2007

Comparison of surface microfouling and bacterial attachment on the egg capsules of two molluscan species representing Cephalopoda and Neogastropoda

Norman SH Lim
*Flinders University*

Kim J. Everuss
*Flinders University*

Amanda E. Goodman
*Flinders University*

Kirsten Benkendorff
*Flinders University*

Publication details


Publisher's version of this article is available at [http://dx.doi.org/10.3354/ame047275](http://dx.doi.org/10.3354/ame047275)
Comparison of surface microfouling and bacterial attachment on the egg capsules of two molluscan species, representing the Cephalopoda and Neogastropoda.

Running head: Surface fouling of molluscan egg capsules

Authors

Sze Hao Norman Lim, Amanda Goodman and Kirsten Benkendorff*.

School of Biological Sciences, Flinders University of South Australia, GPO BOX 2100, Adelaide, SA, 5001, Australia.

*corresponding author: Phone 61 8 8201 3959; Fax 61 8 8201 3015

Email: Kirsten.benkendorff@flinders.edu.au
Abstract: Many organisms naturally defend themselves against microbial attachment and biofouling in the marine environment. In this study, we investigated microbial fouling on two molluscan egg capsules using scanning electron microscopy, two photon laser scanning microscopy with bacterial viability staining and bacterial attachment experiments with the biofilm forming *Pseudoalteromonas* sp. S91 in flow chambers. Results indicate that early stage egg capsules of *Dicathais orbita* are relatively free of surface microorganisms. Egg capsules during the trocophore stage have a regularly ridged microtexture, but as capsules mature, shedding of the outer wall was observed, followed by the extrusion of unidentified droplets, which then accumulate on the capsule surface in association with bacteria. By comparison, the egg capsules of *Sepioteuthis australis* were found to have an irregular surface with many hills and valleys that accommodate colonization by a variety of microorganisms. At the later stages of development these squid egg capsules become heavily colonized by algal spores. Cross sections of egg capsules revealed that *S. australis* capsule walls were about twelve times thicker than *D. orbita* egg capsules. Staining the egg capsules with BacLight™ also revealed a significantly thicker biofilm, with more live and dead bacteria on *S. australis* capsules compared to *D. orbita* (p < 0.05). Flow chamber experiments indicate that the surface of *S. australis* capsules provide a suitable substrate for colonisation by *Pseudoalteromonas* sp. S91, whereas colonization was significantly less on *D. orbita* egg capsules after 24 and 72h (p < 0.01). These experiments indicate that *D. orbita* egg capsules are better defended against fouling microbes compared to the eggs of *S. australis*. *D. orbita* appears to use a combination of physical, mechanical and possibly chemical defense mechanisms to reduce fouling on their egg capsules.
Keywords: Biofilm, egg capsules, mollusc, scanning electron microscopy, two-photon laser scanning microscopy, bacterial attachment.

INTRODUCTION

The economic effects of biofouling on artificial structures, such as boat hulls, nets and pipes, can be enormous. This has resulted in the use of toxic paints that have led to serious environmental problems (e.g. TBT, Champ 2000; Alzieu 2000, Goldberg et al. 2004). Biofilm formation also plays an important role in bacterial pathogenesis. Biofilms are typically resistant to antibiotics, providing a source for persistent infection (Hentzer & Givskov 2003) and/or re-infection from medical tubing and equipment. Understanding the mechanisms by which aquatic organisms naturally regulate fouling of their surfaces should provide a platform for ongoing developments in the control of economically and ecologically important fouling processes (de Nys and Steinburg 2002).

Surface fouling by microorganisms is one of the many threats that face organisms in the marine environment (Davis et al. 1989; Scardino et al. 2003). With an estimated density of $5 \times 10^5$ prokaryotic cells ml$^{-1}$ of seawater (Whitman et al. 1998), sessile invertebrates and algae are exposed to a constant onslaught of potentially detrimental microbes. These include biofilm-forming bacteria, along with single-cell diatoms, that rapidly settle, attach and form colonies on any surface placed in the marine environment (Davis et al. 1989). The formation of a microbial biofilm promotes the attachment of algal spores, protozoa, barnacle cyprids and marine fungi, followed by the settlement of other marine invertebrate larvae and macroalgae (Maki 2000; Callow & Callow 2002). Heavy surface fouling could lead to the accumulation of toxic wastes, a reduction in
oxygen and nutrient availability, as well as increased drag, which can cause sessile organisms to become dislodged from benthic substrates in strong currents. All extant marine organisms have survived for millions of years under the significant selective pressure of surface fouling and therefore must have evolved successful mechanisms for reducing these negative impacts.

The encapsulation of developing offspring within elaborate structures that are deposited on benthic substrata is a common reproductive strategy among marine molluscs. These egg capsules appear to be highly resilient multilaminate biomaterials (Rapoport & Shadwick 2002) requiring substantial maternal energy investment (Stickle 1973). This implies that egg capsules must provide adaptive benefits to increase embryonic survivorship. The functional role of encapsulated development is widely attributed to the protection of developing offspring from environmental stresses, as well as deterring potential predators and pathogens (Thorson 1950; Pechenik 1986; Rawlings 1990; Przeslawski 2004). However, these egg capsules can remain in the marine environment for several months, and thus would also be vulnerable to surface fouling. Nevertheless, previous studies indicate that these egg capsules remain axenic (Lord 1986) and remarkably free of surface macrofouling (Przeslawski & Benkendorff 2005).

