ADHESION, PRESENCE AND ANTIFOULING
OF DEINOCOCCUS GEOTHERMALIS
IN PAPER MACHINE ENVIRONMENT

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Academic Dissertation in Microbiology

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Cover photo: An isosurface projection of confocal laser scanning microscopy image of *D. geothermalis* colony on the steel surface. The biofilm cells were stained with Phaseolus vulgaris lectin (green) and nucleic acid stain, Syto 60 (red). Grids scale 1 µm.
to my family
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List of original publications


The author’s contribution

Paper I:
Minna Peltola participated in the qPCR analyses, interpreted the results, wrote the paper and is the corresponding author.

Paper II:
Minna Peltola designed the lectin analysis and confocal laser scanning microscoping together with CLSM specialists. She carried out all experimental work except for the biofilm staining with microspheres and FESEM imaging. She did the image analysis, interpreted the results and wrote the paper.

Paper III:
Minna Peltola was responsible of the confocal laser scanning microscoping and participated in the writing of the paper.

Paper IV:
Minna Peltola designed the experiments and the instruments together with other authors, interpreted the results, wrote the paper and is the corresponding author. She carried out the experimental work excepting the polarization experiments with the double biofilm analyzer and a part of the statistical analyses.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscope</td>
</tr>
<tr>
<td>DBA</td>
<td>double biofilm analyzer</td>
</tr>
<tr>
<td>DC</td>
<td>direct current</td>
</tr>
<tr>
<td>eDNA</td>
<td>extracellular DNA</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substance</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FESEM</td>
<td>field emission scanning electron microscope</td>
</tr>
<tr>
<td>galNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>gluNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>ITO</td>
<td>indium tin oxide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIA</td>
<td>polysaccharide intercellular adhesin</td>
</tr>
<tr>
<td>PPB</td>
<td>potassium phosphate buffer</td>
</tr>
<tr>
<td>PVC</td>
<td>polyvinyl chloride</td>
</tr>
<tr>
<td>QPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RadBox</td>
<td>radical detection cuvette</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Tfp</td>
<td>type IV pili</td>
</tr>
<tr>
<td>TSA</td>
<td>tryptic soya agar</td>
</tr>
<tr>
<td>TSB/A</td>
<td>tryptic soy broth /agar</td>
</tr>
<tr>
<td>SPW</td>
<td>synthetic paper machine water</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
**Terms and definitions**

aerobic  
air containing atmosphere,  
an organism that favors air containing atmosphere for growth

anode  
positively charged electrode

antifouling  
preventing accumulation of the undesired biotic deposits

appendage  
an external cell surface projection

biofouling  
unwanted accumulation of biomass on surfaces

cathode  
negatively charged electrode

glycoconjugate  
carbohydrate linked to other chemical structure

lectin  
carbohydrate binding proteins

microaerobic  
an atmosphere with low content of air

microaerophilic  
an organism that favours low oxygen content

polarization  
the change of electric potential of solid material

runnability  
continuous operation without any breaks

wet end  
part of paper machine where the pulp slurry is transformed into wet paper sheets and dewatered to be paper

white water  
water from the wet end of the paper machine
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Fig 2. Confocal laser scanning images of biofilms on stainless steel coupons immersed in the water circuit of a paper machine.

Fig 3. Colour differences of D. geothermalis biomass cultivated under microaerobic and aerobic atmospheres.

Fig 4. Amplification DNA of D. geothermalis and nontarget species with primers DgeF627a and DgeR866

Fig 5. Maximum intensity projections of CLSM images from D. geothermalis E50051 biofilm on glass.

Fig 6. Confocal laser scanning (A) and Field Emission Scanning Electron Microscopy (FESEM) (B) images of D. geothermalis biofilm on glass.

Fig 7. Confocal laser scanning image of D. geothermalis (E50051) cells on steel surface, stained with Phaseolus vulgaris (green) lectin and the nucleic acid reactive stain, Syto 60 (red).

Fig 8. The Double biofilm analyzer instrument.

Fig 9. Biofilm of D. geothermalis grown on a stainless steel vial in the DBA instrument.

Fig 10. RadBox.
Abstract

This thesis has two items: biofouling and antifouling in paper industry. Biofouling means unwanted microbial accumulation on surfaces causing e.g. disturbances in industrial processes, contamination of medical devices or of water distribution networks. Antifouling focuses on preventing accumulation of the biofilms in undesired places.

*Deinococcus geothermalis* is a pink-pigmented, thermophilic bacterium, and extremely resistant towards radiation, UV-light and desiccation and known as a biofouler of paper machines forming firm and biocide resistant biofilms on the stainless steel surfaces.

The compact structure of biofilm microcolonies of *D. geothermalis* E50051 and the adhesion into abiotic surfaces were investigated by confocal laser scanning microscope combined with carbohydrate specific fluorescently labelled lectins. The extracellular polymeric substance in *D. geothermalis* microcolonies was found to be a composite of at least five different glycoconjugates contributing to adhesion, functioning as structural elements, putative storages for water, gliding motility and likely also to protection. The adhesion threads that *D. geothermalis* seems to use to adhere on an abiotic surface and to anchor itself to the neighbouring cells were shown to be protein. Four protein components of type IV pilin were identified. In addition, the lectin staining showed that the adhesion threads were covered with galactose containing glycoconjugates. The threads were not exposed on planktic cells indicating their primary role in adhesion and in biofilm formation.

I investigated by quantitative real-time PCR the presence of *D. geothermalis* in biofilms, deposits, process waters and paper end products from 24 paper and board mills. The primers designed for doing this were targeted to the 16S rRNA gene of *D. geothermalis*. We found *D. geothermalis* DNA from 9 machines, in total 16 samples of the 120 mill samples searched for. The total bacterial content varied in those samples between $10^7$ to $3 \times 10^{10}$ 16S rRNA gene copies g$^{-1}$. The proportion of *D. geothermalis* in those same samples was minor, 0.03 – 1.3 % of the total bacterial content. Nevertheless *D. geothermalis* may endanger paper quality as its DNA was shown in an end product.

As an antifouling method towards biofilms we studied the electrochemical polarization. Two novel instruments were designed for this work. The double biofilm analyzer was designed for search for a polarization program that would eradicate *D. geothermalis* biofilm or from
stainless steel under conditions simulating paper mill environment. The Radbox instrument was designed to study the generation of reactive oxygen species during the polarization that was effective in antifouling of *D. geothermalis*. We found that cathodic character and a pulsed mode of polarization were required to achieve detaching *D. geothermalis* biofilm from stainless steel. We also found that the efficiency of polarization was good on submerged, and poor on splash area biofilms. By adding oxidative biocides, bromochloro-5,5-dimethylhydantoin, 2,2-dibromo-2-cyanodiacetamide or peracetic acid gave additive value with polarization, being active on splash area biofilms. We showed that the cathodically weighted pulsed polarization that was active in removing *D. geothermalis* was also effective in generation of reactive oxygen species. It is possible that the antifouling effect relied on the generation of ROS on the polarized steel surfaces.

Antifouling method successful towards *D. geothermalis* that is a tenacious biofouler and possesses a high tolerance to oxidative stressors could be functional also towards other biofoulers and applicable in wet industrial processes elsewhere.
Tiivistelmä


planktisilla soluilla mikä viittaa siihan, että solut käynnistävät niiden tuoton tarttuessaan pintaan. Aiemmin *Deinococcus* suvun bakteereilta tyypin IV pilia ei ole löydetty.


Osoitin Radbox-laitteella, että polarisaation aikana muodostuu happiradikaaleja. Radikaalien todentamiseen kehitämämme menetelmässä hyödynnettiin happiradikaalien kanssa reagoivia fluoresoivia väriaineita, joiden fluoresoivaa signaalia mitattiin reaaliajassa. Happiradikaalit ovat reaktiivisia hapettimia ja sellaisina aiheuttavat solutuhoa. Radikaalien muodostuminen pinnalla on se tekijä, jonka oletamme aiheuttavan deinokokki biofilmien irtomiseen sähköisesti polarisoidulta pinnalta.

Sähkökemiallinen polarisaatio tarjoaakin vaihtoehdon ja/tai lisätehoa biosidien käytölle ja mekaanisella puhdistukselle eri teollisuusympäristöissä tapahtuvaan biofilmin poistoon.
1 Review of the literature

1.1 Biofilm

Biofilms are a common mode of growth for most microorganisms. Biofilm is defined as a community of sessile surface-associated micro-organisms embedded in a self-produced slime or other extracellular polymeric substance (EPS) (Reviewed by: Donlan, 2002; Costerton, 2007). Bacterial colonization starts by the attachment of single cells or cell aggregates onto a surface. The cells grow and develop to three-dimensional micro-colonies and further form complex communities with differentiated cells and interstitial water channels transporting nutrients and wastes (Fig 1.) (Lawrence et al., 1991; Stoodley et al., 2002). Due to the diversity of species and of the requirements of microorganisms for growth, biofilms vary between species (Lemon et al., 2008; for a review, see Branda et al., 2005) although biofilms rarely grow as monocultures in environments other than the tissues of man or animals. Mixed culture biofilms are found from many environments, ranging from slippery river rocks to extreme such as highly radioactive or salty places, surfaces of living organisms or industrial environments and implant devices of patients. Unwanted surface attached microorganisms cause serious disturbance of medical devices, in water distribution networks, and in water using industry (Väisänen et al., 1998; Reviewed by Flemming, 2002 ad by Hall-Stoodley et al., 2004). Multispecies biofilms are ubiquitous as components of many ecosystems. Those involved in the biochemical cycling of elements (Ehrlich & Newman, 2009) can be employed in waste water treatment (Kaksonen et al., 2003), bioremediation (Singh et al., 2006.), in mining (Review of Rawlings & Johnson, 2007). Others are useful in biotechnical applications for production of substances or chemicals (Rosche et al., 2009) or constructed into microbial fuel cells for bioenergy production as reviewed by Lovley (2008).

Biofilm mode of life is beneficial for microorganisms. Important features of biofilms are their high resistance against environmental stressors such as antimicrobials (Nickel et al., 1985; Byun et al., 2007). Compactness and the high cell density have been suggested to facilitate genetic exchange and cell-to-cell signalling (Costerton, 2007).

Bacteria can have competitive interactions in mixed biofilms. Living in tight community can limit space and nutrients. Rapid growers can deplete the nutrient reservoirs and outcompete slow-growers (Reviewed by Nadell et al., 2009). Some bacteria can prey on other bacteria as shown with Bdellovibrios that can attach to surface of other gram-negative bacteria and kill them (Núñez et al., 2005). Certain microbial species can dominate e.g. by producing
antimicrobial compounds. In oral biofilms *Streptococcus gordonii* and *S. sanguis* produce hydrogen peroxide to inhibit the growth of *S. mutans* (Ashby *et al.*, 2009).

