QUALITATIVE ASSESSMENT OF INITIAL BIOFOULING ON FISH NETS USED IN MARINE CAGE AQUACULTURE

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Biofilm formation follows a generally consistent pattern throughout the global range of marine environments (Kerr *et al*, 1998; Higgins *et al*, 2003), consisting of an overlapping sequence commencing with a conditioning layer, followed by bacterial settlement, then Extracellular Polymeric Substance (EPS) build-up and finally microalgae settlement. After, and only after, biofilm formation can macroalgae or macrofauna become established, thus continuing the serial process of biofouling. Biofilm formation and fouling have been well studied on hard substrates such as pipes and ships hulls (de Beer and Stoodley, 1995; Jayaraman *et al*, 1998; Berntsson and Jonsson, 2003), where it causes damage and financial losses through, for example, lower flows or increased fuel consumption. Settlement on more flexible surfaces, such as marine fish culture netting, is less well studied. The costs of fouling for a medium sized company have been estimated at £60K per annum (Willemsen, 2005) but this simply covers the manpower time and processing required removing nets, having them cleaned and copper-based antifoulant re-applied. Additional costs could include reduced fish welfare brought about by low oxygen concentration due to poor water exchange,

increased stress through frequent net changing, increased drag and net deformation (Robinson Swift *et al*, 2006) with the potential for net failure and escapees and the potential to harbour disease pathogens (Douglas-Helders *et al*, 2003). Methods for off- and onshore cleaning of biofouling on cage nets and its physical and economic effects have been investigated at length (Dubost *et al*, 1996; Robinson Swift *et al*, 2006). However, little work has been done to assess the process of initial development of the biofilm. Understanding this is particularly important as it is the biofilm that acts as the mediating layer for later settlement of micro-organisms.

Net panels were deployed at a fish farm on Loch Craignish (56°09.200' N, 05°32.060' W) on the west coast of Scotland. The loch has full seawater with a slow to medium water flow through tidal exchange. Hydrography was not specifically measured during deployment but the site has an average current speed of 0.034 m s^{-1} at the surface (~3m deep) measured in April 1999, the water running in a north-east south-west direction parallel to the shoreline (University of Stirling, unpublished). Nets used here tend to be biofouled with algae and ascidians (Ian Webster, pers. comm.).

Panels (150 mm x 150 mm x 15mm mesh) were made from commercially available uncoated nylon fibre nets, held in wire frames. The deployment method is shown in Figure 1. Four net panels were deployed at a depth of 1m for 8 weeks (23rd January to 20th March 2006). Every 2 weeks a single net panel was removed and from this six 15mm^2 samples where randomly selected and observed using a Scanning Electron Microscope (JEOL JSM6460LV; JEOL (UK) Ltd, Welwyn Garden City, UK) (SEM) (3 samples) and a Confocal Scanning Laser Microscope (Leica TCS SP2 AOBS; Leica Microsystems (UK) Ltd, Milton Keynes, UK) (CSLM) (3 samples).

Samples for SEM observation were fixed in 1% glutaraldehyde in 0.1M sodium cacodylate at 4°C for 1 hour, transferred to 3% glutaraldehyde in 0.1M sodium cacodylate at 4°C for 2 days and after washing in buffer, post-fixed in 1% osmium tetroxide in buffer. Samples were then dehydrated through an ethanol series (30%, 60%, 90% and 100%) and critical point dried. Samples were mounted on stubs using double-faced tape and gold sputter coated (Edwards sputter coater S150B).

Samples for CSLM observation were washed with 2µm filtered seawater, stained using fluorescein sodium, DAPI (4',6-Diamidino-2-phenylindole, 300nM solution) and BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene, 80µM solution) as general, nucleic acid (nuclear) and lipid (*e.g.* biological membranes) stains respectively. Images were captured using the CSLM in conjunction with Leica Confocal Software (v 6.21), using excitation at 405nm, 488nm and 594nm laser lines and three PMT channels: blue 411 – 483nm, green 498 – 587nm, and red 600 – 698nm.