Antimicrobial activity also appears to be widespread in chemical extracts from molluscan egg masses (Benkendorff et al. 2001; Santhana Ramasamy & Murugan, 2005), but it is presently uncertain if the active compounds are located on the surface. Interestingly, a previous study on the egg cases of the dogfish Scyliorhinus canicula, demonstrate that these marine egg capsules are resistant to microbial degradation and performed much better than many commercial antifouling treatments in the field (Thomason et al. 1994).
Mechanisms for deterring fouling on these extracellular structures are not currently understood. The functional mechanisms for preventing detrimental microbial colonisation on marine organisms can include associated chemical, mechanical and/or physical defenses. Chemical extracts from a wide variety of marine invertebrates and algae have demonstrated antimicrobial activity against marine biofilm bacteria (Engel et al. 2001; Steinberg et al. 2001; Santhana Ramasamy & Murugan, 2005) and some secondary metabolites have been shown to regulate biofilm bacteria on biotic surfaces. For example, furanones produced by the red alga Delisea pulchra actively prevent bacterial and larval settlement (Dworjanyn et al. 1999; Steinberg et al. 2001). Secondary metabolites produced by sponges in the Caribbean were also shown to prevent bacterial surface colonization (Kelly et al. 2005). Mechanical methods of fouling prevention are adopted by the macroalga Dilsea carnosa, which shed their epidermis and cuticle to remove any epibionts (Nylund & Pavia 2005). Similarly, moulting in crustaceans would facilitate the removal of epibionts (Dyrynda 1986). Relatively little is known of the role of physical defenses (Steinberg et al. 2001), although surface composition and microtexture can influence the rate of biofouling (Steinberg & De Nys 2002; Baum et al. 2003; Berntsson et al. 2000).

Biofilms cultivated on inanimate and biological surfaces under laboratory conditions have been studied extensively using a wide range of microscopic techniques (Beech et al. 2000), often in combination, to give a clear description of the biofilm. Scanning Electron Microscopy (SEM) allows morphological examination of biofilms established on surfaces (Beech et al., 2000). Bers and Wahl (2004) used SEM to study the
surface properties of different marine organisms, such as the edible crab, *Cancer pagurus* (Crustacea), the blue mussel, *Mytilus edulis* (Bivalvia), the brittle star, *Ophionura texturata* (Ophiuroidea) and the egg case of the lesser-spotted dogfish, *Scyliorhinus canicula* (Chondrichthyes), which are all seldom associated with epibionts. These studies support the evolution of physical antifouling strategies, showing that homogeneous surfaces were capable of deterring attachment by limiting space available for fouling organisms.

Flow chambers such as those designed by Hale and Mitchell (2001), can also be used to study biofilm formation. Bacterial attachment to biological surfaces can be studied using *Pseudoalteromonas* sp. S91, a motile Gram-negative marine bacterium, which secretes hydrolytic enzymes, e.g. chitinases and proteases (Techkarnjanaruk & Goodman 1999) and can utilize squid pen as a nutrient source (Techkarnjanaruk et al. 1997). The derivative strain, S91SB, has a transposon insert containing a copy of the green fluorescent protein (GFP) gene originated from the jellyfish *Aequoria victoria* (Chalfie et al. 1994). The use of GFP in prokaryotes has assisted the study of spatial distribution of microbial cells within a biofilm (Yoshida & Kuramitsu 2002). In addition, the use of GFP in conjunction with two-photon laser scanning microscopy (TPLSM) can facilitate the real time analysis of cells within a living biofilm (Stretton et al. 1998).

This study examines microbial fouling on the surface of egg capsules from two molluscan species; *Dicathais orbita* (Neogastropoda: Muricidae) and *Sepioteuthis australis* (Cephalopoda: Loliginidae) using a combination of microscopic techniques. The recent study by Przeslawski and Benkendorff (2004) on the egg masses of 18 gastropod molluscs revealed that the leathery egg capsules of neogastropods, including *D. orbita*,
were significantly less fouled than a range of gelatinous egg masses. However, in this study protists and algal fouling were only estimated visually under a dissecting microscope. Similarly, Steer et al. (2002) used visual observations to report high levels of algal fouling on the surface of egg capsules from the Southern Calamari, Sepioteuthis australis. These preliminary results suggest that egg capsules from different molluscan species are differentially protected from surface fouling, but further investigation is required to reveal the underlying mechanisms. Therefore, the aim of this study is to compare the physical structure and how this influences the biofouling processes on the surface of egg capsules from a cephalopod and neogastropod. We use scanning electron microscopy to study the surface morphology of the egg capsules, as well as the density and types of microorganisms present on the surface of capsules at different stages of embryonic development. The LIVE/DEAD® BacLight™ Bacterial Viability Assay was used to quantify the amount of live and dead bacteria on the surface. In addition, bacterial attachment experiments are conducted, using S91SB in flow chambers and TPLSM, to quantify bacterial accumulation on the surface of egg capsules.

