacterial protein (AlpP) of *P. tunicata*. *Phaeobacter* sp., which is not known to produce the AlpP protein, was included as a control.

## **Results**

### *Bacterial sensitivity to heat shock*

*Pseudoalteromonas tunicata* was examined for heat shock across the times and temperatures tested. After 30 s of exposure, the cultures at 20 °C produced  $(34 \pm 1) \times 10^9$  Colony Forming Units (CFU), 45 °C resulted in  $(29 \pm 2) \times 10^9$  CFU and 50 °C resulted in  $(23 \pm 1) \times 10^9$  CFU (N = 2,  $\bar{X} \pm$  S.E. for all). *Phaeobacter* sp. is more tolerant to heat than *P. tunicata* (Ruiz-Ponte et al. 1998), hence the immobilisation conditions determined for *P. tunicata* were also employed for *Phaeobacter* sp.

### *Long term survival of bacteria*

Twenty four hours after bead manufacture, the beads become highly pigmented due to the bacterial pigmentation visible through the  $\kappa$ -carrageenan. Table 1 details viability (as determined by plating) of immobilised *P. tunicata* and *Phaeobacter* sp. in  $\kappa$ -carrageenan after storage at various temperatures and in different solutions. Four percent CaCl<sub>2</sub>, a standard hardening agent for  $\kappa$ -carrageenan immobilisation (Bickerstaff 1997), was toxic to *P. tunicata*. Filtered seawater (FSW) displayed improved storage properties, although the inability of FSW to maintain immobilised pure strains of *P. tunicata* under ambient conditions led to the trial of the growth media VNSS as a possible storage solution. Storing the beads in VNSS resulted in contamination at ambient temperatures. However, by renewing the VNSS media every fortnight and straining the beads whilst preventing evaporation by sealing in a petri dish with parafilm, long term storage and survival of immobilised *P. tunicata* and *Phaeobacter* sp. was achieved. The long term storage trial was terminated after 1 year.

Table 1. Storage of immobilised *Pseudoalteromonas tunicata* and *Phaeobacter sp.* (beads) in 4% calcium chloride, FSW-filtered sea water (filter sterilised SW) and VNSS-nutrient rich nine salts solution used as the growth medium.

Bacterium	Storage solution	Storage temp.	Survival status
<i>Pseudoalteromonas tunicata</i>	4% CaCl <sub>2</sub>	-80 °C	21 weeks
	FSW	-80 °C	21 weeks
	VNSS	-80 °C	21 weeks
	4% CaCl <sub>2</sub>	4 °C	dead*
	FSW	4 °C	21 weeks
	VNSS	4 °C	21 weeks
	4% CaCl <sub>2</sub>	20 °C	dead*
	FSW	20 °C	21 weeks <sup>^</sup>
	VNSS	20 °C	21 weeks <sup>^</sup>
	VNSS <sup>#</sup>	20 °C	52 weeks
<i>Phaeobacter sp.</i>	4% CaCl <sub>2</sub>	-80 °C	dead*
	FSW	-80 °C	dead*
	VNSS	-80 °C	dead*
	4% CaCl <sub>2</sub>	4 °C	dead*
	FSW	4 °C	21 weeks
	VNSS	4 °C	21 weeks
	4% CaCl <sub>2</sub>	20 °C	dead*
	FSW	20 °C	21 weeks
	VNSS	20 °C	21 weeks <sup>^</sup>
	VNSS <sup>#</sup>	20 °C	54 weeks

\*- bacteria did not grow on VNSS agar plates after emerging from storage and negative PCR detection

<sup>^</sup> - timepoint when experiments became contaminated

<sup>#</sup> - beads rinsed with VNSS every fortnight, strained and restored in a petri dish sealed with paraffin film

#### *Invertebrate larval and algal spore assays*

*P. tunicata* when immobilised in beads as well as presented as an exposed biofilm significantly inhibited the settlement of *Bugula neritina*, with the bacteria in the beads showing the greatest levels of deterrence comparable to biofilm alone (Figure 2, 1-way ANOVA,  $F_{6,24} = 7.95$ ,  $p < 0.01$ ). *P. tunicata* in beads or as a biofilm also inhibited the alga *Polysiphonia sp.* relative to controls, but for this organism the effect of the bacteria in the beads was not distinguishable from blank  $\kappa$ -carrageenan beads, suggesting an effect from the carrageenan (Figure 2, 1-way ANOVA,  $F_{8,32} = 11.71$ ,  $p < 0.01$ ).

