Pseudomonas-infeksjoner fra vann/miljø - en analog utfordring for både folk og fisk

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Disposisjon

- *Pseudomonas aeruginosa* i dypdykking offshore
  - Kunnskap i 1988 – start av FUDT programmet
  - Opparbeidet kunnskap gjennom 25 års overvåking
    - Biotyping, serotyping og genotyping
  - Biofilm sin rolle for langtidsoverlevelse av infeksjonsagens
- *Pseudomonas fluorescens* i settefiskanlegg – en forstudie 2011
  - Screening av mikrobeflora i vann i settefiskanlegg
    - Biotyping
    - (Genotyping)
- Oppfølging – Strategier og forutsetninger

Deep diving offshore
- Operational saturation diving

- Occupational saturation diving
  - Close to installations

- Pressurized, warm and humid atmosphere

•Living in cramped confined chambers onboard a diving vessel

- Frequent skin infections

In 1977, the world could first read about the specific role of *P. aeruginosa* in infection outbreaks in diving systems

- *Alcock SR*. (1977). Acute otitis externa in divers in the North Sea. A microbiological survey of seven saturation dives. Journal of Hygiene (Camb) 78:395-409

- The same serotype of *P. aeruginosa* (O12) in several divers made up the following hypothesis:
  - **person to person contagion**
  - **the divers ”contaminate the environment”**

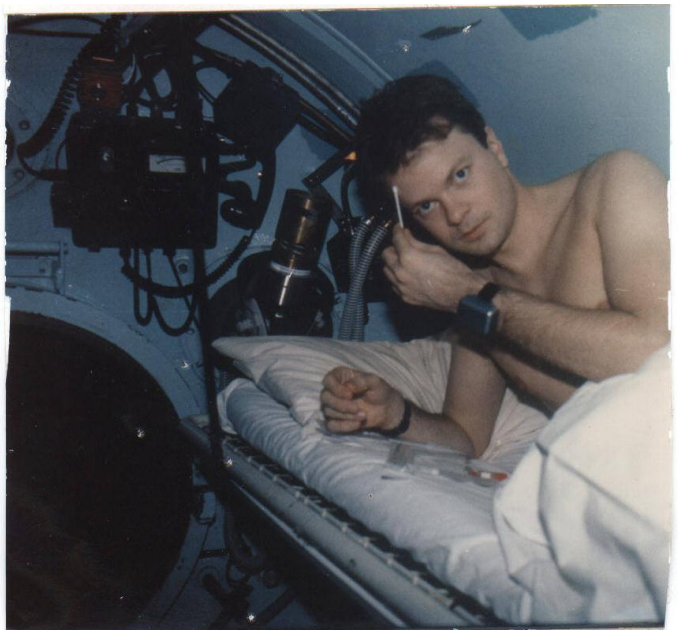
- The study concluded that infections are brought into the systems and are spread by – the divers

- and this was still common sense by the time for the start of the systematic R&D in Diving Medicine 1985

- **HYGIENE**
  - Personal hygiene
  - Daily showering, antiseptic soap
- **PREVENTION**
  - Frequent control of divers
    - Ear samplings pre- and post saturation
  - Profylaxis (ear drops)

- New information: *P. aeruginosa* only <u>post saturation!</u>

- The divers became sampling specialists!



This new information ”demanded” environmental surveys – the first in 1986

- Water (freshwater)
  - Chamber external
  - Chamber internal
  - FW Tanks
- Environment
  - Shower equipment in chambers
  - Divers personal equipment
- For determination of relations to infections
  - Which today is the base of an unique biobank of *P. aeruginosa* isolates

- Typical picture from initial water analyses

Pseudomonas aeruginosa - ”North Sea” Biobank (1)

- Serotyping 1985-1994 – same serotype in infections and water

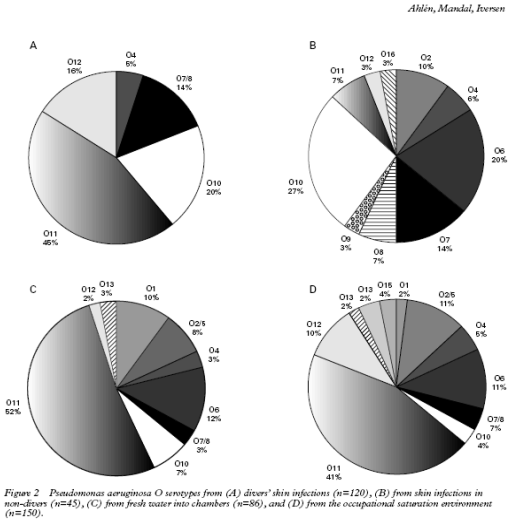
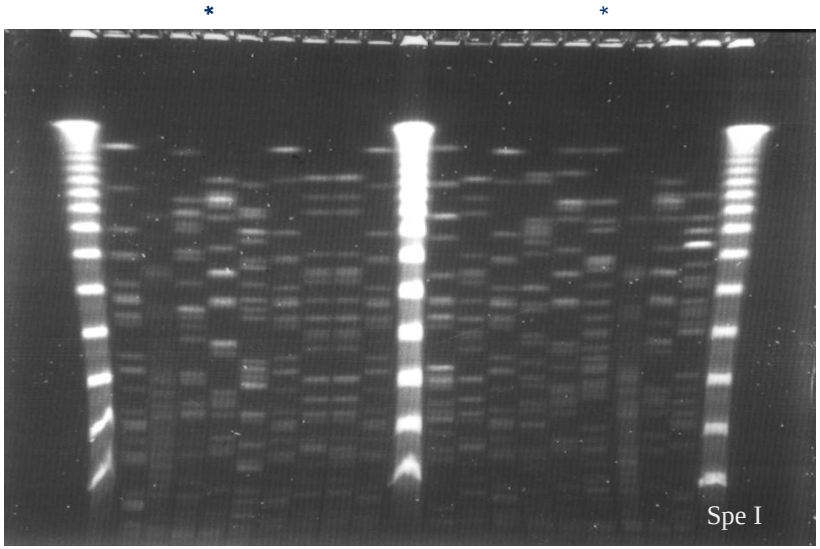


Figure 2 Pseudomonas aeruginosa O serotypes from (A) divers' skin infections (n=120), (B) from skin infections in non-divers (n=45), (C) from fresh water into chambers (n=86), and (D) from the occupational saturation environment (n=150).