**MATERIALS AND METHODS**

**Collection of Dicathais orbita and Sepioteuthis australis egg capsules**

The egg masses of Dicathais orbita were collected from the lower intertidal regions of rocky reefs along the Fleurieu Peninsula, South Australia, from early spring (October) to mid summer (December) 2005. Egg capsules of S. australis obtained from subtidal seagrass habitats at Glenelg, South Australia, and stored at 4°C to prevent deterioration. Egg capsules of both species were separated into different stages of development; for D.
D. orbita fresh (undifferentiated embryos), trocophore (ciliated embryos with no shell) and developed (veligers with pigmented shell); and for S. australis fresh (undifferentiated embryos) and developed (paralarvae) stages were examined. Identification of developmental stage was performed by dissecting capsules from each replicate egg cluster and examining the contents under a dissecting microscope.

D. orbita capsules of known age since deposition were also collected from a recirculating seawater system at Flinders University, to allow SEM images of veliger stage separated into 3, 4 and > 4 weeks old. However, for the quantification of fouling on the surface and in all other experiments egg capsules were collected from the field (exact age uncertain) to ensure representation of the natural status. For the dead/live bacterial staining and the baterial attachment experiments, D. orbita egg capsules were used in the trocophore stage (due to interesting microtopography observed at this stage –see SEM results); whereas fresh squid egg capsules with undifferentiated embryos were used, as these are much closer in age to the D. orbita trocophores than the paralarvae.

**Scanning Electron Microscopy (SEM)**

For each species, five independent egg capsules at each stage of embryonic development were fixed overnight in EM fixative (1.25% Glutaraldehyde, 4% sucrose, 4% paraformaldehyde in Phosphate Buffered Saline (PBS), pH 7.2). After 24 h, the egg capsules were removed from fixative, washed twice with PBS and cut into 0.5 cm x 0.5 cm sections. Samples were then fixed in 2% osmium tetroxide for 60 min before dehydration in increasing concentrations of ethanol (70%, 90%, 95% and 100% for 30 min each). Finally, the samples were dried under pressure with liquid carbon dioxide in a
BalTec CPD030 Critical Point Dryer. The dried samples were placed on metal stubs using adhesive stickers and sputter coated with platinum. Egg capsules were orientated to allow morphological examination and analysis of surface bacterial load. Cross-sectional views of egg capsules were also examined. The samples were viewed using a Philips XL-30 Field Emission SEM.

Cryogenic SEM was also carried out using an Oxford Instruments CT1500 HF Cryo Preparation System attached to the SEM. Egg capsules were attached to the holder using Tissue-Tek OCT compound, then frozen in nitrogen slush, and transferred under vacuum to the preparation chamber. The temperature of the sample was raised to -92°C, and held there for approximately three minutes, to allow any ice on the surface to sublime. After lowering the temperature to -110°C to halt sublimation, the sample was coated with platinum, loaded onto the microscope stage, and examined, whilst being maintained at -150°C.

The percentage of fouling on the surface of egg capsules was determined using images taken from SEM analysis. For each egg capsule, five SEM images were examined at approximately 5000x magnification and the percentage cover per image was estimated visually, and then averaged to obtain one mean value per capsule. A two-factor ANOVA was performed using the statistical package SPSS version 11.0 to determine if species and stage of development (freshly laid and developed) effected surface fouling. A separate one-way ANOVA was also performed on D. orbita (fresh, trocophore & late stage veliger) egg capsule images to determine if the extent of fouling at the trocophore stage differed from the other developmental stages. The assumption of equal variances was tested using Levene’s test and normality was tested using the Kolmogorov-Smirnov
test in SPSS. The alpha level was set at 0.05 for a significant difference, but then Bonferroni corrected to 0.0167 to account for the multiple comparisons using the same data (Quinn and Keough, 2002).

**LIVE/DEAD® BacLight™ Bacterial Viability Assay**

A stock solution was prepared by mixing equal volumes of the reagents SYTO® 9 and propidium iodide (Invitrogen 2004). The working solution was then prepared by adding 3 µl of the stock solution to 1 ml of 0.85% NaCl. Positive and negative controls of live and ethanol killed S91SB cultures were independently stained to optimize the concentration of dyes and parameters for Confocal Laser Scanning Microscopy (CLSM).

Six independent capsules from each species were stained and examined. Under sterile conditions, egg capsules were excised from the cluster and rinsed with MilliQ water before incubation in the working dye solution for 15 min in the dark. After incubation, a medial section of the egg capsule was prepared using sterile forceps and a surgical blade. The apex and basal regions of the egg capsule were removed, and a section of capsule wall (~ 0.5 x 0.5 cm) mounted onto a clean glass slide and viewed under the Olympus BX50F-3 epifluorescence microscope. A Bio-Rad MRC-1000UV CLSM was subsequently used to capture quality images of the stained egg capsules. Images in the green spectra (live) and red spectra (dead) were taken separately for quantification purposes.

The amount of live and dead microorganisms was quantified using Image J 1.32j software. The total area of fluorescence within $xz$ images was quantified under a standardized area of 96.26 µm ($x$) by 96.26 µm ($z$). Paired sample t-tests were used to
compare the amount of green and red fluorescence on the surface of egg capsules within each species (n = 6). One-way ANOVAs were then used to separately compare the amount of green fluorescence and red fluorescence between species. A square root transformation was performed on the data in order to meet the assumptions of equal variances (Levene’s test). After applying a Bonferroni correction (Sokal and Rolf, year), alpha was set at 0.0167.