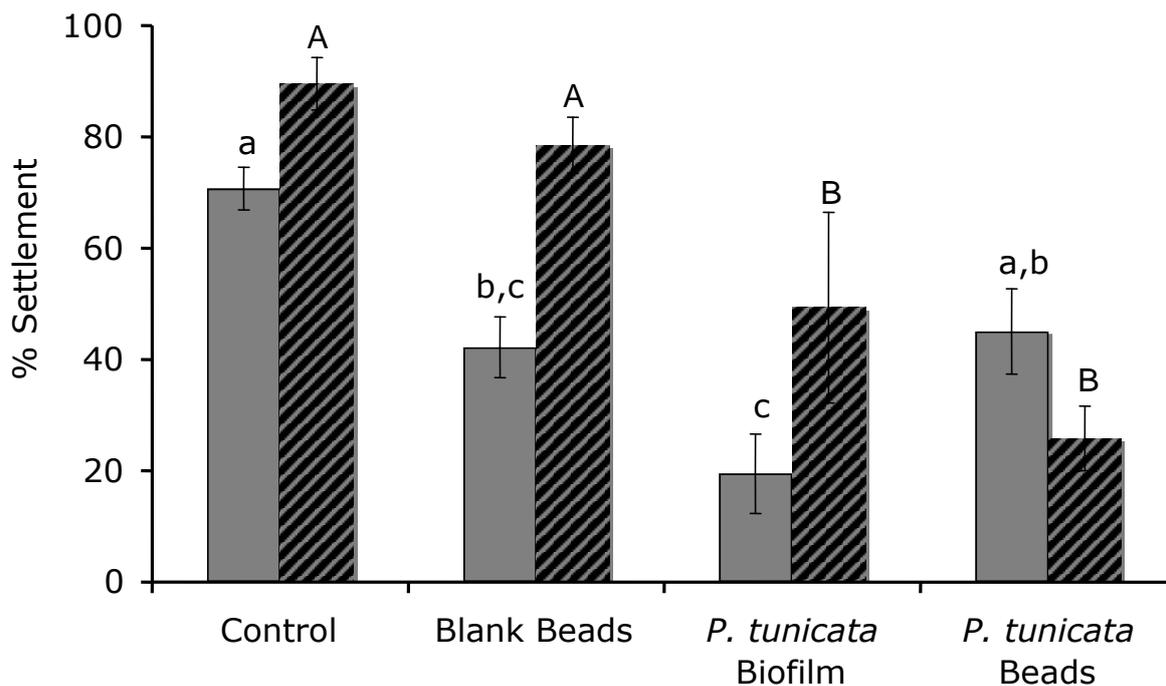


Figure 2. Effect of *Pseudoalteromonas tunicata* immobilised in  $\kappa$ -carrageenan beads on the settlement of red alga *Polysiphonia* sp. spores (grey) and invertebrate larvae *Bugula neritina* (hatched) in a bioassay. N = 9 and 7 per treatment respectively. Treatments were: sterile seawater (control), empty carrageenan beads (blank beads), *P. tunicata* biofilm grown 24 hours prior to test on the base of the petri dish and immobilised *P. tunicata* beads. Significant difference among treatments are indicated by lower- and upper-case letters for alga and invertebrates respectively ( $\alpha = 0.05$ , Tukey's test).

#### Field bioassay

*P. tunicata* immobilised in  $\kappa$ -carrageenan significantly inhibited fouling in the field for a period of up to 7 weeks (Figure 3) relative to the control, sterile  $\kappa$ -carrageenan beads. The outer casing of the field rig became heavily fouled, however the fouling was inhibited at the level of the nylon mesh and just above (~2 - 3 mm) suggesting a slight gradient of emerging antifouling metabolites must have been present for the time of the study up until 7 weeks. Immobilised *Phaeobacter* sp. did not significantly deter the fouling in the field at any sampling date. Overall fouling in the weeks fluctuated substantially over the course of the experiment. At the conclusion of the field trial, the  $\kappa$ -carrageenan beads had almost completely biodegraded. Bacterial growth from harvested beads (a separately deployed rig) was confirmed for *P. tunicata* after 2 and 6 weeks exposure. No growth detected at 12 weeks.