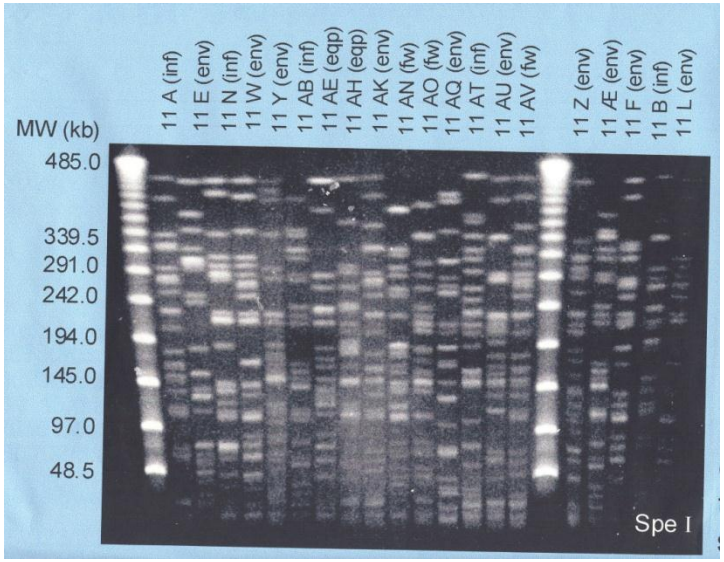
Ahlén C, Mandal LH, Iversen OJ. Identification of infectious strains of Pseudomonas aeruginosa in occupational saturation environment. Occup Envir Med 1998; 55:480-484

- 1995: Genotyping – PFGE
  - Pulsed Field Gel Electroph
    - Fragments of DNA – ”fingerprint”
    - Elektrophoresis-separation



Pseudomonas aeruginosa - ”North Sea” Biobank (2)

- Serotyping versus PFGE
  - 20 Serotype O11 – 20 separate PFGE genotypes

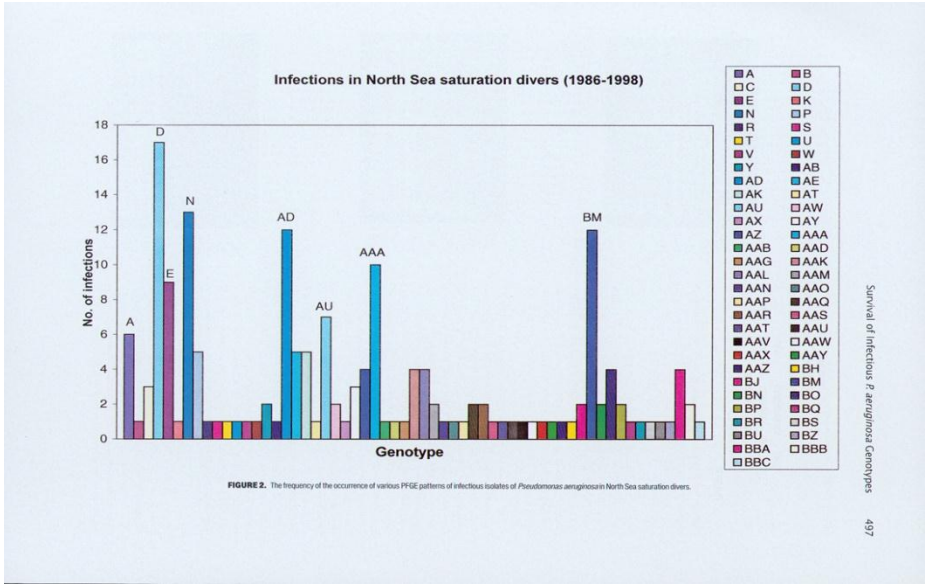


Ahlén C, Mandal LH, Iversen OJ :The Impact of Environmental Pseudomonas aeruginosa Genotypes on Skin Infections in Occupational Saturation Diving Systems. Scand.J Inf Dis.33; 413-419, 2001

- Although serotyping was the gold standard for epidemiology of *P. aeruginosa* by that time
  - the tag ”serotype” showed not to be useable for traceability of infection reservoirs within diving systems
    - and is most likely not usable for any source or reservoir detection
- Since 1995, PFGE has been our method for epidemiology of *P. aeruginosa*
  - and is today the gold standard for epidemiology of *P. aeruginosa* worldwide

Pseudomonas aeruginosa - ”North Sea” Biobank (3)

- Retrospective PFGE 1985 – 2000

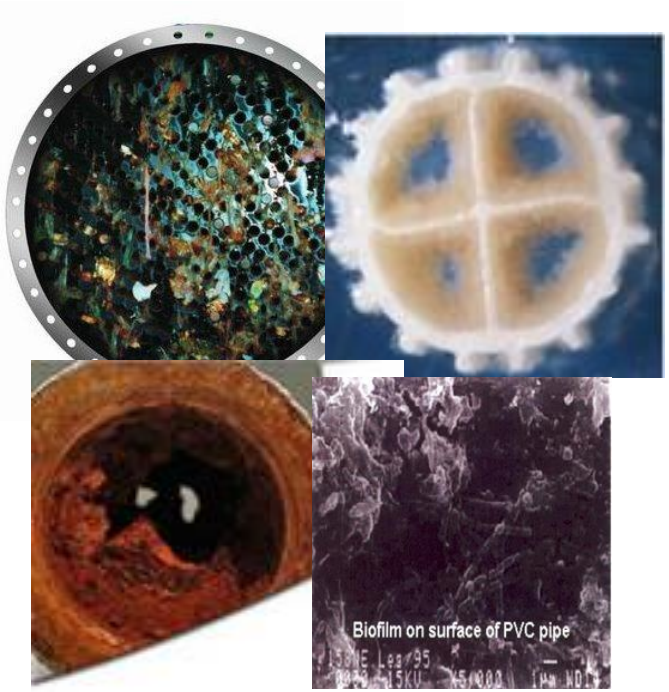


Ahlén C, Mandal LH, Johannessen, L, Iversen OJ. Survival of infectious Pseudomonas aeruginosa genotypes in occupational saturation environments and the significance of these genotypes for recurrent skin infections. 2000. Am. J. Ind. Med.2000; 37:493-500

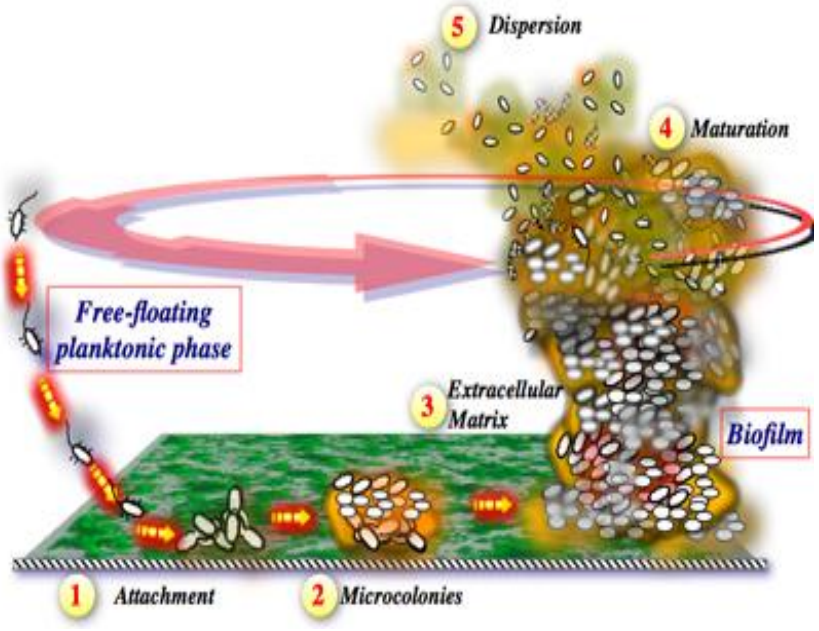
- Only a very few genotypes frequent in infections!
  - some of which seen for more than 15 years
    - despite regular disinfection regimes
  - some causes infections again and again
  - others are never seen in infections
- Documented presence prior to divers entrance
  - i.e not brought in and spread by the divers!!
- Where do they hide?
  - and how?