Bacterial preparation

*Pseudoalteromonas* sp. S91SB were grown in 10 ml of marine minimal medium (MMM; Östling et al. 1991) supplemented with 0.2% glutamate at 30°C and allowed to grow overnight prior to use. Growth of S91SB was screened under a 100W quartz halogen lamp with an infrared and 280nm band-pass filter to check for GFP expression (Andover Corp. part no., FS10-50) (Stretton et al. 1998). Green fluorescence indicates that bacteria were growing healthily.

Bacterial attachment experiment using flow chambers

Flow chamber experiments were carried out in triplicate using independent samples for 0, 24 or 72 hour time periods. Egg capsules were sterilized by dipping into 70% ethanol for 10 min, and then washed with sterile MilliQ water before sectioning into 0.5 cm x 0.5 cm pieces under aseptic conditions. Examination of control samples under the Olympus BX50F-3 epifluorescence microscope confirmed that this procedure was successful for destroying the existing biofilm, without structurally altering the egg capsule walls. A single piece of capsule was placed into a once-through flow chamber, with the exterior surface facing up. Flow chambers used in this experiment were as described by Hale and
Mitchell (2001), and experimental conditions were maintained as described by Fitch et al. (2002). Two round glass coverslips (viewing ports) were sealed together with a rubber O-ring mounted in a stainless steel scaffold. The O-ring contained two needles acting as inlet and outlet ports. A piece of egg capsule was placed between the coverslips before sealing the chamber.

After assembly of the flow chamber, an eight-roller Cole-Palmer Masterflex peristaltic pump (7521-57) was used to pump 70% ethanol through the system for 15 min, followed by sterile MMM for 20 min. After flushing the system with sterile MMM, the peristaltic pump was turned off and 3 ml of a 10^{-1} dilution of S91SB overnight culture was introduced. The chambers were left for one hour to allow settlement and attachment of S91SB to egg capsules. Flow was resumed after one hour. Room temperature and flow rate was maintained at 24°C and 1 ml.min^{-1}, respectively (Delpin et al., 2000). As MMM does not contain any carbon source, egg capsules were the only organic material available for bacterial attachment. After the completion of each time point (0, 24 or 72 h), the chambers were disconnected and taken to Adelaide Microscopy for two-photon laser scanning microscopy (TPLSM) analysis.

Microscopic analysis of biofilm production using TPLSM

A Bio-Rad Radiance 2000MP, in conjunction with a Nikon Eclipse TE300 inverted Multi-Photon Microscope, was used to collect images of biofilms formed in the chambers. The microscope uses a 60x water immersion lens with a numerical aperture of 1.2 and a Coherent Mira900-F titanium:sapphire ultrafast laser, which has an excitation spectrum of 700-980 nm. GFP produced by S91SB was visualized using an excitation
wavelength ($\lambda$) of 800 nm and an emission $\lambda$ of 515 nm. The two photons of 800 nm light is equivalent to 400 nm but without the damage to cells caused by the 400 nm UV light typically used to excite GPF. Autofluorescence produced by the egg capsule was detected as blue at 460 nm. Horizontal ($xy$) and vertical ($xz$) optical sections of biofilm were imaged; within each $xy$ image, three random $xz$ sections were collected. This procedure was repeated three times for each chamber yielding three $xy$ and nine $xz$ images. Confocal Assistant was used to analyse all images and convert them to tif files. Image J 1.32j was used to quantify the coverage of S91SB on egg capsules by calculation of the total area of fluorescence, which is then divided by the mean area for a single S91SB cell (0.56 $\mu$m$^2$) (Everuss 2004). A standardized area of $xz$ sections was used for quantification of fluorescence; 96 $\mu$m ($x$) by 52 $\mu$m ($z$).

One way ANOVAs with Tukey HSD post hoc tests were used to compare differences in the amount of S91SB counts between each time period for $D. orbita$ and $S. australis$ egg capsules. Independent t-tests were also used to compare the difference in S91SB counts between species at 24 h and 72 h time periods. All analyses in this data set were tested more stringently ($\alpha = 0.01$) as assumptions of normality were not met (Underwood, 1997), even after a series of transformations were attempted on the data (e.g. log $x + 1$, square root and cube root).

RESULTS

Scanning Electron Microscopy

Scanning electron microscopy revealed differences in the egg capsule structure of $D. orbita$ and $S. australis$ capsules. Distinct layers of $Dicathais$ egg capsules can be seen in
transverse sections (Figure 1a). The capsule comprises a solid inner wall and porous
sections sandwiching a thick medial section with possible layering. The capsule wall is
approximately 25 \( \mu \)m thick (Figure 1a). By comparison, \textit{S. australis} capsules were
observed to consist of multiple overlapping layers similar to a plywood structure (Figure
1b). The capsule wall of fresh samples was approximately 300 \( \mu \)m thick, and none of the
layers appeared to be porous.