Figure 2 shows a schematic of the succession of species during the 8 weeks of deployment. The major visible constituents of biofilm formation after 2 weeks of immersion were bacteria and EPS, with low abundances of pennate diatoms (Figure 3 A - C) and globules of unidentified "gelatinous" material (Figure 4). The origin of the "gelatinous" material remains unclear but CLSM indicated that it displayed a strong affinity for the lipid dye BODIPY and it is therefore possible that it represents lipid droplets / agglomerations originating from material within the site. Deposition of such material may not be uniform across all sites and will depend upon such variables as the lipid content of the feed being used and general fish health. Regardless of its origin, however, these particles clearly represented a "sticky" surface on which diatoms were able to settle (Figure 4B).

After 4 weeks the abundance of centric diatom strings had increased, these appearing to be attached between net fibres within flocculated particulate material deposited from the water column. This material was stabilised by the presence of EPS (Figure 3D), which allowed the adherence of particles to one another and to the substrate. The flocculated material tended to accumulate within the pleats and crevices created by the net design and consisted of non-cellular substances and detritus. It remains unclear whether deposition of this material mediates diatom settlement or whether EPS excreted by diatoms acts as a sticky substrate that allows flocculated material to adhere. It is likely that both processes are acting simultaneously. Evidence for the fact that initial settlement facilitates further increased settlement comes from observations of, for example, pennate diatom colonisation of centric diatoms (Figure 3F and 3H). This substantiates the idea that the development of biofilm structure over time is mediated by the nature of earlier attachment events, probably through a combination of the provision of an appropriate physicochemical environment for settlement

with an ever-increasing three-dimensional surface area on which to attach. Broken frustules of diatoms were also abundant (Figure 3E), showing that the biofilm includes both living and dead organisms.

Biofilm formation after 6 weeks of immersion showed a heightened biodiversity and a noticeable increase in diatom abundance. Colonial species such as *Fragilaria* sp. were very abundant, along with centric diatom strings (Figure 3 G - I). Bundles of *Rhoicosphenia* sp. were attached by their associated EPS mucilage. There were a variety of stalked diatoms that included *Licmophora* sp., and various (unidentified) species of colonial and non-colonial pennate and centric diatoms.

After 8 weeks of immersion the majority of protists were centric diatom strings, which trapped an increased level of flocculated biogenic and non-biogenic material. *Licmophora* sp. abundance was increased (Figure 3 J - L) and pennate diatom chains were more abundant than at previous times with chains of *Diatoma* sp. and the colonial diatom *Bacillaria* sp. being numerous, although specific abundance was not assessed. The first microfauna (hydroid, Figure 3L) settled after 6 to 8 weeks in the water.

Directly comparing biofilm formation on hard surfaces with that on flexible nets is difficult because of the differing shear forces applicable, increased surface areas available for settlement and the fact that contained fish generate increased localized dissolved nutrient levels that may act to enhance settlement in nitrogen limiting systems. Settlement on fish cage nets, however, follows a similar pattern of succession through conditioning, bacteria and diatoms with associated EPS, and other microscopic algae and fauna. Settlement of diatom types and succession are occasionally reported (Patil and Anvil, 2005), but more generally will be dictated by the species diversity in the water column (Brown and Swearingen, 1998) at the time the net is placed in the water. Early settlement of bacteria and diatoms appears to increase the rate at which further settlement occurs. As the net panels where deployed outside the fish cage it is acknowledged that the rate of biofilm formation would have affected the succession and climax communities present.

The settlement described has specific implications for those developing alternative non-toxic coatings for aquaculture use. Copper sulphate coating on nets allows for the continuous release of toxic copper, where net coverage is less important that having an overall effect on the surrounding micro-environment. Non-toxic coatings are dependent on having an effect over the entire net surface that eliminates crevices and areas of non-coverage, onto which micro-organisms can then settle. At the macroscopic level nets appear relatively smooth, but at the microscopic level they are awash with small crevices and gaps between yarns and net fibres. On the evidence presented such gaps provide spaces within which, flocculent material and debris can be caught, without the need to have specific adhesive qualities and further, provides a massively increased surface area on which to settle.