Long time persistence of infectious *P. aeruginosa* - the role of Biofilm!

- Biofilm is the microbiological society established within water systems

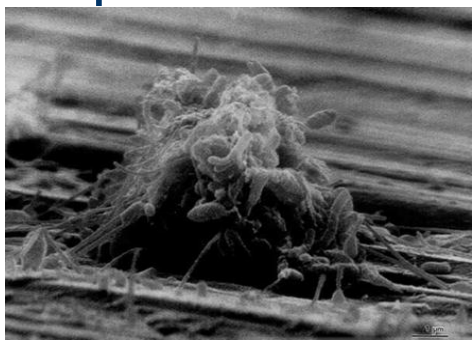


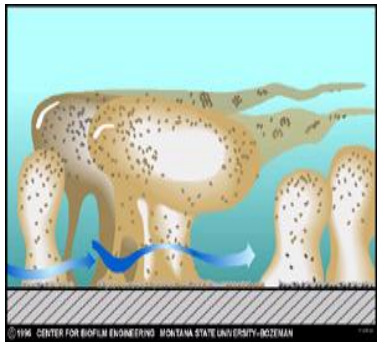
- Biofilm development = changes of growth mode.



Biofilm

- Today we are fully aware that biofilm is a natural part of life……

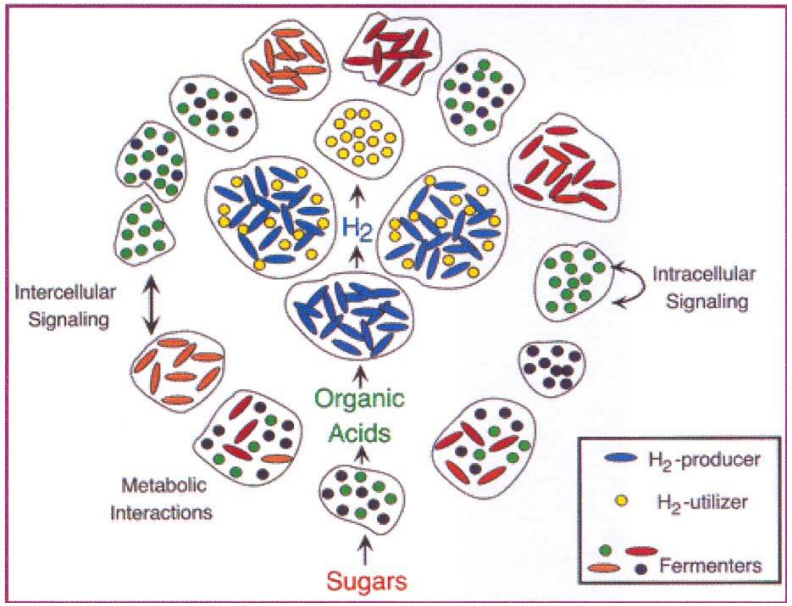


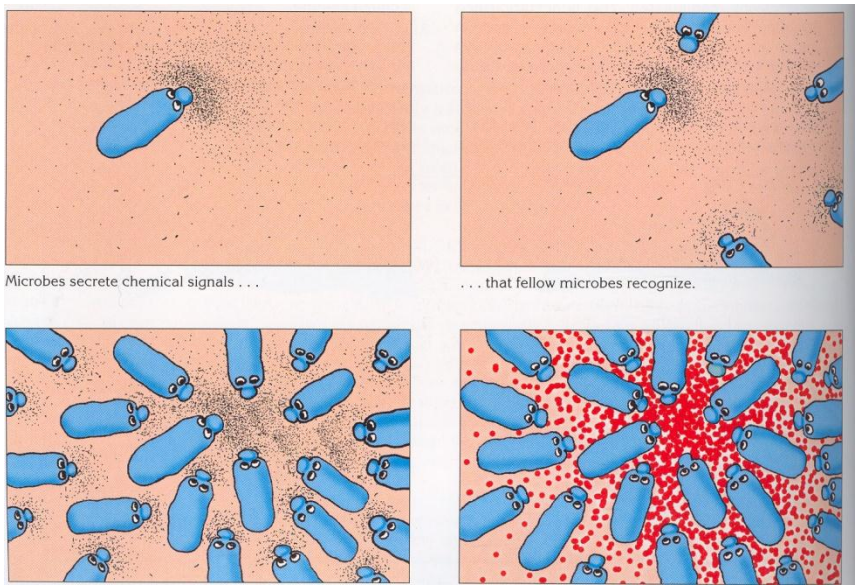


- Biofilm – described as a microbial community in 1943
- Biofilm protects the microbes
  - antibiotics
  - detergents
  - heat
- 30 years of intensive research
  - Gram-negative bacteria
    - model P. *aeruginosa*
    - structure/composition
    - Signal substances
- Medical relevance – after 2000
  - Altered /increased pathogenicity and virulence

Biofilm
- An own society!

- Well organized
  - Pure culture/mix
  - Production and degradation of organic material
- And communicates
  - Signal molecules (Quorum Sensing (QS)
  - Intracellular/intercellular





Det er i Biofilmen det skjer……

- 2006:

  Biofilm - training ground for patogenic microbes

  Maëlle Molmeret M., Horn M., Wagner M., Santic M. and Kwaik Y. (2005) Amoebae as Training Grounds for Intracellular Bacterial Pathogens. Appl. Environ. Micro. Vol.71,No.1.p.20-28.