**Figure 1.** The classical “mushroom” model of the structural heterogeneity of a mature biofilm. Biofilm consists of microcolonies built from cells and EPS and a network of water channels (Courtesy of Center for Biofilm Engineering, Montana State University, Bozeman).

### 1.2 EPS of the biofilm

EPS is believed to act as the glue and molecular sieve in biofilms holding the cells together and interacting with the environment by attaching the microcolonies onto the surfaces (Reviewed by: Branda *et al.*, 2005; Flemming & Wingender 2010). Other main functions of the EPS are listed in Table 1.

Highly hydrated and heterogeneous EPS varies in chemical and physical properties between organisms. The EPS matrix mainly consists of a mixture of polysaccharides but holds also proteins, glycoproteins, glycolipids and extracellular DNA (eDNA) (Flemming *et al.*, 2007). Therefore EPS can contain positively and negatively charged regions (Wolfaardt *et al.*, 1998) as well hydrophobic and hydrophilic regions.

Conventionally exopolysaccharides has been specified as cell surface or capsular polysaccharides or exopolysaccharides but the distinction in biofilms is not very clear (a review by Branda *et al.*, 2005). Production of exopolysaccharides is wide in biofilms for a review, see Sutherland, (2001). Polysaccharides can be homopolysaccharides such as cellulose (Zogaj *et al.*, 2001; Seto *et al.*, 2006), levan (Osman *et al.*, 1986; Laue *et al.*, 2006) and dextran (Leathers & Bischoff 2010) or heteropolysaccharides majority being anionic.
polysaccharide like alginate (Fett & Dunn 1989; Chang et al., 2007) and colanic acids (Rättö et al., 2006) or neutral and cationic such as β-1,6-linked N-acetylglucosamine of the polysaccharide intercellular adhesin (PIA) or related poly-N-acetylglucosamine (PNAG) of Staphylococcus epidermidis and S. aureus (Mack et al., 1996; and a review by Götz et al., 2002).

Extracellular glycolipid production is essential for the adherence of Acidithiobacillus ferreoxidans to pyrite surfaces during biocorrosion/bioleaching (Sand & Gehrke 2006). In Pseudomonas aeruginosa rhamnolipid was suggested to affect the structure of biofilm by maintaining the water-channels open during biofilm development (Davey et al., 2003).

EPS can contain enzymes produced by the biofilm bacteria. These enzymes may be targeted for degrading or modifying EPS (Tielen et al., 2010) for e.g. detachment as in Actinobacillus actinomycetemcomitans cells that produces DspB protein which hydrolyses the 1,4 glycosidic bond of N-acetylglucosamine and may result cells releasing from the biofilm (Kaplan et al., 2003). Enzymes may protect as shown for Pseudomonas aeruginosa that produces antibiotic-degrading β-lactamase in biofilm thus providing resistance towards β-lactam treatment (Bagge et al., 2004).

In the recent years extracellular DNA (eDNA) has been found in EPS of Pseudomonas aeruginosa, Staphylococcus strains and environmental isolate (F8). It has been suggested to have role as the structural stabilizer in biofilm matrix but also an important role in the initial adhesion (Whitchurch et al., 2002; Bockelmann et al., 2006; Das et al., 2010). The complex EPS matrix is stabilized by physicochemical interactions: hydrogen bonds, cation bridging, and van der Waals forces. Repulsive forces are important for the biofilm structure in preventing the polymer network from collapsing (Mayer et al., 1999).

Depending on the environment where biofilms develop the matrix can additionally contain e.g. metal ions, divalent cations, humic substances, and organic or inorganic materials from the environment (Frolund et al., 1995; Jiao et al., 2010). Paper mill slimes may contain non-microbial components such as inorganic or organic process raw materials (fibres), papermaking chemicals, pigments or e.g. alum precipitated as aluminum hydroxide (Eklund & Lindström, 1991; Mattila, 2002; Kanto Öqvist et al., 2008).
**Table 1.** EPS functions in biofilms. Modified from the review of (Flemming & Wingender, 2010).

<table>
<thead>
<tr>
<th>Putative function of EPS</th>
<th>Biomolecules responsible of EPS function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion to the surfaces and other microorganisms</td>
<td>Polysaccharides, proteins, DNA</td>
<td>(Allison &amp; Sutherland, 1987) (Das <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Protective barrier</td>
<td>Polysaccharides, proteins</td>
<td>(De Beer <em>et al.</em>, 1994) (Stewart <em>et al.</em>, 2000)</td>
</tr>
<tr>
<td>Water reservoir</td>
<td>Hydrophilic polysaccharides</td>
<td>(Christensen &amp; Characklis, 1990)</td>
</tr>
<tr>
<td>Sorptive of organic and inorganic compounds, ion exchange</td>
<td>Charged polysaccharides, inorganic substituent</td>
<td>(Wolfaardt <em>et al.</em>, 1998) (Freeman <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Nutrient source (carbon, nitrogen and phosphorus)</td>
<td>All EPS</td>
<td>(Flemming &amp; Wingender, 2010)</td>
</tr>
<tr>
<td>Exchange of genetic material</td>
<td>DNA</td>
<td>(Hausner &amp; Wuertz, 1999)</td>
</tr>
<tr>
<td>Enzymatic activity Binding of enzymes</td>
<td>Polysaccharides and enzymes</td>
<td>(Väisänen <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td>Electron donor or acceptor</td>
<td>Conductive nanowires and pilin for extracellular electron transfer</td>
<td>(Gorby <em>et al.</em>, 2006)</td>
</tr>
</tbody>
</table>
1.3 Factors influencing microbial attachment to abiotic surfaces

Factors and forces that are generally believed to play a role in microbial attachment include hydrophobicity, surface charge, hydrodynamics, cell surface appendages and surface material. These are discussed below.

As soon as any surface faces an aqueous environment it begins to interact with the inorganic and organic substances present in that liquid. The absorbed layer, called the conditioning film can alter the charge, hydrophobicity and the free energy of the substratum (Bakker et al., 2003; Bakker et al., 2004). Organic and inorganic substances as well as cells in the liquid flow approach the surface driven by brownian motion, diffusion, gravitation (sedimentation) or turbulent flow (Characklis, 1990). Motile cells can use their flagelli to approach the surface (O’Toole & Kolter, 1998; Lemon et al., 2007).

The interaction between the cells and the substratum is influenced by different forces. Simplifying, the initial adhesion of the cells is driven by the attractive weak forces Lifshitz-van der Waals forces, hydrophobic interactions and electrostatic forces, which may be repulsive or attractive reviewed by Carpentier et al. (Carpentier & Cerf, 1993). The overall interaction is the sum of these forces and the surfaces either attract or reject each other.

In close proximity to a surface the initially reversible adhesion of microorganisms may change towards the irreversible. The cellular surface structures such as flagelli, fimbriae or self-produced EPS may overcome the electrostatic repulsion and adhesion to the substratum may occur.

1.3.1 Physicochemical properties of the bacterial cell surface

Bacteria are generally negatively charged at environmental pH values due to the presence of functional groups: carboxylic, amine and phosphate residues and proteins on the cell wall, (Plette et al., 1995; Ojeda et al., 2008). Hydrophobicity of a cell surface depends on the exposed residues such as proteins, lipids, polysaccharides.

Physicochemical properties vary between the strains and even between the substrains of the same species as shown for *Listeria monocytogenes* strains (Chae et al., 2006). When 50 strains of *Lactococcus lactis* was studied under the same conditions of those strains the cell surface character was evaluated hydrophilic and electronegatively charged for 52 %, 12 % were hydrophobic and 18 % had low surface charge (Giaouris et al., 2009).
Electrostatic attractive force occurs when e.g. negatively charged bacteria interact with positively charged substratum and repulsive when both surfaces are negatively charged. Electrostatic forces can be affected by the dissolved cations and anions. Electrostatic attraction increases with high ionic concentration but also the adhesion of negatively charged cells to a negatively charged substratum increases suggesting that repulsive forces are attenuated by the ionic strength neutralizing the natural charge of the cells (Jucker et al., 1996; Sheng et al., 2008; Giaouris et al., 2009). Electrorepulsive interaction can be created by manipulating the charge of the substratum by cathodic current (Poortinga et al., 2001).

Van Loosdrecht (van Loosdrecht et al., 1987) proposed that hydrophobicity of the cell surface is the key factor in bacterial attachment to a nonliving surface. In an aqueous media, hydrophobic substances tend to interact with other hydrophobic substances. Several studies have shown that hydrophobicity correlates with the adhesion of different cells; spores and stationary phase vegetative cells of Bacillus cereus, waterborne wild-type of Mycobacterium smegmatis, strains of Listeria monocytogenes and Lactococcus lactis, adhering more effectively to abiotic surfaces than cells that were less hydrophobic or were hydrophilic (Husmark & Rönner, 1992; Peng et al., 2001; Giaouris et al., 2009; Takahashi et al., 2010; Mazumder et al., 2010). On the other hand there are studies showing that hydrophobicity did not correlate with the adhesion or biofilm formation, e.g. L. monocytogenes strains for which the production of EPS was suggested significant in the adhesion (Chae et al., 2006) or Escherichia coli strains which were hydrophilic and adhered effectively to a hydrophilic surface (Rivas et al., 2007).

The interactions between bacterial cells and the substratum are difficult to evaluate on the basis of hydrophobicity or surface charge because there are other properties involved such as surface appendages, EPS and roughness or topography of the substratum and hydrodynamics.

1.3.2 Cell surface appendages; flagelli, fimbriae and pili

Table 2 compiles studies of cell surface appendages involved in adhesion and biofilm formation on abiotic surfaces. Bacterial flagelli are long (15-20 µm) and thin (10-20 nm) appendages extruding from the cell surface located polarly, laterally or peritrichously. Flagelli are used by bacteria for swimming and swarming, multicellular moving along a surface (Jarrell & McBride, 2008). Motility is important for bacteria to approach the substratum and for the initial attachment as reviewed by Harshey (Harshey, 2003).
Proteinaceous, non-flagellar, multi-subunit appendages on the outer surface of the bacteria are called pili (Latin, hairs, hair-like structures) and fimbriae (Latin, threads). They are employed in attachment, virulence, invasion, biofilm formation, twitching and gliding motility and DNA uptake (Fronzes et al., 2008). The review of Fronzes (2008) divided the pili of gram-negative bacteria into five groups based on their assembly pathways; chaperone-usher (CU) pili, Type IV pili (Tfp), curli pili and secretion pili type II and IV. Tfp are found widespread among β- (Neisseria gonorrhoeae), γ- (Pseudomonas aeruginosa) and δ- (Myxococcus xanthus) proteobacteria and the cyanobacteria (Synechocystis sp.) (Nudleman & Kaiser, 2004).