[Insert Figure 1 about here]

Differences in the surface microtopography were observed both between the
species and within species at different stages of development (Figures 2 & 3). Freshly
laid capsules (< 1 week old embryos) of \textit{D. orbita}, possess a thin layer of crust over a
microtopographical structure on the outer most surface (Figure 2a). The surface was
almost free from any bacteria and algae. As the egg capsules mature (1-3 weeks old), the
crust begins to breakdown, exposing the microtopographical features (Figure 2b). This
layer has regular homogeneous ridges ranging from 1-5 \( \mu \)m apart (Figure 2c). Sparse rod
and cocci bacteria were observed on the walls and ridges during the second week of
development. However, during the third week, densities of these bacteria increased, and
other fouling organisms such as filamentous algae were observed, forming a mixed
biofilm community (Figure 2d). In the later stages of development (> 4 week old
veligers), the fouled outer wall structure begins to dissociate and shed from the capsule,
leaving behind a naked capsule without any texture (Figure 2e). This surface was initially
free from microbes, but bacteria began colonization several days later. Unidentified
droplets ranging from 8 -20 \( \mu \)m appeared on the surface of mature egg capsules after the
majority of the outer wall dissociate (Figure 2f). These droplets were either solitarily or
clumped in association with attached bacteria. A greater density of these droplets was
observed on cryostat preparations than on critically point dried capsules. However, fewer
microorganisms were observed on the surface of critically point dried samples (data not
shown).

[Insert Figure 2 about here]

The surface of *S. australis* egg capsules typically had irregular folds resembling
ridges and troughs about 5 μm in width and depth (Figure 3a). Freshly laid capsules
(undifferentiated blastomere stage) collected from the sea contained fewer
microorganisms on the surface (Figure 3a & b) than late stage capsules containing
paralarvae (Figure 3 c-f). Capsules of *S. australis* collected from the field in the later
stages of development were covered with filamentous algae and spores (Figure 3 c &d),
as well as exopolymeric substance and various bacteria, (rod, cocci and spiral forms e.g.
Figure 3e) and diatoms (Figure 3f).

[Insert Figure 3 and 4 about here]

Figure 4 shows the total percentage cover by all fouling organisms on the surface
of *D. orbita* and *S. australis* egg capsules. A two-factor ANOVA was conducted to test
the effect of species and stages of development (fresh & developed) on the level of
fouling (percentage cover). Percentage coverage was significantly higher in the later
stages of development (fresh embryos vs. developed capsules, $F = 25.83$, df = 1, $p<
0.001$). However, species ($F = 1.066$, df = 1, $p = 0.317$) and the interaction term ($F =$
2.296, df = 1, p = 0.149) were not significant. A one-way ANOVA for surface fouling on

*D. orbita* capsules indicates that there is a statistically significant difference between the
three different stages of development (F = 16.318, df = 2, p < 0.001). Post-hoc
comparisons using Tukey HSD test revealed that percent cover of fresh capsules and
trocophore stage capsules did not differ significantly from each other (p = 0.671),
whereas developed capsules were significantly more fouled than both fresh and
trocophore stage capsules (p < 0.001, p = 0.002 respectively, Figure 4a)

**LIVE/DEAD® BacLight™ Bacterial Viability Assay**

Baclight™ was successful in staining microorganisms on the surface of *D. orbita* and *S.
australis* egg capsules (Figure 5). However, the unidentified droplets on the surface of *D.
orbita* capsules were found not to stain with either of these nucleic acid dyes. Control
experiments demonstrated that ethanol-killed bacteria gave the expected red fluorescence,
while viable cells remained green due to retention of SYTO®9. The egg capsules of both
molluscan species were found to have a mixture of live and dead bacterial cells on their
surface (Figure 5 & 6). The thickness of the biofilm on *S. australis* capsules (70µm,
Figure 5b) was approximately four times thicker than the biofilm on *D. orbita* egg
capsules (18µm, Figure 5a). Consistent with this, the total green fluorescence on the
surface of *S. australis* capsules was found to be significantly higher than the green
fluorescence emitted from *D. orbita* capsules (F = 193.61, df = 1, p < 0.001). Similarly,
the total red florescence was significantly higher on *S. australis* than *D. orbita* egg
capsules (F = 75.89, df = 1, p < 0.001).
No significant difference was observed between the amount of live versus dead microbes for either *S. australis* (*t* = 1.747, df = 5, *p* = 0.141) or *D. orbita* (*t* = 0.898, df = 5, *p* = 0.410) (Figure 6). However, the proportion of healthy bacteria was slightly higher (53%) than dead bacteria on surfaces of *S. australis*, whereas a slightly greater proportion of dead bacteria (52.5%) were present on the surface of *D. orbita* egg capsules. Under the CLSM dead bacteria were observed in association with a string of the unidentified droplets on the egg capsules of *D. orbita* (data not shown). Fluorescence microscopy also revealed an association between bacteria and the droplets, although small colonies of live bacteria were sometimes observed around the droplets as well.