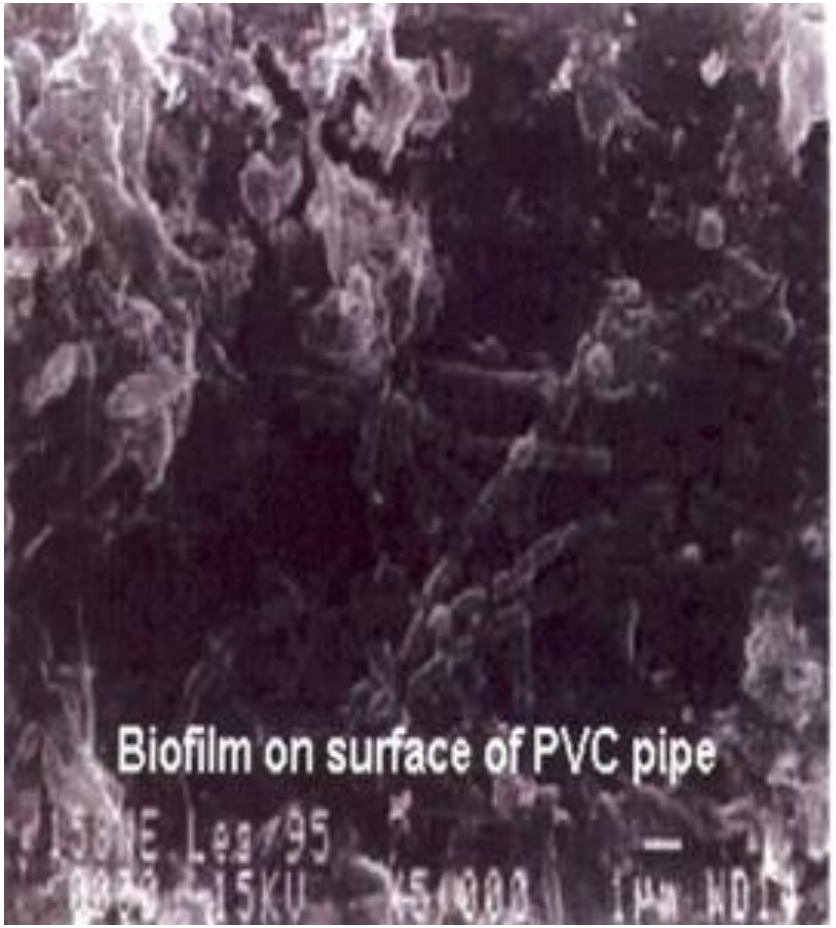
- 2008:

  Biofilm generates genetic diversity!

  Kolter R and Greenberg EP.: The superficial life of microbes. Nature Vol 441. May 18th, 200

Dette var vårt ståsted når vi ble kontaktet av SINTEF Fiskeri og Havbruk v/Yngve Ulgenes

- som spurte om teorien om at PVC kunne virke som vekststimuli for *P. fluorescens* fortsatt gjaldt?
  - i så tilfelle - kunne det være av relevanse for nyhetsoppslag januar 2011 om massedød av settefisk forårsaket av *P. fluorescens?*

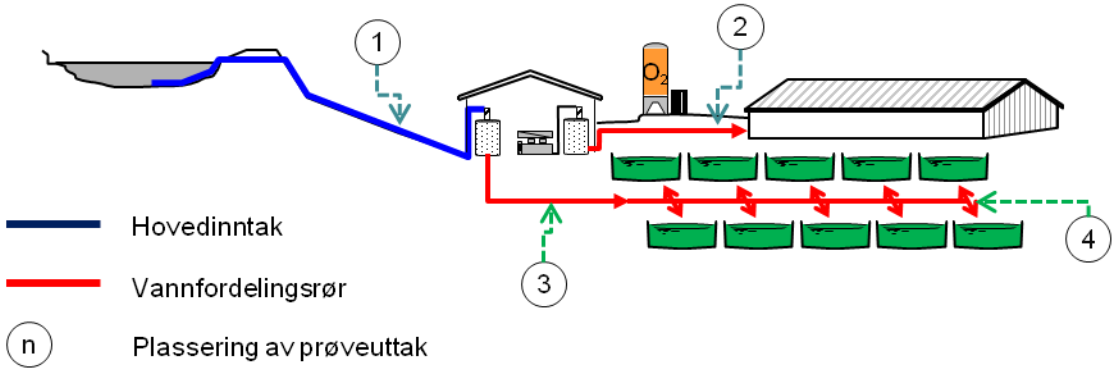


”Screening av mikrobeflora i vanntilførsler til settefiskanlegg med fokus på Pseudomonas sp”

- I januar 2011 melder Veterinærinstituttet at bakterien *Pseudomonas fluorescens* kan være primærårsak til infeksjon og dødelighet hos fisk i settefiskanlegg – dvs en aggressiv patogen bakterie.
  - I løpet av 2010 var det i alt 11 settefiskanlegg hvor bakterien ble rapportert som primærårsak til massiv dødelighet
  - Vanlig forekommende i vann – nærmest sett på som "normalflora”
- Utfordringen analog med dykkerinfeksjoner med bakterien *Pseudomas aeruginosa* som primærårsak
  - Vanlig forekommende i vann – nærmest sett på som "normalflora”
    - Vi har dokumentert at det kun er noen svært få genotyper av bakterien som gir infeksjon
      - Biofilmproblematikk sentral
- Vi vil bruke samme strategi for om mulig å identifisere spesielle infeksjonsgenotyper av *P. fluorescens*

Forprosjekt 2011 Arbeidsbeskrivelse

- Innsamling av vannprøver fra settefiskanlegg med og uten problemer (F&H)
  - 4 vann/4 svabere i pnkt som vist under
  - Samtidig registrering av data fra anlegget



- Mikrobiologiske analyser (T&S)
  - kultivering, biotyping og genotyping

Resultater – *P fluorescens*

- Biotyping ID32 GN (bioMerieux) (1)

Vannprøver fra 41 anlegg:

- *P. fluorescens 1,2* (excellent, very good, good id) påvist fra 18 av 41 anlegg (44%)
  - *P fluorescens* 1 fra 10/18 anlegg ; *P. fluorescens* 2 fra 14/18 anlegg
  - *P. fluorescens 1,2* i vanninntak fra 8 av18 anlegg (44%)
- *P. fluorescens? – (unacceptable/doubtful id) påvist fra 9 anlegg*

Resultater – Andre bakterier
- Biotyping ID32 GN (bioMerieux) (2)

- *Pseudomonas aeruginosa* påvist fra 2 anlegg (NB!)
  - Andre pseudomonasarter
- Andre G-neg: Yersinia (12/41), Serratia (16/41), Aeromonas (18/41), etc
- Koliforme: Enterobacter, E Coli, Morganella, Klebsiella, Proteus i 3/41 anlegg = ”Kloakkanlegg”
- Noen få meget ”rene” anlegg

Genotyping PFGE
(Pulsed Field Gel Electrophorese)

Som for dykkerinfeksjonene så er vi på jakt etter:

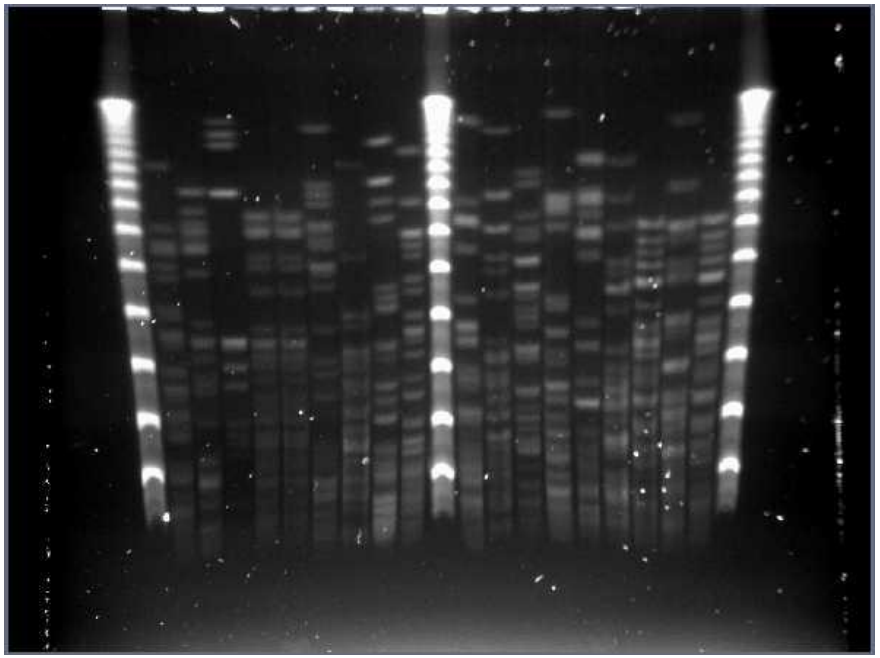
- Spekteret av genotyper av *P. fluorescens* i anleggene
  - Dominante genotyper i "sykdomsanlegg"?
- Referansegenotype
  - Genotype for kjente infeksjonsutbruddstammer av *P. fluorescens*
  - *P. fluorescens* F. nr 6965 av 2009-09-352 L1 fra Vet inst i Oslo
    - vist å forårsake sykdom i deres eksperimentelle systemer

Genotyping krever artsbestemmelse

- I vårt tilfelle biotyping vha. ID 32GN av referansestammen *P. fluorescens* F. nr 6965 av 2009-09-352 L1 fra VI Oslo
  - Referansestammen vill ikke la seg biotype i vårt system
    - *P. fluorescens* ”Unacceptable identification” på ID32 GN
- Mangler sykdomsreferanse for *P. fluoerscens* genotyping
  - Modellstudie mhp. brukt oppsett (PFGE + Spe1)

Vårt genotypingsverktøy fungerer meget bra

- PFGE ser ut å være meget velegnet som verktøy for å separere genotyper også av *P. fluorescens*
- PFGE så langt gjort på
  P. fluorescens 1 og 2
  - Excellent identification ID32 GN
- PFGE –mønster på Fnr 6965 er meget avvikende fra de øvrige



Hjelp!
Forprosjektet kan ikke sluttføres som planlagt

- To umiddelbare utfordringer
  - Artsbestemmelse av utbruddstammen av "*P.fluorescens*"
  - Nå blir vi også usikre på hva vi har av relevante stammer i materialet
    - I vårt innsamlede materiale fins i tillegg til *P. fluorescens ” excellent, very good/good id” også ”doubtful id” og ”unacceptable id”*
      - *Er noen av dem identisk med "referansestammen?"*

Nye muligheter for artsbestemmelse - MALDI-TOF

- En helt ny generasjon analyseutstyr for artsbestemmelse er under utprøving på St Olavs Hospital mikrobiologi
  - Matrix-Assisted Laser Desorption/Ionization (MALDI)-TOF (Time of flight).

- Vi fikk anledning å analysere noen få isolat på dette nye utstyret og valgte da å teste den tilsendte *P. fluorescens* stammen (Fnr 6965 2009-09-352 L1).
  - Denne analysen kom ut med *Pseudomonas antarctica* som første valg.
    - Hvorvidt denne inngår i "*P fluorescens* group" er foreløpig uklart

Foreløbige konklusjoner (1)

- Mikrobiologisk screening med biotyping for å påvise forekomst av *Pseudomonas fluorescens* i vannprøver og biofilmprøver fra 41 settefisk-anlegg for laks og ørret
  - *P. fluorescens* (excellent/very good/good/acceptable identification) er påvist i 18 av 41 anlegg
    - I tillegg ser vi flere anlegg med "P. fluorescens" med usikker identifisering, herunder ”low discrimination", "doubtful" og ”unacceptable”
- Det sykdomsfremkallende *P. fluorescens* -isolatet supplert fra Veterinærinstituttet kommer ut som "unacceptable" i vårt testsystem
  - Analyse med MALDI-Tof gir *Pseudomonas antarctica*

Foreløbige konklusjoner (2)

- Hovedformålet med dette forprosjektet - genotyping av *P. fluorescens*-isolat fra settefiskanleggene for å skille hyppige infeksjonsgenotyper fra ikke-infeksjons-genotyper - kan på bakgrunn av de fremkomne data ikke oppnåes innenfor rammen av dette forprosjektet.

Forslag til oppfølging - strategier og forutsetninger (1)

- All artsbestemmelse må gjøres vha. MALDI-TOF
- Ny artsbestemmelse av kjente sykdomsisolat
  - Veterinærinstitutet har 11 aktuelle isolat (ref. Renate J)
- Genotyping av kjente sykdomsisolat
  - PFGE-Spe1 egnet for *P. fluorescens*
  - for nye arter (for eks. *P. antarctica*) må andre restriksjonsenzym prøves ut'
- Basert på dette - Hva fins av potensielle sykdoms-fremkallende mikrober i materialet fra forstudien?
  - Pseudomonas
  - Andre relevante slekter/arter

Forslag til oppfølging - strategier og forutsetninger (2)

- Ved siden av *P. fluorescens* er det i materialet fra anleggene identifisert (ID32GN) og oppspart en rekke andre bakterier/arter som kan være relevante kandidater for oppfølging mhp. sykdom:

Pseudomonas:	*P aeruginosa, P putida, P alcaligenes,* *P stutzeri, (S maltophila* (fd Pseud)
Yersinia:	*Y kristensenii, Y intermediae, Y ruckeri,* *Y frederiksenii, Y enterocolitica*
Serratia:	*S fonticola, S marcescens, S liquefaciens*
Aeromonas:	*A hydrophila*
E Coli/coliform:	Enterobacter: *E cloacae*, *E gergoviae*; Morganella: *M morganii* Klebsiella: *K oxytoca*, *K pneumonia*; Proteus: *P.vulgari*; Hafnia: *H. alvei* Citrobacter*: C. freundii*,

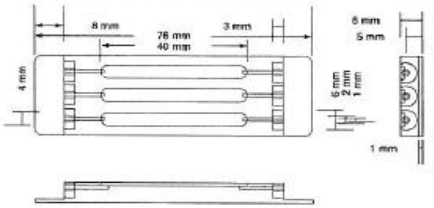
Forslag til oppfølging - strategier og forutsetninger (3)