Interesting is the Tfp of Geobacter sulfurreducens (DL-1) that transfers electrons to insoluble electron acceptors such as Fe(III) oxides, but also have a non-conductive role in attachment to electron-accepting surface and in biofilm formation when the surface is not an electron acceptor (Reguera et al., 2007). Similar conductive “nanowire” has been found in Shewanella oneidensis (Gorby et al., 2006) but the biofilm formation is linked to Tfp (Thormann et al., 2004).

Protein structures can affect the charge and the hydrophobicity of the bacterial cell surface and thus have an influence on the adhesion. Type I pilus of E. coli were shown to increase the hydrophobicity of the cell surface but it did not correlate with the initial adhesion compared under static conditions to non-fimbriated strains whether the substratum was hydrophobic or hydrophilic. However fimbriated E. coli strains were found to strengthen the adhesion to the hydrophobic surface (Otto et al., 1999). In static conditions the flagellar motility of L. monocytogenes was not important in the initial adherence to a surface of hydrophobic polyvinyl chloride (PVC), suggesting that the influence of motility depends on the substratum material, whether it has physicochemical properties similar to the cell surface (Takahashi et al., 2010). Contradictory results were shown with B. cereus, where motility was important for the initial adhesion to glass in static conditions but not in flow, suggesting that the flagelli hindered the interaction between the cell surface and the substratum (Houry et al., 2010). In glucose minimal medium standing cultures of P. aeruginosa type IV pili mutant adhered to abiotic surfaces but microcolony formation failed whereas under flow conditions microcolony formation occurred (Pratt & Kolter, 1998). As a conclusion, bacterial adhesion to the substratum is a complex collection of interactions where everything seems to affect everything.
Table 2. Examples of cell surface appendages involved in the adhesion and biofilm formation on abiotic surfaces.

<table>
<thead>
<tr>
<th>Surface appendages involved in adhesion</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flagella</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>(O'Toole &amp; Kolter, 1998)</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>(Pratt &amp; Kolter, 1998)</td>
</tr>
<tr>
<td></td>
<td><em>Vibrio cholerae</em></td>
<td>(Lemon et al., 2007)</td>
</tr>
<tr>
<td></td>
<td><em>Listeria monocytogenes</em></td>
<td>(Vatanyoopoulos et al., 2000)</td>
</tr>
<tr>
<td></td>
<td><em>Aeromonas</em></td>
<td>(Gavin et al., 2002)</td>
</tr>
<tr>
<td>Type 3 pilus</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>(Di Martino et al., 2003)</td>
</tr>
<tr>
<td>Type I pilus</td>
<td><em>Escherichia coli</em></td>
<td>(Pratt &amp; Kolter, 1998)</td>
</tr>
<tr>
<td>conjugative pili</td>
<td><em>Escherichia coli</em></td>
<td>(Ghigo, 2001)</td>
</tr>
<tr>
<td>Type IV pilus</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>(O'Toole &amp; Kolter, 1998)</td>
</tr>
<tr>
<td></td>
<td><em>Vibrio parahaemolyticus</em></td>
<td>(Shime-Hattori et al., 2006)</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium perfringens</em></td>
<td>(Varga et al., 2008)</td>
</tr>
<tr>
<td></td>
<td><em>Shewanella oneidensis</em></td>
<td>(Thormann et al., 2004)</td>
</tr>
<tr>
<td></td>
<td><em>Acidovorax citrulli</em></td>
<td>(Bahar et al., 2010)</td>
</tr>
<tr>
<td></td>
<td><em>Geobacter sulfurreducens</em></td>
<td>(Reguera et al., 2007)</td>
</tr>
<tr>
<td>Curli fimbriae</td>
<td><em>Escherichia coli</em></td>
<td>(Cookson et al., 2002)</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella enteritidis</em></td>
<td>(Austin et al., 1998)</td>
</tr>
<tr>
<td>Pilus-like filaments</td>
<td><em>Geobacter sulfurreducens</em></td>
<td>(Klimes et al., 2010)</td>
</tr>
</tbody>
</table>

1.4 Microbial growth in paper industry

Microorganisms enter the papermaking process via air, raw materials or chemicals (water, starches, kaolins, carbonates, pulps). The recycled fibers used in paper making may contain $10^8 - 10^{10}$ cfu of aerobic microorganisms per gram d.w (Suihko & Skyttä, 1997).

Microbial growth causes four types of problems in paper industry shown in Table 3. Biofilm formation on undesired surfaces (Fig. 2) may impair the papermaking process in wet end, slimy clumps and pigments endanger the product quality, microbial metabolites spoil raw materials and chemicals and smelling compounds cause odor problems both in end products.
and in the environment. The density of culturable, aerobic heterotrophic bacteria in the wet end circuits have been shown to range from $10^5$ cfu per ml (white water) to $10^8$ cfu per ml (raw material slurries) but is lower in the paper products, $5 \times 10^4$ cfu per g (Väisänen et al., 1991; Väisänen et al., 1998).

In the studies of Granhall (2010) and Lahtinen (2006) cultivation-independent methods was used to analyze microbial composition of biofilms and process water in paper machines. They both found that bacterial profiles in biofilms and process waters differed from each others in different paper machines. They also revealed bacterial 16S rRNA sequences not found before from slimes. According to the authors not all bacteria are responsible for biofilm formation. Those bacteria capable of initiating biofouling have been recognized from steel surfaces of the Nordic paper machine environments; *Deinococcus geothermalis, Meiothermus silvanus, Burkholderia* spp., *Rubellimicrobium thermophilum, Rhodobacter, Tepidimonas* and *Cloacibacterium* (Kolari et al., 2002; Kolari et al., 2003; Denner et al., 2006; Tiirola et al., 2009). The primary-biofilm formers have a key role in assisting secondary biofoulers and when planning antifouling strategy.

Packaging of foods has increased in recent years. This is a concern also for hygiene quality of the end products of paper mill. Gram-positive endospore forming genera e.g. *Bacillus, Brevibacillus* and *Paenibacillus* can survive the high temperature of paper machine circuits, desiccation and as spores the biocidal treatments. These bacteria are found in paper machine slimes and in end products such as food packaging boards (Väisänen et al., 1991; Pirttijärvi et al., 1996; Suihko et al., 2004). Paper products have been shown to contain up to $10^5$ culturable bacilli (*Paenibacillus* and *Bacillus*) spores per g (Väisänen et al., 1991; Pirttijärvi et al., 1996). Ekman et al. (2009) reported that of the $3 \times 10^4$ or $8 \times 10^4$ *Bacillus* spores 0.001 to 0.03 % transferred from the packing paper to dry food (rice and chocolate).

At present the need for protecting the environment directs the paper mills towards reducing the fresh water intake. In paper machines where freshwater intake was very low or the water circuit totally closed high density $\geq 10^8$ / ml of Archaean and low diversity of bacteria were found in slimes and deposits (Kanto Öqvist et al., 2008). Slimes and deposits were not the main problem in these mills, but there were odour problems in the products and in the surroundings of the mills. The circulating waters of the closed paper mills contained volatile fatty acids (lactic, acetic, propionic, butyric) as a consequence of anaerobic bacterial activity (Kanto Öqvist et al., 2008).
Figure 2. Confocal laser scanning images of biofilms on stainless steel coupons immersed in the water circuit of a paper machine for A) 3 d, (thickness of the biofilm 21 µm) and B) 10 d (thickness 37 µm). Biofilm microorganisms (green) were visualized by staining with the DNA dye Sybr Green. Large particles seen as brownish colour (A) or yellowish (B) are wood fibres emitting autofluorescence. Structures visible on the stainless steel surface (grey) result from the reflecting light (panel A). Scale bars 50 µm.
Table 3. Examples of bacteria causing problems in paper machines

<table>
<thead>
<tr>
<th>Problem</th>
<th>Microorganisms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spoilage of starch or chemical additives</td>
<td>Fungi (<em>Aspergillus</em>), <em>Bacillus licheniformis</em>, <em>Sphingomonas</em> sp., <em>Burkholderia</em>, <em>Pseudomonas stutzeri</em>, <em>Ralstonia</em>, <em>Enterobacteria</em>, <em>Brevibacillus</em> spp.</td>
<td>(Väisänen <em>et al.</em>, 1998)</td>
</tr>
</tbody>
</table>
1.5 *Deinococcus geothermalis* in paper machine environment

Paper machine wet end is a man-made ecosystem offering all the requirements needed for microbial growth. Raw materials, degradable starch and cellulose are abundantly available and the chemicals used in papermaking may serve as nutrients as well. Based on analyses of Väisänen *et al.*, (1994) the white water carbon content of five board and paper machine is high, from 30 - 80 mg l$^{-1}$ up to 600 - 800 mg l$^{-1}$, but the content of nitrogen is low, the C:N ratio ranging from 40:1 to 90:1. The content of phosphorus ranged from 0.13 to 0.5 mg l$^{-1}$. Paper machines operate at temperatures from 35 °C to 55 °C at the wet end and with pH 4 to 9. These are optimal for microbial growth (Väisänen *et al.*, 1994; Kolari *et al.*, 2003; Lahtinen *et al.*, 2006; Kanto-Öqvist *et al.*, 2008).

*D. geothermalis* is a tenacious species originally found in geothermal wells (Ferreira *et al.*, 1997), later also from soil of the hot springs area (Kongpol *et al.*, 2008), from deep ocean subsurface (Kimura *et al.*, 2003) and from paper machines (Väisänen *et al.*, 1998; Kolari *et al.*, 2003; Kolari 2003). *D. geothermalis* was identified as a pigmented biofouler of the wet end of neutral and acidic paper machines. It was found by cultivating as well as *in situ*-hybridization mainly from slimes and splash areas of wire sections but also from the circulation water, machine felts, pulp sheets, press cylinder and headbox from different machines (Väisänen *et al.*, 1998; Kolari *et al.*, 2003; Kolari 2003). Pink coloured biofilms of *D. geothermalis* may cause discolouring of paper products as was shown for *Meiothermus* spp. (Ekman *et al.*, 2007). It may also play a role as a primary biofilm-former and assisting other, secondary biofilm formers such as *Bacillus* strains to adhere and form biofilm (Kolari *et al.*, 2001).