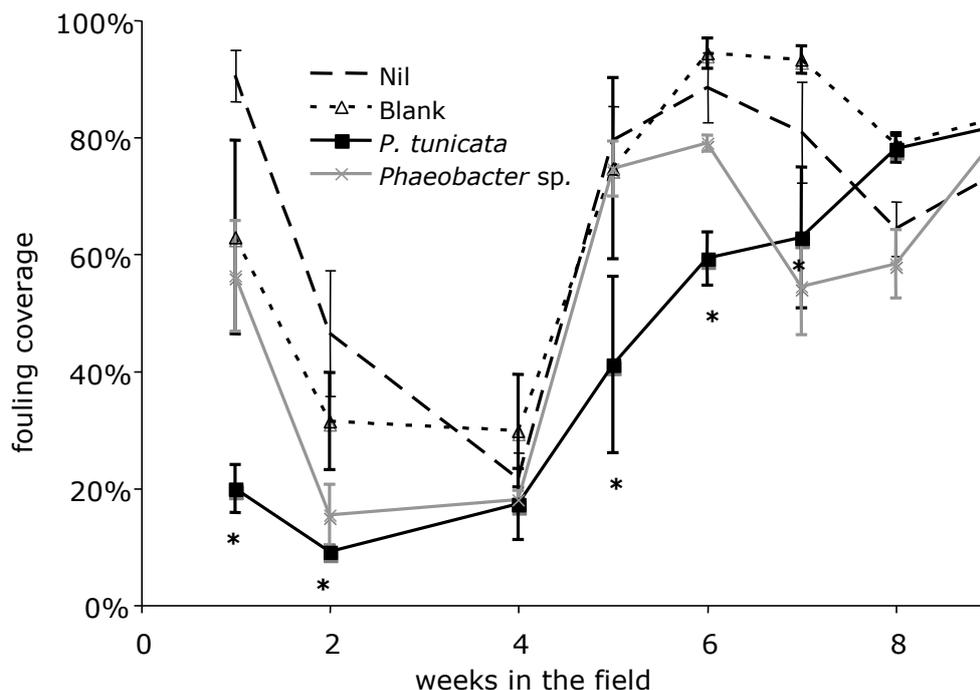


Figure 3. Immobilised bacteria in  $\kappa$ -carrageenan beads exposed to marine field conditions, at Sydney Harbour, Australia. Nil – Procedural control with no polymer, Blank – Sterile polymer beads. *Pseudoalteromonas tunicata* and *Phaeobacter* sp. are the active bacteria used in the immobilised bead treatments. Significant differences ( $p < 0.05$ ) between *P. tunicata* treatments and the sterile bead controls shown by (\*), as determined by a repeated measures ANOVA followed by planned comparisons (Tukey’s test).

#### Detection of bacteria in beads using PCR amplification

PCR analysis using primers specific for the *alpP* gene confirmed the presence of *P. tunicata* from both beads kept in long term laboratory storage and beads exposed at our marine field site. No evidence of the gene of the AlpP protein was found from the *Phaeobacter* sp. control samples.

#### Discussion

To date, the reporting of a “living” surface that employs antifouling marine bacteria as a means of preventing settlement of marine organisms on a surface has been very limited. The present study was aimed at producing a proof of concept in an ocean field setting for such a system that allowed for long term survival of bacteria and a persistent inhibitory effect. This was achieved through immobilisation of the antifouling bacterium *P. tunicata* in a  $\kappa$ -carrageenan hydrogel polymer.