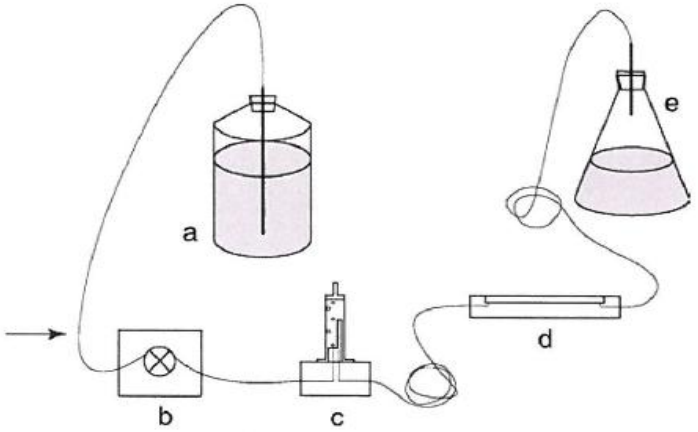
- Vi er fire miljøer i SINTEF som i en konsernsatsing 2008-2011 fått anledning å jobbe sammen om **Biofilm**
  - *"A Systems Biology approach towards the understanding of Microbial Processes in Biofilms"*
  - T&S, Health, Medical technology – Microbiology
    - Senior Scientist Catrine Ahlén (project leader)
  - M&C, Marine Environmental Technology
    - Senior Scientist Odd Gunnar Brakstad
  - M&C, Biotechnology
    - Scientists Inga Marie Aasen and Kolbjørn Zahlsen
  - Marine, Fisheries and Aquaculture, Marine resources Technology
    - Senior Scientist Jorunn Skjermo
- Sammen med Veterinærinstituttet blir vi dynamitt!

Eksempler på verktøy som p.t. er tilgjengelige i gruppen

Flow-celler + konfokal laser scanning mikroskopi CLSM
(T&S- MT- Med Mikro / HUS/UiB – inntil CLSM i Trh)

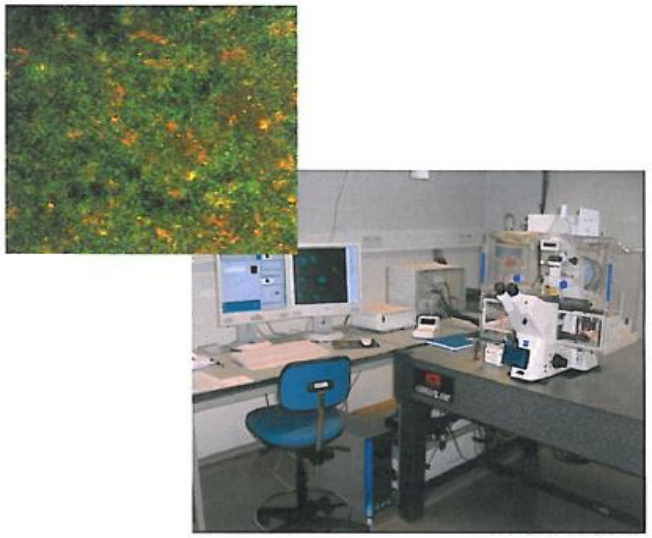
- Flowcelle





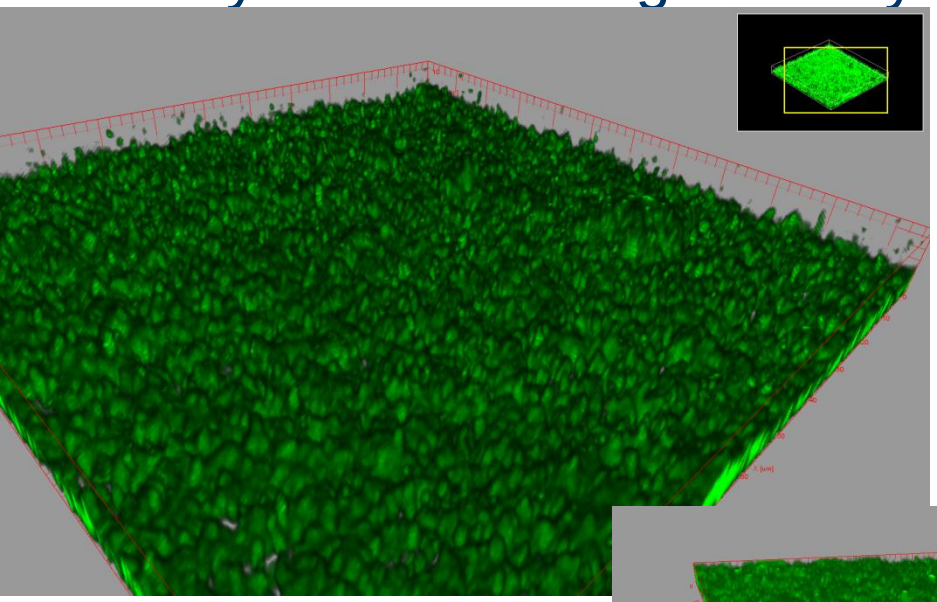
a, medium bottle; b, peristaltic pump; c, bubble trap; d, flow cell; e, waste container.

- CLSM

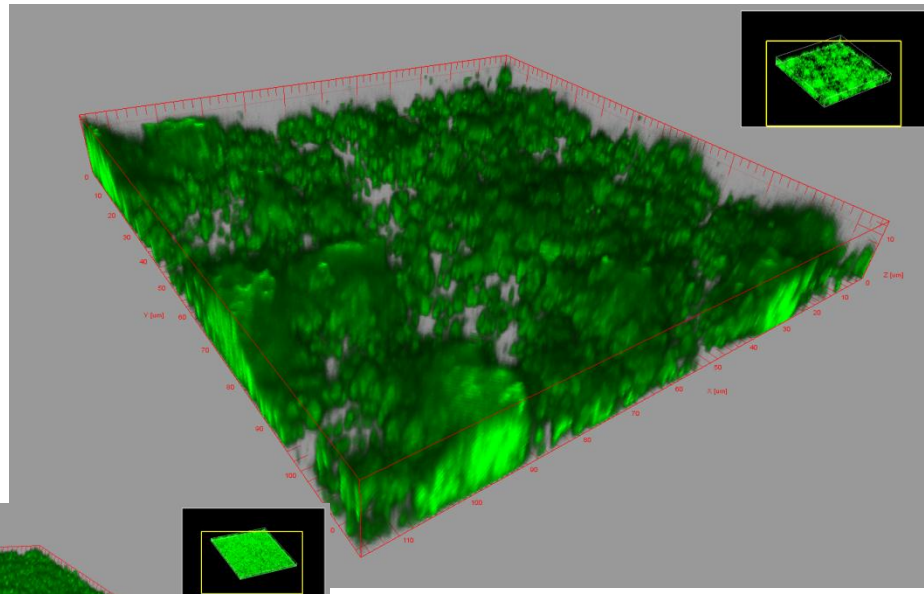


Biofilm formation is characteristic for the particular strains

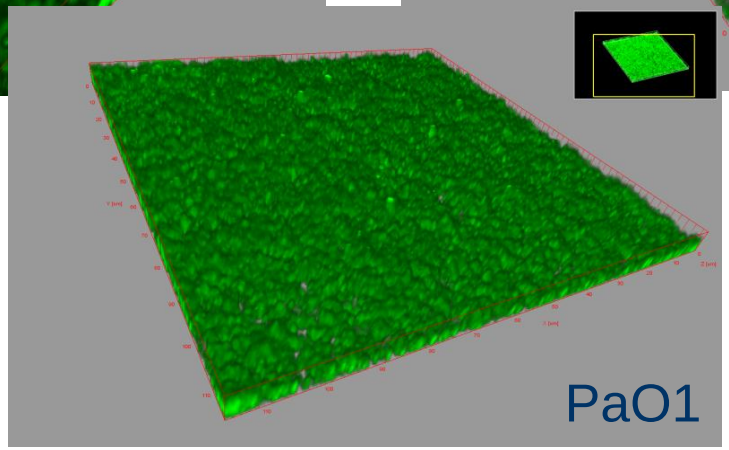
- Cytonine staining of 2 days flow cell culture