*D. geothermalis* belongs to the family of *Deinococcaceae* in the Phylum *Deinococcus-Thermus*. This Phylum is deeply branched in the bacterial phylogenetic tree (Gupta, 1998). The family *Deinococcaceae* comprises over forty validly described species of which many are extremely radiation-resistant, surviving exposure to ionizing radiation (10 kGy), ultraviolet light and desiccation (Mattimore & Battista, 1996). Irradiation resistance of *D. radiodurans* and *D. geothermalis* and also the desiccation resistance of dry-climate soil bacteria were proposed to result from high intracellular content of manganese ions and low concentration of iron ions inside the cells (Daly *et al.*, 2004; Fredrickson *et al.*, 2008). Protein oxidation during irradiation or desiccation is prevented by Mn(II) ions. Thus DNA damage
can be fixed by repair enzymes (Daly et al., 2007) that are multiply present in the proteome of *D. geothermalis* (Liedert et al., 2010). Extensive proteomic analysis of *D. geothermalis* strain E50051 cell envelope and cytosol (Liedert et al., 2010) as well as a limited membrane proteome of *D. geothermalis* strain 11300 (Tian et al., 2010) disclosed many of the most abundant proteins related to stress response (Table 4) e.g. catalase, superoxide dismutase, thioredoxin dismutase. Furthermore, analysis of the proteome and the annotation of the genome revealed 34 unknown proteins and genes that were unique to *Deinococcus* (Makarova et al., 2007; Liedert et al., 2010). Liedert et al. (2010) suggested that these unknown proteins had putative functions related to DNA repair, to stress and halotolerance and to oxidant reduction supporting the genome annotation (Makarova et al., 2007).

**Table 4.** Proteins related to tolerance to environmental stress of *Deinococcus geothermalis*. Collected from Liedert et al., (2010) and Tian et al., (2010).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SodA)</td>
<td>catalyzes disproportionation of superoxide anion to molecular oxygen and hydrogen peroxide</td>
</tr>
<tr>
<td>Catalase (KatA)</td>
<td>catalyzes conversion of hydrogen peroxide to water and gaseous oxygen</td>
</tr>
<tr>
<td>Proteins of Suf FeS assembly</td>
<td>Suf enzymes repair proteins damage under oxidative stress</td>
</tr>
<tr>
<td>Thioredoxin, thioredoxin reductase</td>
<td>involved in the reduction of disulfides and of methionine sulfoxides exposed to oxidative stress</td>
</tr>
<tr>
<td>Heat-shock proteins DnaJ, Hsp70</td>
<td>protect against deleterious effects under stress</td>
</tr>
<tr>
<td>Chaperone proteins</td>
<td>assist folding/unfolding of proteins</td>
</tr>
<tr>
<td>S-ribosylhomocysteinase (LuxS)</td>
<td>involved in bacterial communication, biofilm formation</td>
</tr>
<tr>
<td>S-layer proteins</td>
<td>cell envelope proteins, environmental protection</td>
</tr>
<tr>
<td>Chlorite dismutase</td>
<td>enzyme converting chlorite to chloride</td>
</tr>
<tr>
<td>ABC transporter</td>
<td>multidrug transport protein, toxic chemical cleaning</td>
</tr>
</tbody>
</table>

The success of *D. geothermalis* as a biofouler in paper machine environment is supported by its multiple tools to battle against oxidative stressors, ability to form biofilm, adhesion threads responsible for the firm attachment to nonliving surfaces and crosslinking to
neighbouring cells (Kolari et al., 2002; Raulio et al., 2008). *D. geothermalis* did not detach when washed 1 h with alkaline and acidic liquids, such as 0.2% NaOH 0.5 % SDS (sodium dodecyl sulphate) and even 1M HCl (Kolari et al., 2002; Kolari, 2003). Alkaline aqueous solutions are used as washing agents to remove microbial deposits in paper industry (Alén, 2007). Genome annotation of *D. geothermalis* has revealed an additional set of genes involved in xylose utilization (xylanase, xylose, isomerase xylose kinase) (Makarova et al., 2001). Xylose is a component of plant hemicellulose and is released into the waters during pulping (Bjarnestad & Dahlman 2002).

### 1.6 Strategies of antifouling

Antifouling aims at controlling biofouling, harmful microbial growth on surfaces. The strategies used include prevention of biofilm growth or attachment and/or promoting detachment or mechanical removal of the biofilms. In wet industry e.g. in paper machines, the methods used for controlling biofouling are mainly biocides (microbicides or slimicides) and mechanical cleaning.

Environmental restrictions in uses of biocides and the resistance of biofilm bacteria towards the biocides have motivated to search for methods complementary to or replacing biocides. Biocides may impede biofilms employed for biological waste water treatment respectively. Large industrial complexes such as paper mills have plenty of surfaces and high volume of in the wet-end, limiting the application of chemical antifouling treatment. Biodispersants have been used to improve the efficacy of biocides, to reduce the accumulation of microbial deposits and to improve penetration of biocides through the EPS (Blanco et al., 1996; Alén, 2007). Enzymes have been used to hydrolyze EPS (Eklund & Lindström, 1991; Chaudhary et al., 1997; Rättö et al., 2005). Novel techniques such as electrical (Matsunaga et al., 1998; Perez-Roa et al., 2009) or ultrasound (Lambert et al., 2010) modulation have been applied to disturb biofilms or to prevent biofilm formation. Alternatively, designing new surface materials or coatings with antifouling properties have been in focus for wet industrial processes. Surface properties such as hydrophobicity, surface topography or antimicrobial coatings have been studied for industrial application (Raulio et al., 2006; Murata et al., 2007; Raulio et al., 2008). The photocatalytic TiO₂ coatings were shown to destroy *D. geothermalis* biofilms on the coated steel surface when exposed to 360 nm light, for 20 h (Raulio et al.,...
Photocatalytic effect is based on reactive oxygen species generated during illumination of TiO$_2$ with UV light (Cho et al., 2004).

### 1.6.1 Biocides used in paper mills

The active substances of biocides vary having different antimicrobial activity (Maillard, 2002). Oxidizing as well as non-oxidizing biocides are presently in use at paper machines to control microbial deposits (Paulus, 1993; Simons & da Silva Santos, 2005). The oxidizing biocides used in pulp and paper industry include halogenated compounds such as brominated alkylhydantoin, BCDMH (1-bromo-3-chloro-5, 5-dimethylhydantoin), ammonium-bromide (NH$_4$Br), as well as non-halogenated oxidizing biocides, peracetic acid and hydrogen peroxide (Simons & da Silva Santos, 2005). Oxidizing biocides inactivate enzymes containing sulfhydryl groups or disulfic bridges and damage non-specifically the organic matrix and weaken the biofilm and also are effective against bacterial spores (Paulus, 1993). Non-oxidizing biocides have several different modes of action. Thiazols e.g. BIT (1, 3-benzisothiazolin-3-on) react with cell nucleophiles (Paulus, 1993), nitriles can form complexes with Fe$^{2+}$ disrupting the function of cytochromes preventing the transport of electrons, 2, 2-dibromo-3-nitrilopropionamide (DBNPA) is electrophilic with activated halogen group (Paulus, 1993; Rossmoore, 2001). Glutaraldehyde interacts with amino and thiol groups of proteins or lipoproteins. It destroys a broad spectrum of microorganisms including bacterial and fungal spores, mycobacteria and viruses (Scott & Gorman, 2001; review of Maillard, 2002). Cationic surfactants, e.g. quaternary ammonium compounds (QACs) neutralize the negatively charged cell surface causing distortion of the cell membrane and lysis of the cell (Simoes et al., 2005). Carbamates such as dimethyldithiocarbamate chelates metal ions essential to the microbial metabolism.

Unsuccessful biocide usage may increase biofilm formation by the resistant biofoulers in a paper machine (Kolari et al., 2003). The type of machine, chemical composition of the recycled waters and input of additives, pH, temperature, organic matter content and the microbiota varies and contribute to the success of the biocide program selected. Due to resistance and the limited number of usable biocides the trend is to combine biocides for enhanced effect (Sriyutha Murthy & Venkatesan, 2009). Biocides are in EU regulated by the Biocidal Product Directive (BPD) 98/8/EC.
1.6.2 Electrochemical antifouling of biofilms

High electric field is lethal on microorganisms. Electric current was reported to kill *Pseudomonas aeruginosa* infecting ulcers when direct current of 200 to 1000 µA was used (Rowley et al., 1974). Application of the cathodic current was found to suppress the infection and the anodic current stimulated the healing. A few years later Gordon (Gordon et al., 1981) demonstrated that cathodic polarization of platinum and copper electrodes enhanced attachment of marine bacteria and anodic polarization reduced attachment.

In the recent years electrochemical polarization has been exploited to detach biofilm, to inhibit biofilm growth and to prevent adhesion of microorganisms on pipelines and heat exchangers of cooling systems and ship hulls in seawater environment as well as on medical instruments. Table 5 introduces these electrochemical antifouling studies.

Biofilms growing on the medical instruments, implants or percutaneous pins may cause serious infections and complications that can lead to replacement of the implant. At best 95% of the implant-associated *Staphylococcus aureus* and *S. epidermidis* strains (1 × 10⁷ bacteria per cm²) could have been detached from the stainless steel surface by direct cathodic electric current in 2.5 hour (van der Borden et al., 2004). However, in the further studies van der Borden et al., (2005) realized that electric block current do not cause tissue damage to the patients and detached 76% of the adhered *S. epidermidis* cells in 1.5 h, whereas direct current detached 64%. In addition, the number of viable bacteria persisting on the surface decreased to one-tenth with block current. Costerton et al., (1994) and Blenkinsopp et al., (1992) have shown in their studies that electric current can enhance the effect of antibiotics and biocides. By combining antibiotic (tobramycin) with electric current left only <100 viable *P. aeruginosa* cells/cm² of the sessile cells in 48 h whereas antibiotic (5 mg L⁻¹) alone remained 5 × 10⁵ per cm² bacteria viable (Costerton et al., 1994). Industrial biocides (isothiazolone, glutaraldehyde and quaternary) significantly decreased viability of *P. aeruginosa* biofilms even though the biocide concentrations were lower than those killing planktic cells indicating synergistic effect of biocide and electric pulsed current (Blenkisop et al., 1992).

With high current density (0 to 167 mA/cm², 1 to 4 V) electrochemical oxidation of water generates reactive oxidative species (ROS) hydroxyl radical (·OH), ozone (O₃), superoxide
anion ($\text{O}_2^-$) and hydrogen peroxide. Each of these has been shown to disinfect planktic cells (Kerwick et al., 2005; Jeong et al., 2006; Jeong et al., 2009). This has been seen as a promising alternative for disinfecting drinking water as well as industrial process waters. Reactive oxygen species are powerful oxidants and therefore short lived (nanoseconds) in organic matter containing environments. Small amounts of ROS are naturally generated during aerobic metabolism.

For long term use electrochemical antifouling has been shown effective in environments where it is more important to maintain surface clean than to kill fouling organisms. In marine environment cathodic current with alternating potentials accumulated less than 100 g wet wt per m$^2$ fouling organisms whereas on the corresponding control surface wet wt was 10 kg per m$^2$ in 2 years (Wake et al., 2006).