[Insert Figure 5 and 6 about here]

**Bacterial attachment**

*Pseudoalteromonas* sp. Strain S91SB was able to survive and attach to egg capsule surfaces within flow chambers. Auto fluorescence of egg capsules was observed as blue, which allowed differentiation of GFP fluorescing bacteria. S91SB did not attach well to the surface of the *D. orbita* egg capsule (Figure 7), although some small micro-colonies of bacteria were observed after 24 h. Higher densities of bacteria were occasionally seen attached to the glass slide as a layer of floating GFP producing bacteria above the surface of the capsule (data not shown). TPLSM images also showed the unidentified droplets on the surface of *D. orbita* egg capsules, but these were not associated with S91SB attachment. In contrast, S91SB was almost always observed to colonize the surface of *S. australis* egg capsules in high densities (Figure 7).
The number of S91SB bacteria attaching to the surface of egg capsules from the two species was quantified by the area of fluorescence. Analysis of variance revealed a significant difference between the three time points in *D. orbita* (F = 7.717, df = 2, p = 0.005). Post-hoc analysis revealed that the 0h time point has significantly fewer attached S91SB than 72h (p = 0.004) (Figure 7). The number of attached S91SB at the 24hr time point did not differ significantly from 0h (p = 0.062) or 72 h (p = 0.370). For *S. australis*, increasing numbers of S91SB clearly attach to the surface over the 3 day period (Figure 7) and significant differences were observed between the time points (F = 151.871, df = 2, p < 0.001). However, post-hoc analysis revealed that there was no significant difference between 0h and 24h (p = 0.146), whereas bacterial load significantly increased after 72 h, with p < 0.001 for both 0h and 24h time points. Independent t-tests were performed to compare between species at time point 24h and 72h. This revealed significantly higher bacterial counts on the surface of *S. australis* compared to *D. orbita* egg capsules after both 24 (t = 9.765, df = 6.2, p < 0.001) and 72h (t = 12.45, df = 5.3, p < 0.001) in the flow chamber (Figure 7).

**DISCUSSION**

Egg capsules, being sessile in the marine environment, are susceptible to microbial assault (Shields 1990; Benkendorff et al. 2001). Therefore, they require some form of protection against fouling organisms. From this study, it is evident that the egg capsules of *D. orbita* have anti-fouling defense mechanisms to reduce the impacts of microbial foulers on the encapsulated embryos until they are released as pelagic larvae. This was
shown by comparatively low microbial densities observed on samples taken directly from
the field and after experimental induction of bacterial attachment in the laboratory. By
comparison, the eggs of *S. australis* appear to provide a favourable environment for
fouling organisms, but may be more resistant to microbial penetration due to the
overlapping of multiple layers in the capsule wall, which is 15 times thicker than the *D.
orbita* capsules (Figure 1).

As expected from the longer exposure times, the percentage cover of fouling
viewed on the egg capsules of both species increased as embryonic development
progressed (Figure 4). Percent coverage of fouling organisms on the surface was
significantly higher in developed capsules compared to fresh capsules (*p* < 0.001). A
greater range of fouling organisms were observed on the squid egg capsules (e.g. Figure
3) compared to *D. orbita*, including several bacterial morpho-types, as well as micro and
macroalgae. The presence of filamentous algae on well developed *S. australis* egg
capsules is consistent with independent observations on a Tasmanian population of this
species by Steer et al. (2002), who showed that algal fouling promotes synchronized
development of *S. australis* egg capsules. The beneficial effects from fouling by
photosynthetic algae could include increased oxygen levels for encapsulated embryos
during the day (Cohen & Strathmann 1996), protection from the effects of UV radiation
(Biermann et al. 1992) and possibly a reduction in settlement by other fouling organisms,
as many algae are known to produce antifouling compounds (Schmitt et al. 1995;
Dworjanyn et al. 1999; Maréchal et al. 2004).

The capsules of *S. australis* are multi-laminated, with layers arranged like a
plywood structure (Figure 1b), which helps explain their tough and elastic nature. The
multiple layers and overall thickness of these capsules could enable periodic shedding of built up biofilm, although this was not observed in any of the specimens examined here.

Rather, the irregular folds on the surface of *S. australis* capsules (Figure 3) appear to provide suitable habitat for bacterial settlement (refer to Verran & Boyd 2001). Field studies on micro-textured surfaces have also revealed that surfaces with 5μm wide ridges favor the settlement of green alga spores (Callow et al. 2002), due to the valleys and sidewalls of these structures providing an energetically stable attachment site. The complex structure of *S. australis* egg capsules could facilitate microbial settlement to enable biodegradation of the thick multi-laminated capsule wall to eventually enable release the juvenile squids. Unlike *Dicathais orbita* capsules, cephalopod egg capsules do not have an escape aperture and the juveniles must therefore break through the wall to be released. Molluscan species that lack escape aperture can typically only emerge once the capsule wall starts to deteriorate (Rivest 1983).

SEM images also provided detailed images of the changes in *D. orbita* egg capsule morphology over the course of encapsulated development. A thin layer of white crust was seen on the outer surface of fresh capsules (Figure 2a). This starts to deteriorate after approximately one week, exposing the micro-ridged structure of the capsule (Figure 2b &c). The mechanism driving this aging process is unknown, although it may simply be eroded under the influence of wave action or currents. Erosion of the periostracum in molluscs (Almeida et al. 1998) has been reported to lead to increasing amounts of boring organisms and settlement of barnacles and algae (Harper & Skelton 1993; Mao-che et al. 1996; Kaehler 1999; Cerrano et al. 2001). However, after removal of the crust from *D. orbita* capsules, a regular microtopographical feature of ridges and pores was observed.
throughout the trocophore stage (1-3 weeks) (Figure 2b). This surface texture is consistent with a physical defense strategy involving minimal contact points for attachment by biofouling organisms. Scardino et al. (2006) provide support for the “attachment point theory”, whereby reduced adhesion strength occurs for fouling organisms that are larger than the scale of microtexture. Nanoridges and pores at a scale suitable for the reduction of microbial attachment have been found on the skin of the pilot whale (Baum et al. 2002) and bivalve shells (Scardino et al. 2003). The spacing between ridges on D. orbita capsules (1-5µm, Figure 2c) compare favorably to the relatively unfouled shells of Mytilus galloprovincialis (1-2 µm, Scardino et al. 2003). Nevertheless, after only three weeks, D. orbita egg capsules maintained in a recirculating seawater system had still become heavily fouled (Figure 2d).