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LIST OF FIGURES

Figure 1

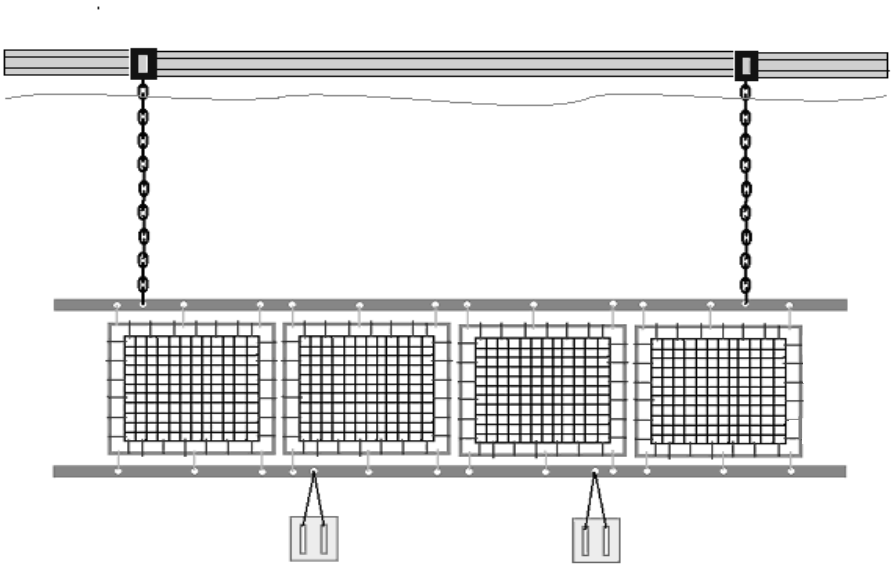


Figure 2

2 weeks	Extracellular polymeric substance (EPS), bacteria, pennate diatoms
↓	
4 weeks	Flocculated material, increased abundance of pennate diatoms, centric diatoms.
↓	
6 weeks	Increased biodiversity and abundance inc. *Fragilaria* sp *Rhoicosphenia* sp *Licmophora* sp
↓	
8 weeks	Increased biodiversity and abundance inc. *Diatoma* sp., *Bacillaria* sp., hydroids.