Why electrochemical antifouling is effective against bacteria?
Costerton et al., (1992) and Blenkinsopp et al., (1994) hypothesized that low electric current does not kill biofilm bacteria but offers an electrophoretic force for antimicrobial agents to overcome the diffusion barrier. Other main hypotheses that come up from research the paper cited in Table 5 were:

- Electrorepulsive,-static and -phoretic forces. External electric field induces motion of charged objects. Motility depends on strength and charge of the electric field. Electrorepulsive and/or -static force result when two object of like charge repels each other.
- Repetitive current changes disrupt the cell membrane. Exposure to fast changes of current (anodic, cathodic) may cause motility of charged cell membrane
- Production of toxic compounds e.g. ROS
### Table 5. Studies of electrochemical antifouling.

<table>
<thead>
<tr>
<th>Electrochemical program</th>
<th>Environment</th>
<th>Target biofilm</th>
<th>Efficacy</th>
<th>Result of the electric effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathodic direct current (DC) 15-125 µA (1.5 – 1.7 V), electrode (21 cm²) stainless steel (AISI 316)</td>
<td>Flow chamber, 0.5 - 150 mM potassium phosphate buffer (PPB)</td>
<td><em>Staphylococcus epidermidis</em>, <em>Staphylococcus aureus</em></td>
<td>Highest detachment 95 % after 2.5 h with 100 µA in 1 mM PPB</td>
<td>Electrorepulsive forces</td>
<td>(van der Borden <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>Cathodic block current 15, 60 and 100 µA, (1.5 – 1.7 V) changing frequency (0.1 – 2 Hz), electrode (21 cm²) stainless steel (AISI 316)</td>
<td>Flow chamber 0.5 - 150 mM PPB</td>
<td><em>Staphylococcus epidermidis</em> on stainless steel (AISI 316)</td>
<td>Detachment 76 % with 100 µA (0.1 Hz) after 150 min. Viable bacteria decreased 10 fold.</td>
<td>Electro-osmotic fluid flow, block current may have disrupted the cell membrane</td>
<td>(van der Borden <em>et al.</em>, 2005)</td>
</tr>
<tr>
<td>Current 50 – 100 mA /m², alternating potentials, titanium electrode</td>
<td>Field experiment in seawater cooling pipelines</td>
<td>Seawater fouling organisms</td>
<td>Average fouling of the material decreased 100 fold compared to non protected surface</td>
<td>Most probably the generation of ROS</td>
<td>(Wake <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Currents ranging -800 (-0.9 V) to + 800 µA (0.9 V), glass electrode (21 cm²) coated with indium tin oxide (ITO)</td>
<td>Flow chamber, human whole saliva</td>
<td><em>Streptococcus oralis</em> (J22), <em>Actinomyces naeslundii</em> T14V-J1, <em>Streptococcus oralis J22</em> detachment when currents increased whether positive or negative current, no</td>
<td></td>
<td>Electrostatic and electrophoretic forces</td>
<td>(Poortinga <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>Currents; cathodic, anodic, block (cathodic and anodic in turns) of 15 µA/cm², ITO coated glass electrode (6.5 cm²)</td>
<td>Flow cell, 20 mM potassium phosphate (pH 7.1)</td>
<td>Actinomyces naeslundii 147</td>
<td>effect on Actinomyces naeslundii</td>
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<tr>
<td></td>
<td></td>
<td>Flow cell, 20 mM potassium phosphate (pH 7.1)</td>
<td>Detachment 80% with cathodic and block current after 40 min, anodic and block current inactivated the remaining bacteria on the surface</td>
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<tr>
<td></td>
<td></td>
<td>Pseudomonas aeruginosa (PAO1)</td>
<td>Electrostatic and electrophoretic forces of cathodic current. Inactivation may have been caused by the repetitive electric current changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC, potential 10 V, &lt; 100 mA/cm², polarity changed every 64 s, stainless steel (AISI 316), polarization treatment was combined with tobramycin (5 mg/L)</td>
<td>Flow cell, salts medium</td>
<td>Pseudomonas aeruginosa (UR-21)</td>
<td>Polarization treatment combined with tobramycin resulted almost complete kill, &lt;100 viable cells/cm²</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polarization treatment combined with tobramycin resulted almost complete kill, &lt;100 viable cells/cm²</td>
<td>Electrophoretic force allowed antimicrobial agents to overcome the diffusion barrier</td>
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<td></td>
<td></td>
<td>Pseudomonas fluorescens (ATCC 17552)</td>
<td>Electrorepulsive forces</td>
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<tr>
<td></td>
<td></td>
<td>Flow cell, 0.01 M and 0.1 M NaCl</td>
<td>negative potentials inhibited adhesion</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Pseudomonas fluorescens (ATCC 17552)</td>
<td>electrorepulsive forces</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>-0.5 and -0.2 V, electrode glass covered with thin film of gold</td>
<td>(Busalmen &amp; de Sanchez, 2001)</td>
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</tr>
</tbody>
</table>
2 Aims of the study

This study focused on biofouling by and antifouling of *Deinococcus geothermalis* in paper machine environment. This work represents an extension of earlier studies that had shown the presence of *D. geothermalis* in paper machine biofilms and the firm adhesion of this species to abiotic surfaces. I expanded these studies by characterizing the adhesion tools mapping the EPS architecture and the true prevalence of *D. geothermalis* in biofilms on paper machines. To eradicate *D. geothermalis* we developed a method based on electrochemical antifouling.

Specific aims were to:

1. To get an overview of the prevalence of *Deinococcus geothermalis* we measured DNA of this species in biofilms collected from different locations in paper and board machines in many different mills.

2. To describe *in situ* the architecture of *D. geothermalis* biofilm on abiotic surfaces.

3. To characterize the molecular tools that *D. geothermalis* uses to anchor on surfaces.

4. To develop an antifouling method based on electrochemical polarization effective on deinococcal biofilms as the target.

5. To reveal the mechanisms of the antifouling effect of electrochemical polarization in non-saline environment we developed a device to document the generation of ROS in real-time.
3 Materials and methods

3.1 Methods used in this thesis work.

The methods used in this thesis research were as compiled in Table 6.

Table 6. Methods used in this study.

<table>
<thead>
<tr>
<th>Method</th>
<th>Described in paper</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microscopy methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescent staining of biofilms (nucleic acid and lectins) for CLSM</td>
<td>II, III</td>
<td>(Neu et al., 2001)</td>
</tr>
<tr>
<td>Confocal laser scanning microscopy</td>
<td>II, III</td>
<td></td>
</tr>
<tr>
<td>Field emission scanning electron microscopy</td>
<td>II, III</td>
<td></td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td></td>
<td></td>
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<tr>
<td>Designing of PCR-primers specific for D. geothermalis</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>DNA extraction and purification</td>
<td>I</td>
<td>(Ekman et al., 2007)</td>
</tr>
<tr>
<td>Quantifying bacterial biomass using QPCR with universal primers for bacteria</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td><strong>Methods and tools developed for this thesis</strong></td>
<td></td>
<td></td>
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<tr>
<td>DBA and RadBox instruments</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>ROS detection with scanning fluorometry</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td><strong>Other methods</strong></td>
<td></td>
<td></td>
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<tr>
<td>Sampling</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Growing of biofilms on abiotic surfaces</td>
<td>II, III, IV</td>
<td></td>
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<tr>
<td>Image analysis</td>
<td>I, II, III and IV</td>
<td></td>
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<tr>
<td>Scanning fluorometry</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Analysis of biocide susceptibility of biofilms</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Detection of biofilm removal after electrochemical antifouling</td>
<td>IV</td>
<td>(Kolari et al., 2003)</td>
</tr>
</tbody>
</table>
3.1.1 Instruments and methods developed in this thesis

Double biofilm analyzer (DBA)

The double biofilm analyzer (DBA) was designed and built to screen for antifouling effects by electrochemical polarization on biofilms grown on stainless steel vials of the DBA. The 12 detachable stainless steel vials (AISI 316L, depth 30 mm, diameter 45 mm, holding volume 40 cm$^3$) were mounted on DBA platform (Fig 8). Six of the vials could be individually polarized. The electric current was fed into each vial via a control unit and Pt-coated Nb-wire-electrodes, one for each vial (Savcor Group Ltd, Mikkeli, Finland). The wire electrode and a reference-electrode (Savcor model 1) were implanted in the lid and became immersed inside each of the six vials when the lid was closed. Each vial itself worked as the counter electrode. DBA instrument was designed to simulate paper machine conditions by placing the DBA instrument in the incubator (45 °C) that provided rotation to match liquid flow of 0.3 ms$^{-1}$ in each vial. The polarization programs used in the DBA instrument were commercial trade ware delivered by Savcor Group Ltd (Mikkeli, Finland). The recipe for the synthetic paper machine water (SPW) was designed based on analytical data of white water of paper machines in Finland as shown in Table 1 of Paper IV.

Radical detection cuvette (Radbox) and real-time detection of oxygen radicals

The RadBox instrument was built for detecting reactive oxygen species formed during the polarization of the Radbox cuvette (10 mm × 25 mm, holding volume 4 ml). The cuvette was equipped with a working electrode (wire Ø 2 mm of steel AISI 316L), longitudinally inserted into the cuvette. The side walls (23 mm × 18 mm, AISI 316L) of the cuvette worked as the counter electrodes. The potential of the working electrode was measured against a reference-electrode (Savcor model 2). The principle of the Radbox function was that ROS sensitive fluorescence dye (Tempo-9-Ac) was mixed into the SPW medium and polarized. The ROS generated by polarization was detected as fluorescence emission from the reaction of ROS with Tempo-9-Ac (50 µM) (Molecular Probes, Eugene, Oregon USA). The detection was done with a scanning fluorometer (Fluoroskan Ascent, Thermofisher, Finland). The fluorescence output, ex 355 nm and em 425 nm, by Tempo-9-Ac, continuously recorded for 300 s from 72 locations inside the cuvette (one round of 72 locations each 7.5 seconds). The
readings obtained were integrated for six blocks of longitudinal sections (12 locations each). SPW medium was fortified with low melting agarose (0.25 % w/v, Sigma Aldrich, St. Louis, USA) to attenuate the liquid flow.

3.2 Methods other than those described in papers I-IV

3.2.1 Culturing D. geothermalis in microaerobic conditions

The ability of D. geothermalis (E50051) and of Meiothermus silvanus (B-R2A5-50-4) to grow in low oxygen and high carbon dioxide was tested. D. geothermalis and M. silvanus were grown on plates of R2A, TSA and TSB/A for 2-3 d at 45°C in an atmosphere with 1 % O₂ and 1% CO₂ in 98 % N₂ in a cell culture incubator (HERAcell 150i, Thermo Scientific, USA).
4 Results and discussion

4.1 Microaerobic growth of *D. geothermalis*

*D. geothermalis* (strain E50051) and *Meiothermus silvanus* (strain B-R2A5-50-4) as a reference strain from the same phylum were cultivated in a microaerobic atmosphere (1 % O$_2$ and 1 % CO$_2$) and in normal atmosphere. *D. geothermalis* grew on R2A, TSA and TSB/A plates similarly under atmospheric air and in microaerobic conditions. *M. silvanus* did not grow under microaerobic conditions. During microaerobic growth *D. geothermalis* colonies lost their pink pigment and turned yellowish or almost colourless (Fig 3). The genome of *D. geothermalis* contains genes for nitrite NAD(P)H reductase (Dgeo_2392) and for molybdopterin-cofactor-dependent nitrate reductase (Dgeo_2389) (Makarova *et al.*, 2007) which are known to express in anaerobic conditions. Cytochrome bd ubiquinol oxidase and a cytochrome d ubiquinol oxidase, usually involved in electron transfer under low oxygen (Junemann, 1997) were recently reported by Tian et al., (2010) to be present in *D. geothermalis* proteome. I suggest that the trait of growth under low oxygen concentration could reflect the evolutionary origin of *D. geothermalis*. It belongs to a branch in the bacterial phylogenetic tree older than the cyanobacteria (Gupta, 1998) and thus is older than the oxygenated atmosphere of the earth.