PaNS1



PaNS2



PaO1

Biofilms from flow-cells - A model study with *Pseudomonas aeruginosa*, Confocal Laser Scanning Microscopy (CLSM) and Metabolic fingerprinting

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[2] University of Bergen-HUS, N-5020 Bergen
[3] SINTEF Materials and Chemistry, N-7465 Trondheim, Norway
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Introduction

Intercellular communication is crucial for establishment, proliferation and disintegration of biofilms. In this study, we use *Pseudomonas aeruginosa* as model system. Three different strains are included in the study; PAO1 (reference strain) and two genetically identical isolates NS1 (1992) and NS2 (2007) representing one of the most persistent and widespread genotypes in North Sea deep divers' working environment.

The overall aim of our work is to prevent establishment of harmful biofilms and the identification of key compounds in the establishment of such biofilms will be a sub-goal.

The objective of the presented work is to establish and evaluate an MS-method for direct analysis of extracellular metabolites formed by biofilms, as a way to characterize the various stages of a biofilm.

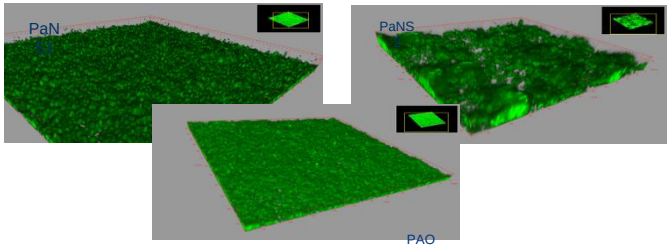


Figure 1: Biofilm formation in Pao1, PaNS1 and PaNS2 visualized by cytonine staining and CLSM after 2 days of flow cell culture. The pictures show the characteristic features of the three strains.

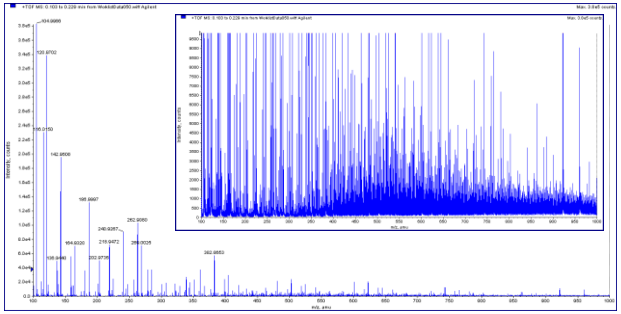


Figure 2: Typical mass spectrum (TOF-MS) from a biofilm sample. Insert panel at upper right has a reduced (zoomed-in) abundance scale to illustrate spectrum complexity; 4163 masses have abundance > 1000 in this particular sample.

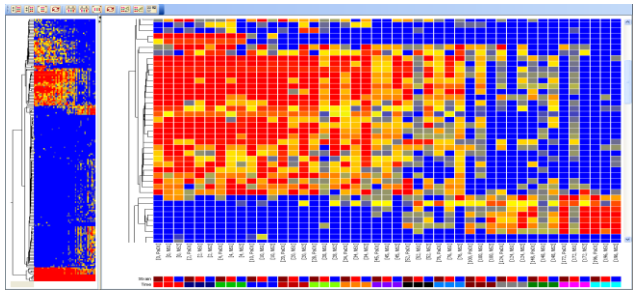


Figure 3: Hierarchical clustering (heatmap) of detected masses that show significant variation with time (selected by 2-way ANOVA, p-value < 0.05 on sets of four replicates). Right panel is an expanded subsection of left panel. Each row represents one mass; each column represents one condition (one set of replicates) annotated as **[Sampling time (hours), Strain]**. Blue = low abundance, red = high abundance. Masses that show similar abundance profile across the sample set are clustered together.

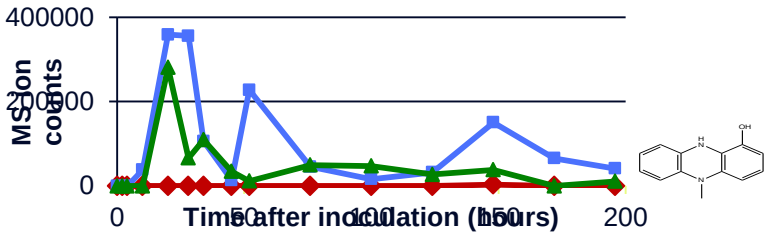


Figure 4: Abundance (ion counts) for compound with Mw = 210.08501 across the sample set. Each data point represents mean of four replicate samples. The compound was verified to be pyocyanin (right).

Summary

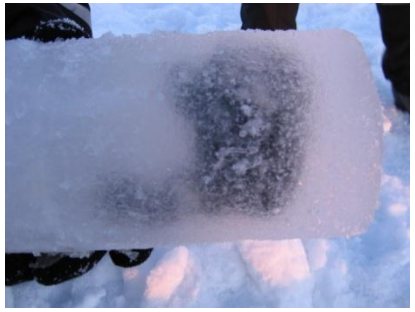
- Metabolic fingerprinting by high resolution (TOF) MS can be used to identify both complex and specific changes in extracellular biofilm metabolite patterns.
- The potential of the method is demonstrated through significant changes in metabolite patterns as shown in Figure 3 as well as identification of specific compounds as demonstrated in Figure 4.

In conclusion, the presented method seems to be a promising starting point for identification of new components of importance for biofilm establishment.

SINTEF Materials and Chemistry

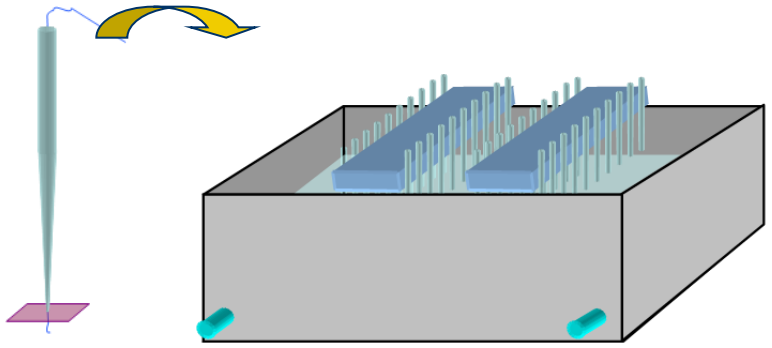
Marine Environmental Technology

- Studies of biofilm communities and processes on oil surfaces
  - Offshore oil reservoirs
  - Oil spills in cold environments (Seawater, seabed sediments, marine ice)
- Fouling studies
  - Detection and characterization of biofilms in marine installations
  - Prevention and treatment of biofilms



