BIOFOULING METHODS

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[Figure 4.1 Analysis of 16S amplicon data sets. After](#page--1-197) the researcher has decided on a set of specific

barcoded 16S primers, the samples are amplified, mixed, and sequenced on a 454 pyrosequencer. Subsequent assignment to samples based on barcodes, trimming of barcodes, and primer and lowquality read removal give rise to a set of unique sequences that will be used in all downstream analyses. Here, two possible analysis paths are shown. The first is taxon based, and all sequences are clustered into operational taxonomic units (OTUs) with a specific cutoff (here: 3%). These data can be used to calculate metrics for the alpha and beta diversity. The phylogenetic identity of the OTUs can be determined by searching a representative sequence in a 16S database. The second analysis is phylogeny-based. Here, the first step is the calculation of a phylogenetic tree from all sequences. This tree is the basis for estimation of beta diversity [measures. Alpha diversity is estimated in combination](#page--1-197) with the taxonomic assignment of the single reads.

Figure 4.2 Example chromatogram. Each peak [represents a base position and each "color" \(depicted](#page--1-198) as gray levels) represents a base. The height of the peaks shows the light intensity as the florescent marked fragment passes the detector.

Figure 4.3 An overview of the different steps involved in microbial community analysis by DGGE, T-RFLP and ARISA techniques. Nucleic acids from an environmental sample are extracted, PCR amplified and the obtained bands are then analyzed. Note that the used primers amplify 16S rRNA in the case of [DGGE and T-RFLP but its region in the case of ARISA.](#page--1-199)

[Figure 4.4 Workflow indicating differences between](#page--1-200) BAC and Fosmid Clone libraries. This figure helps

[determine what type of clone should be used, as well](#page--1-200) as special instructions for construction.

Chapter 05

[Figure 5.1 Example of biofilm staining using different](#page--1-201) fluorescent markers. (A) Staphylococcus aureus ATCC 27217 biofilm stained with Syto9 and propidium iodide (Invitrogen). Green correspond to total cells and red/yellow correspond to membrane altered cells and also extracellular nucleic acids. (B) Staphylococcus aureus biofilm stained using Syto9 (total cells in green) and two lectins: ConA (red) and WGA (blue) (Invitrogen). (C) Amyloid fiber TasA stained with Thioflavine in Bacillus subtilis biofilms. (D) Bacillus subtilis 24-h biofilm of strain 168 carrying a GFP-hag transcriptional fusion and stained using the lipohilic marker FM4-64, which dye the cytoplasmic membrane in red (Invitrogen).

Figure 5.2 (a) Quantification of Chemchrome V6 fluorescence intensity loss (membrane [permeabilization\) during benzalkonium chloride C14](#page--1-199) treatment (0.5% w/v) at five different depths in a S. aureus ATCC 6538 biofilm. (b) Representation of fluorescence loss in the biofilm during the biocide treatment after 0, 30 s, 1 min, 1 min 30 s, and 2 min of application. Each image corresponds to the 3D reconstruction of fluorescence in biofilm using the IMARIS software (Bitplane®).

[Figure 5.3 Pathways leading to GacS/GacA-mediated](#page--1-19) gene expression. In all γ-proteobacteria GacS/GacA orthologs control "housekeeping" genes and horizontally acquired virulence genes regulating behaviors such as stress responses, attachment, motility, biofilm formation, virulence, and quorum sensing behaviors through the csr posttranscriptional regulatory system. GacS, a transmembrane sensor kinase, perceives an environmental signal (likely acetate [30]) and autophosphorylates. Phosphorylated GacS then transphosphorylates a response regulator GacA, [which binds to the promoter region upstream of the](#page--1-19) csrB sRNA gene to regulate its expression. The csrB regulatory RNA can sequester up to 18 CsrA molecules. Free CsrA protein binds to mRNA of target genes to either stabilize or de-stabilize messages. Stabilized messages are translated (flhDC) and de-stabilized messages are targeted for degradation (rpoS).

Figure 5.4 Expression of E. coli pMT41 (csrBluxCDABE) promoter reporter. To reconstruct the UvrY-csrB pathway of E. coli, the promoter of E. coli K-12 *csrB* sRNA was cloned upstream of a promoterless luxCDABE cassette (pMT41). Regulation of the reporter was tested in E. coli MG1655 uvrY33::Tn5 mutant (gacA orthologous mutant) in the presence of gacA from Serratia marcescens PDL100 expressed from an arabinoseinducible promoter on p BAD18-gacA. The gacA plasmid was constructed as follows. Firstly, genomic gacA from S. marcescens PDL100 was amplified with Taq polymerase using primers ACATCTCAGGCTATAACAGAGGCTG and TCGTCACGCAAAAGAACATTATATC. The resulting \sim 1000 bp PCR fragment was gel purified and cloned into pCR2.1-TOPO PCR cloning vector, from which it was excised with EcoRI and subcloned into pBAD18, which was completely digested with EcoRI and treated with CIAP. The resulting construct was confirmed by sequencing. pBAD18-gacA carries resistance to ampicillin. Strains contained the

promoter reporter in the wild type E. coli MG1655 (), or gacA (uvrY) mutant RG133 (), with pBAD18-gacA in the presence of 50 mM arabinose () or with the [pBAD18 vector control in the presence of arabinose](#page--1-19) (). The substitution of glucose for arabinose eliminated complementation by gacA borne on p BAD18-gacA().