The carotenoids in *Deinococcus* responsible for the pink or red pigments are linked to scavenging ROS (Tian & Hua, 2010). *D. geothermalis* has also catalase to decompose hydrogen peroxide and superoxide dismutase to catalyze disproportionation of superoxide anion to molecular oxygen and hydrogen peroxide. A colorless mutant has been described of *D. radiodurans* sensitive to environmental stressors such as desiccation and ROS (Tian *et al.*, 2007). My results show that low oxygen concentration prevented the expression of pigments in *D. geothermalis*. Interesting is that *D. geothermalis* has been shown to reduce Fe (III) and Cr (III) under anaerobic conditions at 45 °C (Brim *et al.*, 2003). *D. geothermalis* biofilm on steel surface thus may change the electrochemical properties of the stainless steel generating anode-cathode pairs (Dickinson & Lewandowski 1998). The ability to use metals as electron acceptors could promote *D. geothermalis* to adhere and to grow on steel surface.
Figure 3. Colour differences of *D. geothermalis* biomass cultivated under microaerobic and aerobic atmospheres on solid media R2A, TSA and TSB/A (from the left to the right).

### 4.2 *D. geothermalis*-specific real time qPCR

In this study we quantified *D. geothermalis* biomass present in the wet end of paper and board machines, process waters and end products, by quantifying the density of *D. geothermalis* 16S rRNA genes in colored deposits collected from 24 paper and board machines (Paper 1, Table 1). The numbers of *D. geothermalis* 16S rRNA genes were compared to those of the domain “Bacteria” and to that of *Meiothermus* spp. measured by the same method but using primers of different specificities (Table 8). Primers used in the QPCR for this thesis work are shown in Table 7.

The primers DgeF627a and DgeR866 had 100 % match to 16S rRNA gene sequences of the type strain of *Deinococcus geothermalis* (DSM11300ᵀ) and to those of the paper machine isolates, strains E50051, E50053. The amplification product sized 256 bp and had a melting temperature of 90 ± 0.5 °C. Calibration curve (Fig 2A in Paper I) was used for quantification of 16S rRNA genes in unknown samples. The curve was log linear from 50 fg to 5 ng of genomic DNA from *D. geothermalis* E50051. Amplification was placed between the crossing points 32 to 13.8. Fifty femtograms of the template DNA corresponded to 28 and 5 ng to 280 0000 16S rRNA gene copies with the genome size of 3.27 Mb and 2 copies of the 16S rRNA gene per genome.
Table 7. Primers used for the QPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5´-3´)</th>
<th>Target region</th>
<th>Length of the amplified sequence (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DgeF627a</td>
<td>GGA GTG GGT TGG AGA CTG GCT</td>
<td>627-647</td>
<td>256</td>
</tr>
<tr>
<td>DgeR866</td>
<td>CCA GGC GGC ACG TTT CTC GC</td>
<td>866-885</td>
<td></td>
</tr>
<tr>
<td>pE</td>
<td>AAA CTC AAA GGA ATT GAC GG</td>
<td>908-928</td>
<td>165</td>
</tr>
<tr>
<td>pF</td>
<td>ACG AGC TGA CGA CAG CCA TG</td>
<td>1053-1073</td>
<td></td>
</tr>
<tr>
<td>MeioF692</td>
<td>GAA ATG CGC AGA TAC CGG A</td>
<td>692-711</td>
<td>147</td>
</tr>
<tr>
<td>MeioR821</td>
<td>TGT CGG ACA CCC AGC ACT</td>
<td>821-839</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) E. coli numbering  
\(^b\) (Edwards et al., 1989)  
\(^c\) (Ekman et al., 2007)

Specificities of the primers (Table 7) were tested with six type strains of species within the genus *Deinococcus*; *D. grandis* DSM3963\(^T\), *D. murrayi* ALT-1\(^b\)\(^T\), *D. proteolyticus* DSM20540\(^T\), *D. radiophilus* DSM20551\(^T\), *D. radiodurans* DSM20539\(^T\) and *D. radiopugnans* ATCC19172\(^T\). These species had ≥3 mismatches in their 16S rRNA gene sequences with the forward primer DgeF627a and 2 mismatches with the reverse primer DgeR866. In addition, species from phyla other than *Deinococcus-Thermus* were tested; *Burkholderia cepacia* F28L1, *Pseudoxanthomonas taiwanensis* jk-M, *Roseomonas gilardii* ATCC499956\(^T\), *Geobacillus stearothermophilus* DSM 1550 and *E. coli* MT102. The strains of species other than *D. geothermalis* showed (1 ng DNA) crossing points ≥30 cycles and the melting temperatures of the amplification products deviated by 0.5 to 3 °C from that of *D. geothermalis* (Fig. 4). Fifty femtograms of *D. geothermalis* DNA exceeded the same crossing point as *D. radiodurans* (1 ng) (Fig 4).
Figure 4. Amplification of DNA of *D. geothermalis* and nontarget species with primers DgeF627a and DgeR866. The figure shows *D. geothermalis* 1-6 (1 ng – 0.00005 ng) and type strains of other species of *Deinococcus*, (7-12) (1 ng), *Meiothermus ruber* DSM1279 (13), of proteobacteria (14-16, 18) *Burkholderia cepacia* F28L1, *Pseudoxanthomonas taiwanensis* jk-M, *Roseomonas gilardii* ATCC499956*, Escherichia coli* MT102 and of *Geobacillus stearothermophilus* DSM 1550 (17).

Spiking 1 ng DNA of non-target species with 0.1 ng of *D. geothermalis* DNA resulted in amplification of *D. geothermalis* DNA with the same crossing point, at 20 cycles, as without spiking (Paper I, Fig 2B).

We conclude that the number of *D. geothermalis* can be reliably calculated when the amplification product with the primers DgeF627a and DgeR866 was obtained within 30 cycles and had the correct melting temperature.
4.3 *D. geothermalis* DNA in slimes and deposits of paper machines

Based on earlier work by Kolari *et al.*, (2001, 2003) and the PhD thesis of Kolari (2003) it was clear that *D. geothermalis* was a primary-biofilm former. It was also frequent in paper mill slimes of the wire section splash areas, as was shown by 48 isolates of *D. geothermalis* from six different machines and *in situ*-hybridizations done with samples from three other machines (Väisänen *et al.*, 1998; Kolari *et al.*, 2003; Kolari, 2003).

In our studies *D. geothermalis* DNA was found by QPCR from 9 machines and in 16 of the 120 independently collected mill samples, equally from both paper and board machines. In addition, seven samples contained detectable but not quantifiable amount of *D. geothermalis* DNA (Table 8). Sampling was carried out from 15 different areas of the machines (Fig 1 in Paper I). *D. geothermalis* was most frequently found in the slimes of the splash areas and the inside walls of tanks. Tolerance to oxidative stress and desiccation are advantageous in colonizing such areas. Only two paper machines and one board machine yielded multiple (up to three) *D. geothermalis* positive samples. In a study a majority (73 %) of the isolated bacteria were identified as *D. geothermalis* strains in one machine (Kolari *et al.*, 2003). It was found that the pulp used by that machine was contaminated by *D. geothermalis*. It was concluded that the pulp further contaminated other paper machine where it was used as the raw material, explaining the high frequencies in those machines.

In the samples in this thesis (Paper I) the total content of bacterial 16S rRNA gene copies ranged from $10^7$ to $3 \times 10^{10} \text{ g}^{-1}$. *D. geothermalis* represented a minor fraction of this, 0.03 – 1.3 %. Similar amounts reported by Kolari (2003), using *in-situ*-hybridization method with DgeoF-probe $<$ 0.05 % to $< 2$ % of all cells, but 10 % in one slime sample. Based on our results *D. geothermalis* thus was not an abundant in the deposits of those examined machines compared to the other pink biofouler, *Meiothermus* spp. (Table 8) of which the 16S rRNA gene copy content ranged from 0.5 to 100 % of the bacterial deposits. *Meiothermus* was shown a dominant biofouler in several machines (Ekman *et al.*, 2007) and in headbox water (Prince *et al.*, 2009). Nevertheless, *D. geothermalis* may endanger paper quality as it was positively recognized in a coloured spot of an end product (Table 8).

The experimental data accumulated thus far, allows suggesting explanations why quantities of *D. geothermalis* were so low in most of the sampled biofilms. *D. geothermalis* adheres firmly onto surfaces and forms flat colonies (only 10 µm in height) growing in a patchy
manner (Paper II and Kolari et al., 2001). Therefore in sampling most of this bacterium may remain unsampled even the surface looks clean after the biofilm has been harvested. It is tempting to speculate that *D. geothermalis* may have other niches as well. We showed that *D. geothermalis* can grow in low oxygen atmosphere whereas *Meiothermus silvanus* did not. Locations under a thick slime are depleted of oxygen (Rasmussen & Lewandowski, 1998). In such environments *D. geothermalis* may form colorless biofilm. This might skew its real significance in paper machines and that its prevalence may be higher than our results show.
Table 8. QPCR measurements targeted to the 16S rRNA gene of eubacteria and of *Meiothermus* spp. in samples positive for *D. geothermalis*.