Egg capsules of D. orbita in the late veliger stage (> 4 weeks) contained the highest percent cover of fouling organisms (Figure 4a) and appear to be a lot smoother (Figure 2e &f) than the earlier stage capsules (Figure 2a & b). Ablation of the microtextured outer wall layer was observed under the SEM (Figure 2e). Mechanical shedding of the biofilm has also been reported in the algae Dilsea carnosa during late autumn and early spring (Nylund & Pavia 2005). The effectiveness of mechanical shedding in limiting surface fouling on D. carnosa was suggested to be especially important in temperate regions, where huge variations in fouling organisms arise as a result of seasonal change (Nylund & Pavia 2005). The egg capsules of D. orbita are deposited on temperate reefs in late spring to summer. Thus shedding of the outer capsule wall may be an effective strategy for preventing biofilm buildup at a key time in their embryonic development, when there are heavy microbial loads in the water column.
After shedding of the fouled ridged layer, the surface of D. orbita capsules was found to be relatively free from microbial fouling (Figure 2e), but areas with increasing densities of bacteria were observed in older capsules, accompanied by large unidentified droplets (Figure 2f). The droplets appear to be secreted through pores in the wall, as the outer capsule degrades. These droplets are clearly not cellular because they did not stain green or red when incubated with the Baclight™ nucleic acid probes. They also do not appear to be membrane bound vesicles, but remain globular rather than dissolving onto the surface. This suggests that they are hydrophobic, which is further supported by the lower densities of droplets observed using standard SEM after critical point drying in ethanol, compared to cryostat SEM. Soaking the egg capsules in organic solvents such as dichloromethane was also observed to remove the droplets from the surface (unpublished data), suggesting that they may contain lipophlic compounds, such as the indole dimer tyriverdin, which is a potent bacteriostatic agent previously reported from extracts of D. orbita egg capsules (Benkendorff et al. 2000). The droplets were observed to aggregate with bacteria on the capsule surface (Figure 2f) and fluorescence microscopy revealed they were frequently associated with dead bacteria (data not shown) suggesting they may have antimicrobial properties. Several species of marine macroalgae have the ability to produce secondary metabolites and release them onto the surface to prevent epibiont settlement (e.g. Ragan & Glombitza 1986; Clayton & Ashburner 1994; Dworjanyn et al. 1999). Further studies on the chemical defense of D. orbita capsules are clearly required.

Bacterial attachment experiments undertaken in laboratory flow chambers further support the idea that D. orbita egg capsules provide a less favorable environment for
biofilm formation than *S. australis* capsules. *Pseudoalteromonas* S91SB are motile marine bacteria commonly found in biofilm communities in temperate Australian waters are unlikely to have any specific interactions with molluscan egg capsules, thus providing a good model system for random biofouling. They secrete chitinases and proteases to break down particulate organic material (POM) (Techkarnjanaruk & Goodman 1999). Since molluscan egg capsules consist mainly of proteins and carbohydrates (Hunt 1966, Flower *et al.* 1969, Rapoport & Shadwick 2002), it was predicted that S91SB would preferentially attach to the egg capsules and utilize them as a carbon source, unless the capsules have properties that prevent bacterial attachment. However, S91SB were found to adhere preferentially to the glass coverslips than to *D. orbita* capsules (data not shown). Bacterial attachment was clearly higher at 72 h compared to 0 h; however there was no significant difference between the 24 h and the 72 h time periods in *D. orbita* capsules, indicating that the biofilm build-up becomes quickly stagnant (Figure 7). Conversely, bacterial attachment experiments on *S. australis* showed that S91SB was able to attach to these egg capsules and form a thick layer of biofilm (Figure 7). A significant increase in S91SB counts at both 24 h (p < 0.001) and 72 h time points (p < 0.001) was observed (Figure 7), again supporting the idea that antifouling protection is absent from *S. australis* capsules.

The removal and prevention of biofilm formation using physical, mechanical and chemical means has been described in other organisms, but typically these defense mechanisms are studied in isolation. Our studies indicate that the neogastropod *D. orbita* egg capsules may use a combination of all these defense mechanisms to defend its egg capsules, including a surface texture not suitable for bacterial attachment, followed by
shedding of the outer layer to remove existing microbial colonization and then exudation of unidentified chemical droplets that aggregate and possibly interfere with bacterial growth on the surface. By comparison, the cephalopod capsules had comparatively high loads of live bacteria, and their microtexture appears to provide good habitat for microbial colonization. The difference in the surface structure and microbial fouling properties of these two molluscan egg capsules contributes to our understanding of antifouling defense mechanisms in marine organisms.