Figure 5.5 Typical results generated from an initial lux screen. E. coli MG1655 pMT41 serves as a positive control. E. coli MG1655 pTIM2442 is a control for non-csrB specific luminescence. E. coli RG133 pMT41, LB media only and blank wells all serve as negative controls. The graph shows luminescence activity of nine compounds which were selected for additional study from the 1280 compound Library of Pharmaceutically Active Compounds (LOPAC). Compounds with no inhibition or less than 1 log(CPS) counts were not considered. Most show an intermediate level of luminescence with similar counts between the pMT41 and [pTIM2442 reporters indicating nonspecific inhibition.](#page--1-202) Only sanguinarine shows a significant csrB specific inhibition although log(CPS) counts are well below the E. coli RG133 pMT41 negative control.

Figure 5.6 Typical Results generated from a dilution series time course lux screen. E. coli MG1655 pMT41 serves as a positive control. E. coli MG1655 pTIM2442 is a control for non-csrB specific luminescence. E. coli RG133 pMT41 serves as the [negative control. Eight threefold dilutions were used,](#page--1-203) 150–0.07 μM. (a) Results of the 3′-Azido-3′ deoxythymidine dilutions series, which represents strong nonspecific lux inhibition; these compounds are likely to inhibit bacterial growth. (b) Results of the stavudine dilution series, which represents high

dilution(s) only nonspecific *lux* inhibition; these [compounds are likely to inhibit metabolism and/or](#page--1-203) luminescence.

Figure 5.7 Effects of DMSO on biofilms formed by S. Typhimurium. DMSO is a volatile solvent commonly used to dissolve candidate compounds in these assays. To determine if DMSO impacts biofilm formation, liquid cultures of Salmonella were incubated in the presence of DMSO at increasing concentrations for 24 hours in microtiter plates. [Bound biofilms were stained with 0.1% crystal violet](#page--1-204) and subsequently solubilized with 33% acetic acid. White bars represent uninoculated CFA media with DMSO incubated for 24 hours at 37 °C, stained and solubilized in 33% acetic acid. Absorbance measurements were made at 595 nm using a microtiter plate reader.

Figure 5.8 Background binding of dye and DMSO to Corning 96-well polystyrene plates. The staining process involves crystal violet, 33% acetic acid, [ethanol, and potentially other solvents. This results in](#page--1-205) significant background binding of crystal violet to polystyrene microtiter plates. For this reason, solubilized biofilms and controls are transferred to new 96-well polystyrene plates prior to absorbance measurements. This reduces background staining and variability.

Chapter 06

Figure 6.1 (A) Schematic plan view of a slide rack [showing a randomized and replicated arrangement of](#page--1-206) slides. Only one batten is shown, though another one at the bottom can be used for security of the slides in turbulent waters. (B) Side of view of rack showing how neoprene pinches the slide in place as the bolt is

<u>tightened. (C) and (D) show how the deployment</u> ropes should be tied with a weighted bridle to minimize excessive swinging in currents and waves. [The buoys \(D\) maintain the rack at a constant depth](#page--1-206) in a tidal regime. (E) shows a rack attached to a Tboom for deeper water deployment. (Not to scale.)

Figure 6.2 (a) Example data presentation that is not correct as the bars are means across all levels of replication, in this case that includes field of view, replicate and block. The error bars are standard deviations. (b) This is also incorrect as the bars are means of all levels of replication and the use of 95% confidence intervals is therefore misleading and encourages the reader to eyeball the data and make subjective and, therefore, erroneous interpretations. This is the most common form of data display. (c) Probably the best way to present these data as the bars represent the estimated means from the generalized linear model and the errors indicated are simply standard deviations. (d) Treatment group [centroids of two discriminant functions summarize all](#page--1-19) variables measured, including species data, for this experiment. There is a clear grouping of some treatments with the rest scattered across the plot. Interpretation requires examination of the two function matrices.

[Figure 6.3 Ballast Organic Biofilm \[BOB\] sampler](#page--1-207) used to acquire biofilm samples in ballast water tanks.

[Figure 6.4 Photo of tray of test plates \(left\). As shown](#page--1-208) on the right, two of these trays fit into the BOB sampler.