Figure 3

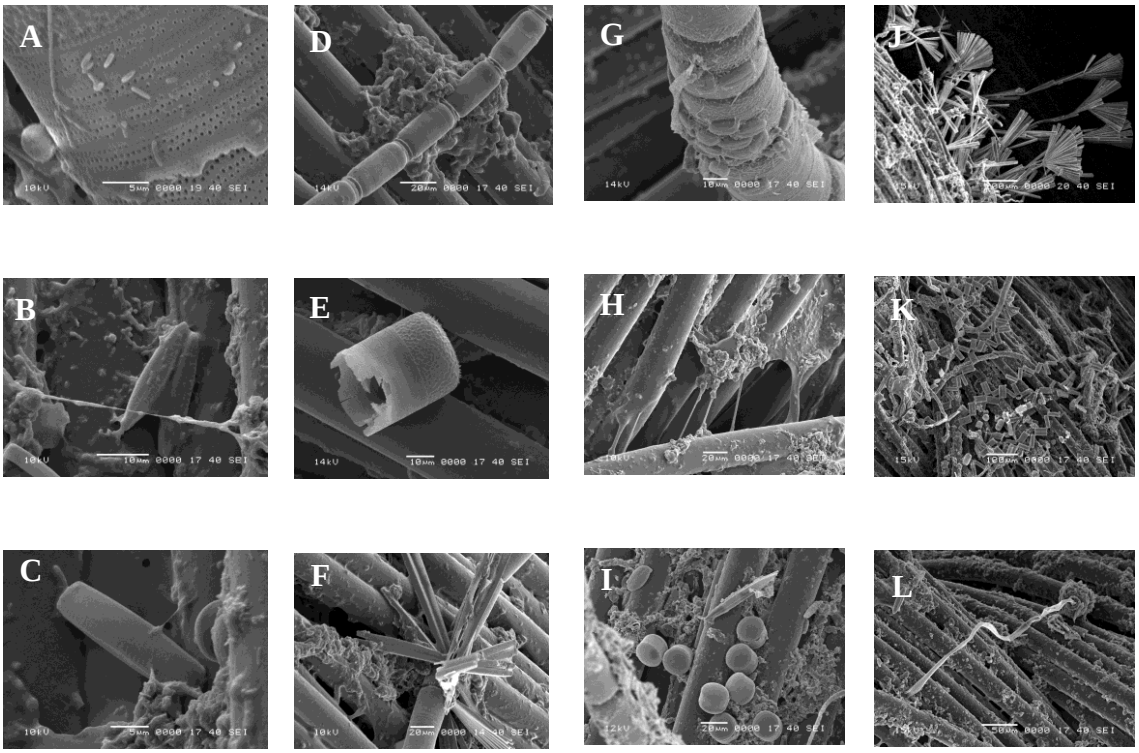
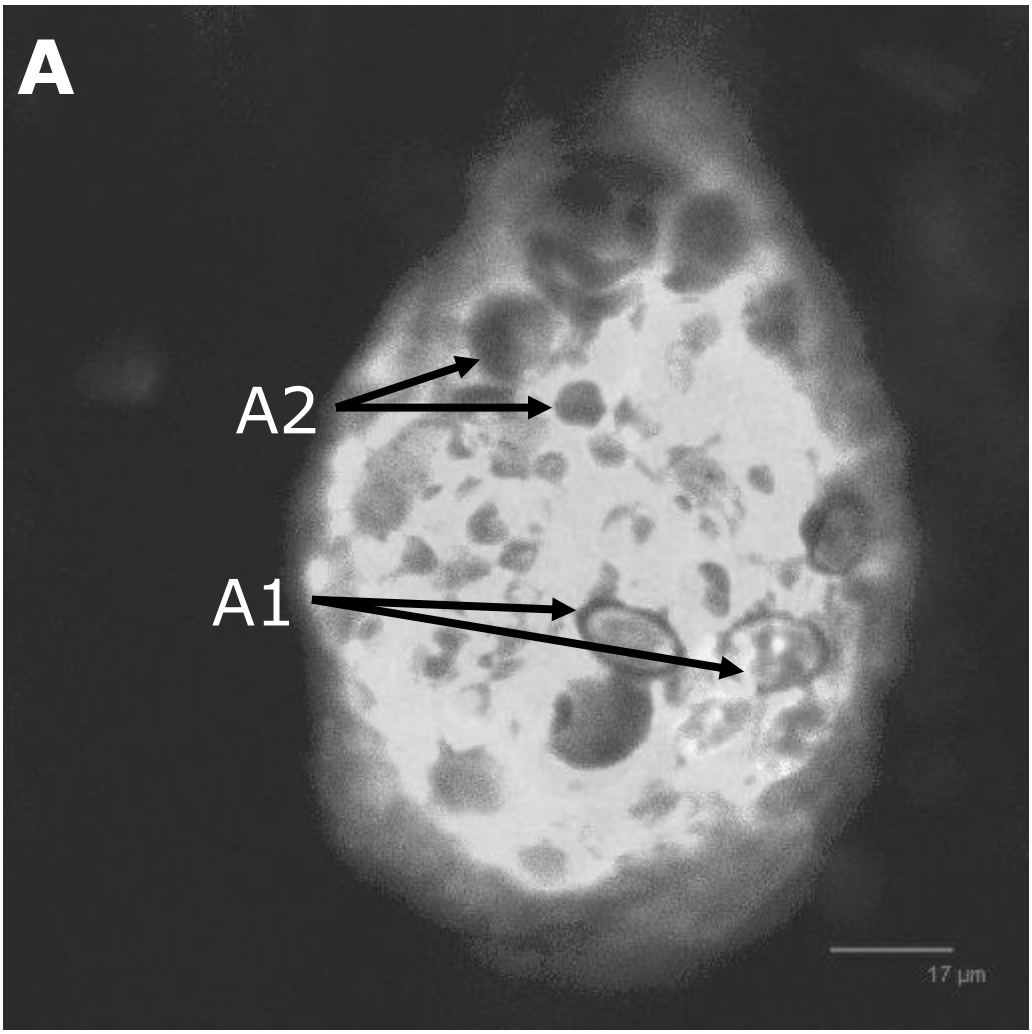