$\kappa$ -carrageenan was selected for immobilisation and long term survival of the bacteria. The selection of this polymer was based on its low toxicity to prokaryotes and ability to maintain long term immobilisation of bacteria for up to 4 years in other studies (Cassidy et al. 1997). The ability to maintain *P. tunicata* in an immobilised form for a period of up to 1 year is a substantial improvement over previous attempts. For example, *P. tunicata* was immobilised in polyacrylamide for 6 days (Gatenholm et al. 1995) which was attributed to the toxic acrylamide monomer within the polyacrylamide gel system employed. The 14 days of survival achieved by Holmström et al. (2000) was in a relatively less toxic poly vinyl alcohol immobilisation system. Holmström et al. (2000) also postulated that the anti-bacterial protein produced by *P. tunicata* might not be released from the polyvinyl alcohol polymer hence causing autolysis of the bacteria. In the present study, bacterial agar overlays were performed on newly made  $\kappa$ -carrageenan beads and beads that had been recovered from the field trial. The overlay bacteria used were the marine strains *P. undina* and *P. nigrifaciens* previously shown to be sensitive against *P. tunicata* (Holmström et al. 2002). In these experiments, 2 – 3 mm zones of inhibition were observed suggesting that the anti-bacterial protein is emerging from within the beads (data not shown). Further more, it was demonstrated that *P. tunicata* cells in stationary phase of growth became resistant to the antibacterial protein (James et al. 1996). Given that the immobilised cells in this study were only rinsed every fortnight in the growth medium we suggest that the cell growth is insignificant and that most of the immobilised cells are in stationary phase and therefore resistant to the AlpP protein. In summary, this increased resistance plus the observations on release of the antibacterial compound, coupled with the low toxicity of the  $\kappa$ -carrageenan polymer collectively suggest why *P. tunicata* can survive for periods of up to 12 months (under laboratory conditions) in this  $\kappa$ -carrageenan immobilisation system, in contrast to a few days or weeks of earlier attempts. To determine if the immobilisation system reported in this paper would be effective against marine fouling, laboratory bioassays were conducted using the marine fouling organisms *Bugula neritina* and *Polysiphonia* sp. The successful reduction of invertebrate larval settlement by the immobilised *P. tunicata* in  $\kappa$ -carrageenan beads strongly suggests that the anti-larval metabolite is able to diffuse out from within the  $\kappa$ -carrageenan bead.

There was no significant antifouling effect against *Polysiphonia* sp. for the *P. tunicata* immobilised in beads. On three previous bioassays (two against *Polysiphonia* sp., once against the green alga, *Ulva australis*), a similar result was observed in which there was no significant difference in the inhibition of algae using  $\kappa$ -carrageenan alone versus  $\kappa$ -

carrageenan containing *P. tunicata* (Yee, personal communication). However, fouling of both algae and invertebrates were significantly inhibited in our field trial for up to 4 weeks. These differences between laboratory and field results could be due to a variety of factors including the presence of different fouling organisms in the lab or field, or the greater choice of surfaces available to propagules in the field.

Variation in fouling over time in the field trial was substantial and in particular fouling decreased strikingly at 2 – 4 weeks before building back up. However, even against this background of temporal variations in fouling, *P. tunicata* immobilised in  $\kappa$ -carrageenan beads significantly reduced fouling for up to 7 weeks in the field. Photographic images indicated the succession of fouling to be initially green and brown algae, followed by bryozoans and eventually small *Hydroides* sp. The second bacterium used in our field trial, *Phaeobacter* sp. is effective against algae and larvae in laboratory assays (Rao 2005) but was not effective in this field trial. We do note *Phaeobacter* sp. was included in the field trial primarily as a bacterial control and not as an alternative antifouling treatment.

This study represents one of the first such antifouling field experiments conducted using a “living” antifouling system employing immobilised marine bacteria. A previous field trial experiment was conducted by Powers et al. in US Patent 6,342,386 (Powers et al. 2002). In example 11 of this patent, they described 3 different coatings (a liquid polymer, a silicone resin and an acrylic latex paint) combined with two different non-marine microorganism mixtures the first of which contained *Bacillus polymyxa*, *B. subtilis* and *B. lichenformis* and where the second of these bacterial mixtures contained *B. subtilis*, *Pseudomonas aeruginosa*, *P. putida*, *P. fluorescens* and *Escherichia hermannii*. Powers et al. (2002) reported a reduction in fouling coverage on their panels from 30% on the control to 2% fouling on their treatments after 4 months, though no statistical data was shown.

Subsequent to our field study, a preliminary degradation trial was conducted in our sea water aquarium facilities where degradation rates of the unmodified  $\kappa$ -carrageenan beads were observed at similar rates to that of the field trial samples reported here. This suggests that unmodified carrageenan alone would not be an appropriate matrix for a marine product but could possibly be used as an interface between a cytotoxic coating and the bacterial cells.

In summary, significant improvements have been achieved with longer lifetimes for the immobilised bacteria and antifouling efficacy in the field compared to previous studies, highlighting the potential application of “living paint” technology as an alternative to current antifouling strategies. Technological advancements that would further improve performance

could include the use of alternative curing agents and prolonging the lifetime of the immobilisation polymer.

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