Figure 6.5 Amount of chlorophyll (μg cm−2) on the [rock surface in a single plot across sampling times:](#page--1-209) (a) before removal of macro-algae $-$ day 0 ; (b) day 1 where algae were removed at top left and bottom right quadrants; (c) day 44 ; (d) day 75 ; (e) day 117 ; [\(f\) day 160. Elevated amounts of chlorophyll at 'A' and](#page--1-209) 'B' are due to growth of macroalgal sporelings or cyanobacteria [14].

Figure 6.6 Reflectance spectra (350–850 nm) of macro- (top panel) and microalgal (bottom panel) biofilms on a rocky substratum. The horizontal black lines at the top of each graph show the wavelength [regions covered by a multispectral \(CIR\) camera \(G =](#page--1-210) $green; R = red; NIR = near infrared. Absorptions by$ pigments are shown: $PE = phycoerythrin$; $PC =$ phycocyanin.

[Figure 6.7 Reflectance spectra of a marine biofilm](#page--1-211) grown on a sandstone tile (350–850 nm).

[Figure 6.8 Second- and fourth-order derivatives \(400–](#page--1-212) 750 nm), respectively, of biofilms of green micro- α lgae (a, b) , cyanobacteria (c, d) , and diatoms (e, f) . The original reflectance spectrum is shown in gray.

Chapter 07

[Figure 7.1 Schematic and photograph of the parallel](#page--1-213) plate flow cell (U.S. Patent No. 4,175,233). Many other types of test plates can be substituted for the germanium prisms indicated in the drawing on the left. The rigid outer shells of Plexiglas, are shown in the photo on the right.

Figure 7.2 Views of the Portable Biofouling Unit [PBU] showing two different styles of manifolds. In [the photograph on the left, six parallel plate flow cells](#page--1-214) are installed, with water directed to the flow cells from the manifold on the opposite side of the unit.

[The electrical cord from the small submersible pump](#page--1-214) is seen at the lower right corner of the PBU.

[Figure 7.3 Examples of microscopic views of ocean](#page--1-215) biofilms after immunofluorescent staining: (a) Comamonas terrigena; (b) Vibrio alginolyticus; (c) Achromobacter; (d) Pseudomonas putrefaciens.

Figure 7.4 Mixed population fermentor system [schematic. Details of the component systems are](#page--1-216) given in Table 7.2.

Figure 7.5 Biofilms grown in the mixed population fermentor on a single, standard nonbiocidal coating [but in four separate weeks \(columns\) over the course](#page--1-217) of a few months differ in coverage, color, and structure.

Chapter 08

[Figure 8.1 Boat-based pumping system with water](#page--1-19) being pumped though the corrugated hose into a small plankton net suspended from a PVC frame inside a shipping drum. Note the webbing straps securing the drum to the rail of the boat.

Figure 8.2 Blow-up schematic of larval trap with (A) rubber cap with the top cut out and secured with hose clamp that holds the funnel and plankton net in place, (B) funnel and ball valve, (C) small plankton net into which the funnel nests (opening is upward); the trap body is composed of a (D) large diameter PVC pipe with drainage holes glued to (E) a flatbottomed PVC end cap. The trap is secured to the substrate with stainless steel brackets. A sleeve (not shown) can be sewn into the side of the plankton net and loaded with a formalin-impregnated chalk block to kill and fix captured larvae. For simplicity, the [plankton net is illustrated as a shallow cup shape but](#page--1-19) should actually be deep enough that about 3 cm of the top edge can be folded down over the rim of the [trap body \(D\) so that the net is firmly held in place by](#page--1-19) the rubber cap and hose clamp (A).

Figure 8.3 Cylindrical tube trap constructed from [conical tubes and flexible PVC tubing \[19, 20, 32\].](#page--1-218)

[Figure 8.4 PVC crosses for deployment of cylindrical](#page--1-143) tube traps on moorings [32].

Figure 8.5 A small cleared area has been scraped in the mussel bed to allow attachment of settlement collectors: a 10 \times 10 cm PVC barnacle settlement plate and an orange plastic pot scrubber (Tuffy[™]) for mussel settlement.

[Figure 8.6 \(a\) Binarized input, \(b\) labeled cyprids and](#page--1-126) (c) all cyprid tracks processed from color AVI sequence in ImageJ. In this video a total of 75 cyprids are tracked in the ROI.

Figure 8.7 (a) Mean and error bar (95% confidence interval) plot of standard derived variables for larvae exploring seven surfaces. Note how difficult it is to understand the difference between the surfaces. (b) [Discriminant function plot for the same data showing](#page--1-220) clearly the separation between two groups of surfaces, with surface G quite different to all others.

[Figure 8.8 Radar plots of the same surfaces shown in](#page--1-221) Figure 8.7. The plots take much more space but identifying differences is quite straightforward.

Chapter 09

Figure 9.1 (a) Example of a fouling community on a PVC panel submerged for six months. (b) With [overlaid grid points to estimate percentage cover. \(c\)](#page--1-222) Removal of all but one species using threshold color