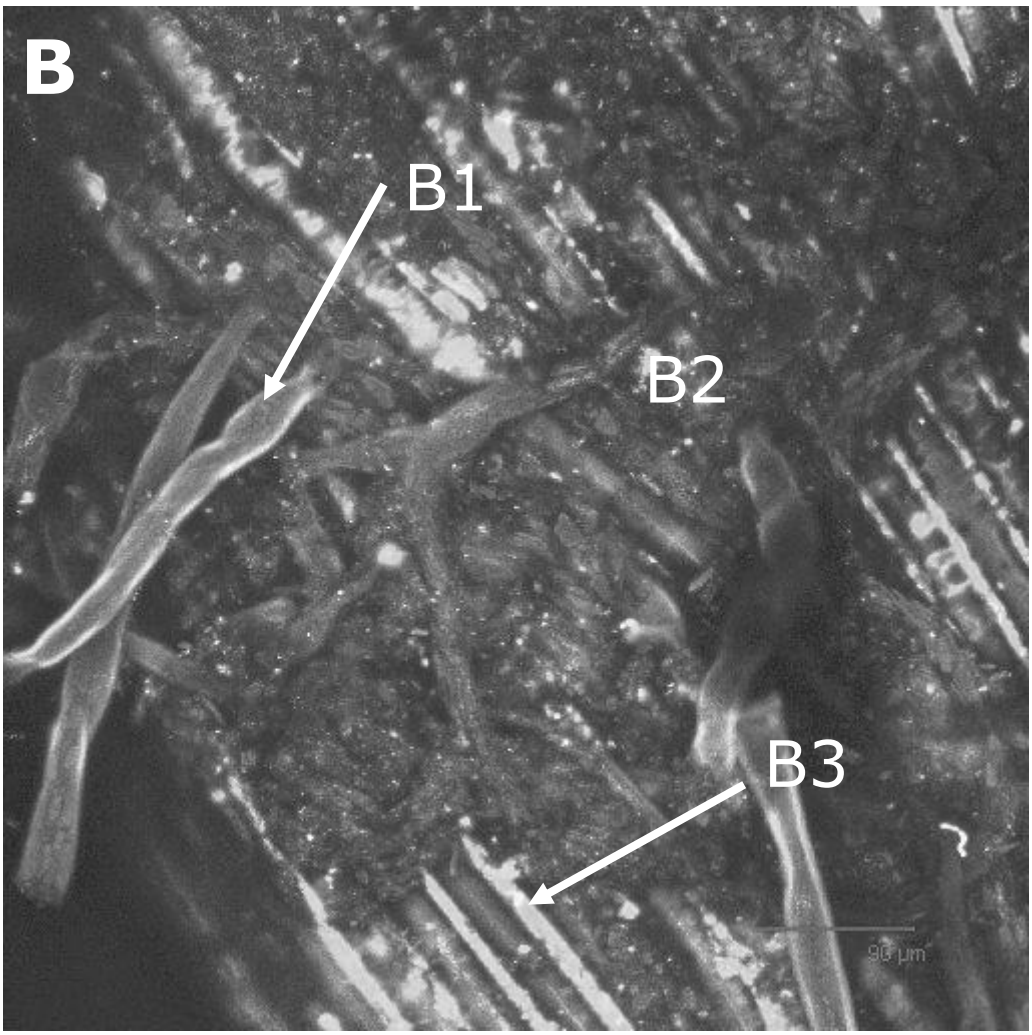


Figure 4

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