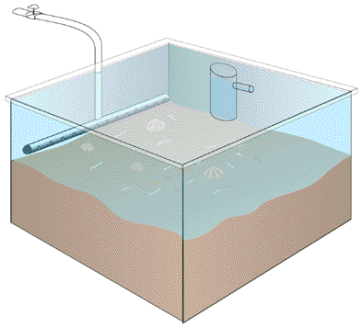
Experimental systems – laboratory
SINTEF Marine Environmental Technology

- Seawater flow-through surface systems for biofilm generation



Seawater flow-through system for generation of biofilms growing on immobilised oil films

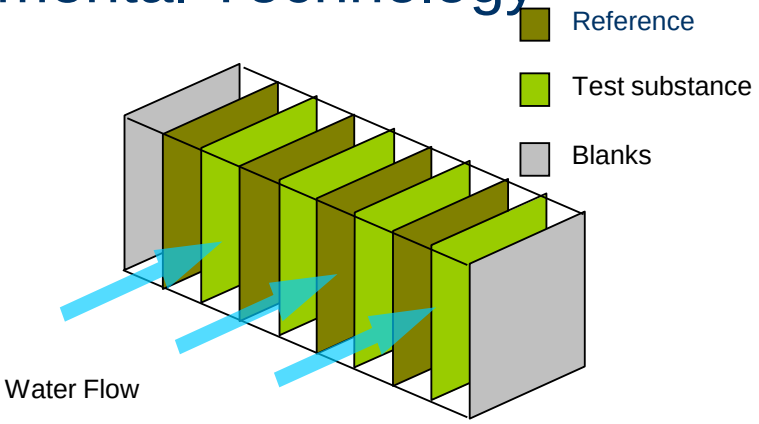
- Flow-through systems for studies of biofilms in marine porous sediment systems



Experimental systems – Field

SINTEF Marine Environmental Technology

- Field testing panels for and nets for studies and prevention of biofouling on steel steel surfaces



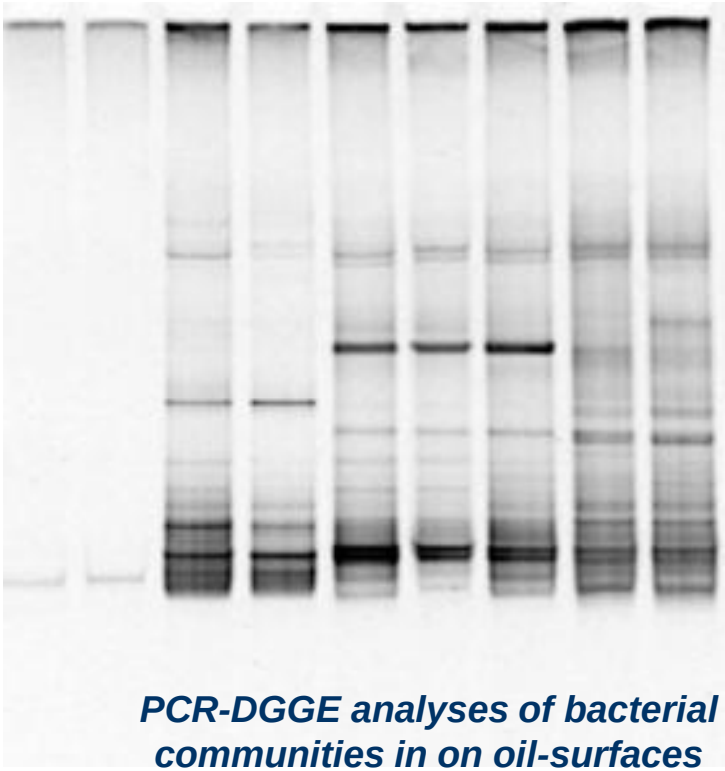
Steel panels for biofilm tudies

- Field testing of marine growth on fish farm nets

Field test systems for marine growth on fish farm nets (Photo T. Nordtug)

Analyses of biofilms
SINTEF Marine Environmental Technology

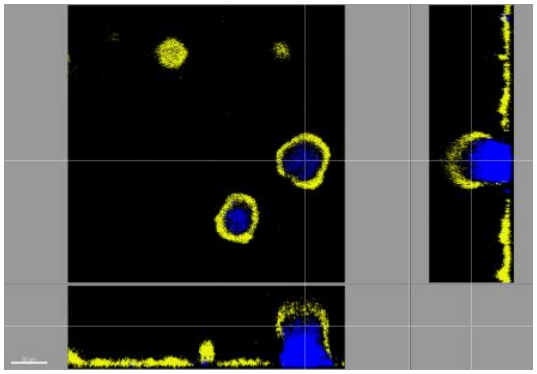
- **Analyses**
  - ***Fluorescence microscopy***
  - ***Changes in microbial communities (PCR-DGGE; cloning-sequencing) analyses)***
  - ***Expression systems in biofilms***
    - ***qPCR***
    - ***mRNA-based supression-subtractive hydbridization (SSH) libraries***
  - ***Qurum sensning analyses with luminescent bacterial cultures***
  - ***Chemical analyses of microbial degradation products***
    - ***GC-FID, GC-MS, LC-MS***



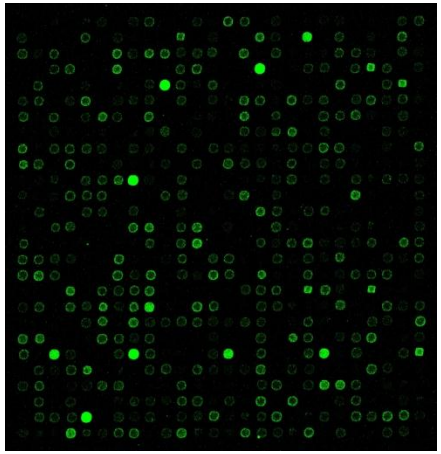
PCR-DGGE analyses of bacterial communities in on oil-surfaces (Photo: O.G. Brakstad, SINTEF)

Internal cooperation (SINTEF/NTNU)

- **SINTEF**
  - Chemical expertise
    - ***Synthetic anti-bacterial molecules***
    - ***Molecule binding technology to surfaces***
  - Material surface expertise
    - ***Polymer and surface coating technology***
    - ***Corrosion mechanisms and analyses***
  - ”Geminisenter - Medical Microbiology” (SINTEF/NTNU/St Olav’s)
    - Biofilm
    - Genomics
- **NTNU**
  - FUGE Node
    - Microarray Technologies
  - Microbial ecology
  - Microscope technology
  - Master and PhD students



Flow-cell grown Biofilm analysed by CLSM (Photo C Ahlén, SINTEF)



Microarray analyses of bacterial oil reservoir community (Photo Vidar Beisvåg, NTNU)

Exploring the Biofilm secrets requires a great specter of disciplines and expertice - there's no way to combate them but through cooperation ☺