**Acknowledgements**

We sincerely thank Kim Everuss (Flinders University), as well as Dr M. Wallwork, Dr J. Terlot and Ms L. Waterhouse (Adelaide Microscopy Unit) for their assistance with this study. We are also grateful to Dr M. Steer (SARDI, Aquatic Sciences) for supplying the *Sepioteuthis australis* egg capsules. This project was supported by a philanthropic grant to KB, and an Australian Research Council to AEG.

**References**


rejection of micro-textured surfaces and implications for recruitment by the barnacle


costs and benefits. Sci Total Environ 285:21-71


Everuss K (2004) Competition or cooperation between marine biofilm bacteria recycling POM. [Honours thesis]. Flinders University of South Australia


Harper EM, Skelton PW (1993) A defensive value of the thickened periostracum in the
*Mytiloidea*. Veliger 36:36-42


Hentzer M, Givskov M (2003) Pharmacological inhibition of quorum sensing for the

Hunt S (1966) Carbohydrate and amino acid composition of the egg capsule of the whelk,
*Buccinum undatum*. Nature 210:436-437

product information. [http://www.invitrogen.com](http://www.invitrogen.com)

Kaehler S (1999) Incidence and distribution of phototrophic shell-degrading endoliths of
the brown mussel *Perna perna*. Mar Biol 135:505-514


Lord A (1986) Are the contents of egg capsules of the marine gastropod *Nucella lapillus* (L.) axenic? Am Malacol Bull 4:201-203


Östling J, Goodman AE, Kjelleberg S (1991) Behaviour of InP-1 plasmids and a miniMu


23 Rapoport HS, Shadwick RE (2002) Mechanical characterization of an unusual elastic
biomaterial from the egg capsule of marine snails (*Busycon* spp.) Biomacromolecules 3:42-50


Stickle WB (1973) The reproductive physiology of the intertidal prosobranch *Thais lamellose* (Gmelin) I. seasonal changes in the rate of oxygen consumption and body component indexes. Biological Bulletin 144


Figure Legends

Figure 1. Scanning electron micrographs showing the transverse section of (a) *Dicathais orbita*, (b) *Sepioteuthis australis*, egg capsules. OW = Outer Wall.

Figure 2. Scanning electron micrographs of the surface of *Dicathais orbita* egg capsules at different stages of development; (a) undifferentiated embryonic stage, < 1 week old; (b) trocophore stage, 1-3 weeks old, (c) trocophore stage showing microtopographical features; (d) veliger stage, >3 weeks old, showing increasing densities of microbial foulers; (e) veliger stage, > 4 weeks old, with shedding of the fouled microtopographical structure, OW = outer wall; (f) late veliger stage, > 4 weeks, showing the presence of unidentified droplets (UD) on the surface.

Figure 3. Scanning electron micrographs of the surface of *Sepioteuthis australis* egg capsules in two stages of development; (a & b) fresh capsules (undifferentiated embryos) showing complex microtopography; (c & d) heavily fouled capsules containing well developed juveniles (paralarvae); (e) different bacteria and exopolymeric substances on the surface of a well developed capsule; (f) a diatom on the surface of a well developed capsule.

Figure 4. The percentage surface covered by fouling organisms on molluscan egg capsules at different stages of development in (a) *Dicathais orbita*, (b) *Sepioteuthis australis*. Error bars indicate the standard error of the means. The different letters above the bars indicate significant differences within species.
Figure 5. $x\z$ images of molluscan egg capsules stained with Baclight™ live/dead viability stain taken using confocal laser scanning electron microscopy; (a) live biofilm (green fluorescence) on *Dicathais orbita* egg capsule; (b) dead biofilm (red fluorescence) on *D. orbita* egg capsule; (c) live biofilm (green fluorescence) on *Sepioteuthis australis* egg capsule; (d) dead biofilm (red fluorescence) on *S. australis* egg capsule. The images were merged together using 36 individual scans taken at 2µm intervals. Ec = egg capsule. Scale bar = 60µm.

Figure 6. Mean amount of green and red fluorescence seen on the surface of molluscan egg capsules after staining with Baclight™ cell viability stain. Error bars indicate the standard error of the means. Different letters above the bars indicate significant differences between the groups.

Figure 7. Comparison of *Pseudoalteromonas* sp. S91SB counts on *Dicathais orbita* and *Sepioteuthis australis* egg capsules at different time periods after exposure in a flow chamber. Error bars indicate standard error of the mean. Letters above the bars indicate significant differences.
b) Percentage fouled (%)

Fresh (undifferentiated embryo)  Trocophore  Developed (late stage veliger)

Stage of development

b) Percentage fouled (%)

Fresh (undifferentiated embryos)  Developed (paralarvae)

Stage of development
**D. orbita** S. australis

Area of fluorescent (µm²)

- Green (live bacteria)
- Red (dead bacteria)

<table>
<thead>
<tr>
<th></th>
<th>D. orbita</th>
<th>S. australis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green (live)</strong></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td><strong>Red (dead)</strong></td>
<td>a</td>
<td>b</td>
</tr>
</tbody>
</table>
S91 counts

Sepioteuthis australis
Dicathais orbita

0h 24h 72h