<table>
<thead>
<tr>
<th>Analyzed sample (machine)</th>
<th>numbers of 16S rRNA gene copies</th>
<th>Total bacterial</th>
<th>% of the bacterial gene copies identified as</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deposits</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wire section splash area (A)</td>
<td>$10^9$</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>Wire section splash area (MK)</td>
<td>$3 \times 10^8$</td>
<td>0.03</td>
<td>2</td>
</tr>
<tr>
<td>Wire section splash area (D)</td>
<td>$8 \times 10^9$</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>Wire section of inner layer (M)</td>
<td>$5 \times 10^9$</td>
<td>&lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>Shower water tank (K)</td>
<td>$1 \times 10^{10}$</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Circulating water tank (N)</td>
<td>$2 \times 10^8$</td>
<td>&lt; 0.001*</td>
<td>100</td>
</tr>
<tr>
<td>Cloudy filtrate tank (K)</td>
<td>$3 \times 10^{10}$</td>
<td>&lt; 0.001*</td>
<td>67</td>
</tr>
<tr>
<td>Cloudy filtrate tank (R)</td>
<td>$8 \times 10^9$</td>
<td>0.005</td>
<td>8</td>
</tr>
<tr>
<td>Reject storage tank (R)</td>
<td>$4 \times 10^9$</td>
<td>0.05</td>
<td>7</td>
</tr>
<tr>
<td>AES screen splash area (USc)</td>
<td>$3 \times 10^{10}$</td>
<td>0.03</td>
<td>13</td>
</tr>
<tr>
<td>Bow screen (R)</td>
<td>$3 \times 10^{10}$</td>
<td>0.007</td>
<td>11</td>
</tr>
<tr>
<td>Unspecified, slime (E)</td>
<td>$1 \times 10^{10}$</td>
<td>&lt; 0.001*</td>
<td>0.5</td>
</tr>
<tr>
<td>Press felt (K)</td>
<td>$10^7$</td>
<td>&lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>Water tank, process water (A)</td>
<td>$2 \times 10^8$</td>
<td>&lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td><strong>End products</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defect paper 1 (A)</td>
<td>$5 \times 10^9$</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Defect paper 2 (A)</td>
<td>$2 \times 10^7$</td>
<td>&lt; 0.001*</td>
<td></td>
</tr>
</tbody>
</table>

*positive but the amount was non-quantifiable. The sampled machines are indicated in parentheses.*
4.4 Adhesion of *D. geothermalis* to a surface

4.4.1 Architecture of *D. geothermalis* biofilm

*D. geothermalis* forms tenacious and compact colonies on abiotic surfaces (Saarimaa *et al.*, 2006; Raulio *et al.*, 2008) but is not a major slime-producer and shows no capsular polysaccharide material on the surface.

I used 66 fluorescently (Paper II, Table 1) labelled carbohydrate-specific lectins to explore the architecture of *D. geothermalis* biofilms. Biofilms were grown on glass or on stainless steel (AISI 316) and stained with the fluorescently labelled lectins and counterstained with a nucleic acid reactive fluorogenic dye. Binding of these lectins to the biofilms was visualized by CLSM. This experimental approach enables studying the EPS structure of fully hydrated living bacterial microcolonies *in situ* when the lectins with different carbohydrate specificities bind to the bacterial glycoconjugates demonstrating the arrangement and the compositions of the glycoconjugates relative to the cells in the biofilms. Table 9 shows the lectins that interacted with glycoconjugates in deinococcal biofilms. Fig 5 and Figs 1-5 in Paper II show CLSM images of the binding locations in and on the deinococcal colonies grown on abiotic surfaces.

The lectin study disclosed that *D. geothermalis* in monocultures form no classical “mushroom” type of biofilm structure (as described in Fig 1.) on an abiotic surface. The microcolonies were porous, compact and flat (height 10 to 20 µm).

The EPS in *D. geothermalis* microcolonies on glass or on steel bound to several lectins indicating it was a composite of different glycoconjugates. We identified five functionally different binding locations in *D. geothermalis* biofilms for the lectins 1) the glycoconjugates surrounding each cell individually, 2) the intercellular matrix, 3) the skeleton structures inside the biofilm, 4) biofilm interfaces with the nonliving substratum and 5) adhesion threads linking the cells to neighbouring cells or to the substratum. The EPS content was thus heterogeneous, but the binding patterns showed that the EPS was organized to diverse glycosylated entities, not just the capsular and the biofilm exopolymers. In natural heterogeneous biofilms the EPS regions have also been shown to arrange in to zones (Lawrence *et al.*, 2007).
The functions of EPS are multiple (Table 1). The lectin staining showed that glycoconjugates containing α-galNAc and galNAc(α1,3)galNAc residues had a structural role as building blocks in the skeleton-like matrix inside the biofilm and also in the heterogeneous intercellular matrix substance that filled the spaces between the cells and also covered the colonies (Fig 5A, Paper II Fig 1). These structures may also function as water reservoirs for *D. geothermalis*.

The internal space of the microcolonies was occupied by hydrophilic substances but outer surfaces of the colonies were hydrophobic, as indicated by adhesivity to small (≤ 20 nm) hydrophobic microspheres (Paper II, Fig 6). Hydrophobicity is suggested important for the adhesion onto nonliving surfaces and also for a *D. geothermalis* microcolony the hydrophobicity of its outer layer could explain why these biofilm colonies function as pedestals promoting adhesion of other bacteria, e.g. *Bacillus* bacteria (Kolari et al., 2001). It has also been shown that bacteria accumulating on air-liquid interfaces possess more hydrophobic surfaces than bacteria on water-submerged area (Dahlback et al., 1981). For *D. geothermalis* that favours to colonize air-liquid interfaces, the hydrophobic outer shelter of the microcolony could also work as a protective layer against desiccation.

Galactose containing glycoconjugates were prevalent throughout the EPS content of *D. geothermalis* biofilms. Analysis of EPS has revealed galactose in biofilms of other bacteria (Verhoef et al., 2005; Mata et al., 2006; Jiao et al., 2010). EPS of *Pseudomonas aeruginosa* (WFPA801) was shown to contain galactose and mannose with the chemical analysis and this was confirmed with galactose and mannose specific lectins (Ma et al., 2007). Galactose containing glycoconjugates can function also as nutrient reservoir for biofilm bacteria. *D. geothermalis* can utilize e.g. galactose (Ferreira et al., 1997) and possess β-galactosidase enzyme that can hydrolyse galactose-containing oligosaccharides (Lee et al., 2010). The differences in binding locations are due to differences in glycoconjugate compositions or arrangements (Lawrence et al., 2007). Sequences of β-1, 3 and β-1, 4 were common lectin specificities in *D. geothermalis* (Table 9). Sutherland (2001) suggested that these sequences may confer rigidity in the EPS structure.

I agree with the view of my co-author Thomas Neu that the precise consistency of the EPS or the nature of the glycoconjugates is difficult to interpret based on the lectin bindings alone in complex glycoconjugate systems (Neu et al., 2001). A novel method where CLSM technique
is combined with Raman microscopy has given promising results on the chemical structure and arrangements in EPS (Wagner et al., 2009).

The architecture of *D. geothermalis* biofilm, the flat and compact microcolonies as well as the numerous different glycoconjugates of EPS, may contribute its survival and adaptation to extreme conditions prevailing in paper machines e.g. high water flow and temperature, and the presence of many different antimicrobial compounds.
Table 9. Fluorescently labelled lectins that bound to the biofilm of *D. geothermalis* strain E50051 were imaged with CLSM. The data are presented in Paper II unless otherwise referred to. Gal; galactosyl, neu; neuraminyl, glc; glucosyl, NAc; N-acetyl, fuc; fucosyl, man; mannosyl

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Binding location</th>
<th>Target glycoconjugates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agaricus bisporus</em></td>
<td>cell surface</td>
<td>β-gal, gal(β1,3)galNAc</td>
<td></td>
</tr>
<tr>
<td><em>Arachis hypogaea</em></td>
<td></td>
<td>β-gal, gal(β1,3)galNAc</td>
<td></td>
</tr>
<tr>
<td><em>Triticum vulgaris</em></td>
<td></td>
<td>glcNAc&lt;sub&gt;2&lt;/sub&gt; glcNAc&lt;sub&gt;3&lt;/sub&gt;, sialic acid (neuraminic acid)</td>
<td>(Kolari, 2003)</td>
</tr>
<tr>
<td><em>Amaranthus caudatus</em></td>
<td>adhesion threads</td>
<td>gal(β1,3)galNAc, gal(β1,3)neu5Ac</td>
<td></td>
</tr>
<tr>
<td><em>Dolichos biflorus</em></td>
<td>skeleton matrix of biofilm</td>
<td>terminal α-galNAc, galNAc(α1,3)galNAc</td>
<td></td>
</tr>
<tr>
<td><em>Maclura pomifera</em></td>
<td></td>
<td>α-gal, α-galNAc, gal(β1,3)galNAc</td>
<td></td>
</tr>
<tr>
<td><em>Erythrina cristagalli</em></td>
<td>biofilm interface</td>
<td>gal(β1,4)glcNAc, galNAc, gal</td>
<td></td>
</tr>
<tr>
<td><em>Phaseolus lunatus</em></td>
<td></td>
<td>galNAc(α1,3)[L-fuc(α1,2)gal], galNAc</td>
<td></td>
</tr>
<tr>
<td><em>Phaseolus vulgaris</em></td>
<td></td>
<td>galNAc</td>
<td></td>
</tr>
<tr>
<td><em>Robinia pseudoacacia</em></td>
<td>intercellular matrix</td>
<td>No information available</td>
<td></td>
</tr>
<tr>
<td><strong>Lens culinaris</strong></td>
<td>α-man, fucose linked to chitobiose core of oligosaccharide, α-glc, α-glcNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lathyrus odoratus</strong></td>
<td>intercellular matrix, α-man end groups, glc, glcNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lyopersicon esculentum</strong></td>
<td>α-glucosyl(β1,4)glcNAc oligomers</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Helix aspersa</strong></td>
<td>galNAc, glcNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trifolium repens</strong></td>
<td>2-deoxyglucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wisteria floribunda</strong></td>
<td>terminal galNAc(β1,4), terminal galNAc(β1,3), terminal galNAc(α1,3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Abrus precatorius</strong></td>
<td>D-galactose (Kolari, 2003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Limulus polyphemus</strong></td>
<td>fetuin, D-glucuronic acid, sialic acid, NeuAc (Kolari, 2003)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The specificities are indicated as given by the suppliers.
Figure 5. Maximum intensity projections of CLSM images from *D. geothermalis* E50051 biofilm on glass. Green colour shows the locations of the lectin bound to the glycoconjugates and red shows the fluorogenic staining responding to nucleic acid. In panel A) *Maclura pomofera* lectin bound to the matrix of the biofilm, but did not interact with the cells. B) *Wisteria floribunda* lectin found binding sites on the exterior of biofilm colonies and the spaces between the cells. C) *Erythrina cristagalli* lectins interacted with the glycoconjugates of the cell surface. Scale bars 10 µm.

4.4.2 The adhesion threads of *D. geothermalis*

Many studies have shown that *D. geothermalis* adheres firmly to an abiotic surface; steel, glass or plastics, and when doing so it extrudes cell surface appendages anchoring the cell to the abiotic surface and to other cells (Papers II and III; Kolari *et al*., 2002; Raulio *et al*., 2006; Raulio *et al*., 2008). Fig 6B shows *D. geothermalis* biofilm where thin adhesion threads form a network between the cells. We characterized these adhesion threads of *D. geothermalis* E50051 and found them to contain protein. Several N-terminal sequences from the deinococcal cell surface material were found similar to four pilin components of the type IV pili (TfP) (Paper III Table 1). TfP has been detected in many \( \beta \), \( \gamma \) and \( \delta \)-proteobacteria (O'Toole & Kolter, 1998; Thormann *et al*., 2004; Shime-Hattori *et al*., 2006; Varga *et al*., 2008; Bahar *et al*., 2010). Our work was the first demonstration of these pilins in the phylum *Deinococcus-Thermus*. These appendages were missing in planktic cells of the same strain (Paper III, Fig 1, 3) indicating that the threads have a role in adhesion and in the formation of microcolony architecture. Raulio *et al*. (2008) showed that these adhesion threads were thick and numerous when *D. geothermalis* (E50051) cells adhered on stainless steel (AISI 316L). Whereas on hydrophobic fluoropolymer coated stainless steel surface the adhesion threads were thin, short and few in number. They suggested that the volume of biofilm on a given surface depended on the thickness and the number of the adhesion threads.

Recently, Tian *et al*. (2010) reported that planktic *D. geothermalis* cells expressed TfP assembly protein PilO, PilM and a fimbrial assembly protein suggesting that TfP components may have also other functions beyond the biofilm formation.

When we stained *D. geothermalis* biofilm with the lectin *Amaranthus caudatus* specific for gal(\( \beta 1, 3 \))galNAc and imaged using CLSM, the adhesion threads (Fig 6A) were visible as
puffy spots around the cells. In addition, periodic acid-Schiff (Paper III, Fig 2) staining also indicated protein glycosylation. Glycosylation is a common posttranslational modification of proteins. So far this is the first study that demonstrated that Tf p like surface appendages were covered with specific glycoconjugates. The sugar moieties on the adhesion threads may protect the protein threads and increase “stickiness” of the cells in adhesion. A web-like structure formed by the adhesion threads and sugar covering may contribute to the tenacious adherence in high liquid flow prevailing in the paper mill.

Figure 6. Confocal laser scanning (CLSM) (A) and Field Emission Scanning Electron Microscopy (FESEM) (B) images of D. geothermalis strain E50051 biofilm on glass. Panel A shows the fully hydrated biofilm stained with fluorescently labelled Amaranthus caudatus lectin (green) and counterstained with the nucleic acid stain, Syto 60 (red) to locate the cells. White lines show a zoomed image of two cells. In panel B the biofilm was fixed and dehydrated for FESEM (by courtesy of Mari Raulio). Both images show the adhesion threads, in (B) forming a network-like structure and in (A) the threads are covered with glycoconjugates detected with Amaranthus caudatus. Fixation and dehydration shrinks the adhesion threads that are visible as chubby spots in the CLSM image which was taken from cells with no prior treatment like fixing or drying.
4.4.3 Glycoconjugates contributing to the adhesion

Staining with fluorophore conjugated lectins were used as a tool to visualize the glycoconjugates of *D. geothermalis* growing as biofilms on steel or on glass (Paper II).

The glycoconjugates connecting cells of *D. geothermalis* to the substratum reacted with the *Phaseolus vulgaris* lectin. The glycoconjugates bridging *D. geothermalis* to glass differed from those connecting the cells to stainless steel surface (Paper II, Fig 4). Hydrophilic and hydrophobic coated glass substrata were shown to induce a modification of the cell surface of *Pseudomonas aeruginosa* as demonstrated with different lectin stainings (Bejarano & Schneider, 2004). A similar modification may have resulted into the differences observed in the binding patterns of *Phaseolus vulgaris* lectin to the glycoconjugates on *D. geothermalis* surface.

*Phaseolus vulgaris* lectin reacted with glycoconjugates encircling horizontally cells adhered. In addition, this lectin stained also glycoconjugate tracks on the steel surface distant to the cells (Fig 7, Paper II Fig 4C and D). These tracks may represent foot prints emanating from the glycoconjugates that mediated slippery adhesion of *D. geothermalis* (E50051) to steel demonstrated by Kolari *et al.* (2002) using the atomic force microscopy. The phenomenon could resemble that of the gram-negative bacterium *Myxococcus xanthus*, leaving slimy tracks when it glides over a surface. *M. xanthus* exhibits two different surface gliding motilities: an adventurous (A)-motility and social (S)-motility. The pilus-independent A-motility was suggested to rely on slime extruded from nozzle-like structures similar with those observed of cyanobacteria (Wolgemuth *et al.*, 2002). Interesting in this context is the discovery reported by Li *et al.* (2003) on the S-motility of *M. xanthus*, mediated by an extracellular matrix combined with polar type IV pili. Interactions between the Tfp and the extracellular matrix on the surface of another cell may induce retractions of the Tfp and thus S-type motility (Li *et al.*, 2003). *D. geothermalis* possibly also possesses a motility mechanism linked to its Tfp, to polysaccharides or both.

Kolari (2003) reported that the N-acetylglucosamine specific *Triticum vulgaris* lectin bound to the cell surface glycoconjugates of *D. geothermalis* (E50051) and that the *Limulus polyphemus* lectin with specificity for D-glucuronic acid and sialic acid bound to glycoconjugates located in the intercellular space of this strain. Those studies were done with *D. geothermalis* biofilm grown in wire water on stainless steel. These lectins were negative
when I used the same strain of *D. geothermalis* grown in R2 medium on glass surface. This and our findings in Paper II (Fig 4) can be interpreted to indicate that *D. geothermalis* biofilm interacts with its environment, modulating its cell carbohydrate composition to match the substratum. Factors such as the growth cycle, sources of calcium or of carbon and the presence of antimicrobial agents may influence the composition and yield of EPS produced by bacteria (Uhlinger & White, 1983; Strathmann *et al.*, 2002; Dynes *et al.*, 2009).

![Figure 7. Confocal laser scanning image of *D. geothermalis* (E50051) cells on steel surface, stained with *Phaseolus vulgaris* (green) lectin and the nucleic acid reactive stain, Syto 60 (red). As a monolayer the cells of *D. geothermalis* were lined by a glycoconjugate matrix. A dense network of gliding tracks is visible in the middle, between the cells. Scale bar 20 μm.](image)

### 4.5 Strategies for studying electrochemical antifouling in simulated paper mill environment

Antimicrobial agents (biocides) dispersed into the bulk of the water flow do not necessarily reach the locations where biofouling occurs. An alternate option is to generate antimicrobial agents directly on the fouled surface. Electrochemical polarization follows this strategy. Strategy to generate ROS or free chlorine or chlorine containing radicals underneath a biofilm has been applied to kill and remove
biofilm from the surfaces (Wood et al., 1996; Rabinovitch & Stewart, 2006). We developed novel tools to explore the effects of electrochemical polarization on biofilms growing under conditions prevailing in paper mills: warm temperature (45 °C) and high water flow (> 0.3 m s⁻¹) and rich organic contents. The medium, synthetic paper machine water (SPW) was constructed for these experiments to model the authentic white water of a paper machine: high in organic matter (> 500 mg L⁻¹) and low in chloride concentration (0.01 % w/v) (Paper IV Table 1). In DBA experiments SPW that contained 10 % of R2 medium was used to provide the substrate needed for biofilm growth. D. geothermalis (E50051) was used as the test organism. The test surface was stainless steel that is the common material used in wet industry.

4.6 Antifouling of D. geothermalis biofilm by electrochemical polarization

4.6.1 The novel tool to study electrochemical antifouling: the double biofilm analyzer

The instrument, double biofilm analyzer (DBA) (Fig. 8) was designed and built during this thesis work. It is a platform that holds twelve stainless steel vials (holding volume 40 ml each) of which six were in situ polarizable. It thus enabled simultaneous testing of different electrochemical polarization programs in the presence or absence of biocides. The polarization potentials were online controlled and recorded with a reference electrode and a data recording computer. Each of the vials worked as the counter electrode when polarized.

Stainless steel is not as durable as e.g. titanium, platinum or gold (Siitonen, 2004). Therefore the range of applicable potentials was limited compared to studies where disinfectants were produced by electric polarization to quantities sufficient to kill planktic cells (Jeong et al., 2006; Jeong et al., 2007; Jeong et al., 2009).
Figure 8. The Double biofilm analyzer instrument. In use mode (A), the lid is closed. The electrodes were mounted to the lid (polarizable and reference). When the lid was closed the electrodes immersed into the liquid in the steel vials. Open DBA (B) shows the 12 steel vials and the lid-mounted working and reference electrodes.

Steel surfaces in the paper machine wet end are never totally clean. We simulated this situation by pregrowing \( D. \) geothermalis (E50051) without any antifouling. After growth for 24 h in SPW (with 10 vol % R2) there were mature biofilm the steel vials. The DBA vials were then emptied and refilled to submerge the pregrown biofilm and polarization was started. The polarization extended 4 to 48 h after which the vials were emptied and inspected to see whether the electrochemical treatments had removed the submerged biofilm and/or prevented growth of new biofilm on the upper air-liquid-interface. (Fig 9). The duration of the experiments had to be limited to 48 h because of the evaporation of water at 45 °C, reduced the liquid volume in the vials.

Figure 9. Biofilm of \( D. \) geothermalis grown on a stainless steel vial in the DBA instrument. Biofilms were grown in the air-liquid interface in two steps (upper and lower) during cultivation with two different fillings volume. The biofilms were visualized by staining with crystal violet (blue). Diameter of the vial 4.5 cm.
After experimenting with many different polarization programs that were tested, we concluded that a direct cathodic and a cathodically weighted pulsed polarization were effective in removing submerged biofilms of *D. geothermalis* within 48 h from steel surfaces (Paper IV Fig 3). Biofilm removal was first observed after 24 h (Paper IV Fig 5), reflecting the firm adhesion and the high resistance of *D. geothermalis*. Cathodic polarization of the substratum means that it becomes negatively charged. This generates an electrorepulsive force between the surfaces of the deinococcal cells if these are negatively charged. The electrorepulsive force may result into detachment of the bacteria from the substratum. During pulsed polarization the anodic and the cathodic polarizations were alternated with high frequency. According to published literature, this may inactivate the microbial cells by distorting the integrity of the cell membrane (Liu *et al.*, 1997; van der Borden *et al.*, 2005). Pulsed current may also destabilize the EPS structure by affecting physicochemical interactions between the biomolecules and prevent ions from accumulating on the substratum.

Although direct cathodic current was as effective as cathodically pulsed polarization up to 48 h, we noticed that over-time the current attenuated (data not shown). This means that in real life the antifouling efficiency will decrease with time. Our results are supported by recent publications where it was suggested that pulsed and block current could be the solution to overcome the disadvantages of direct currents (anodic and cathodic) (van der Borden *et al.*, 2005; Hong *et al.*, 2008).

Our experimental results showed that electric polarization resulted into effective antifouling of the steel surfaces below the air-liquid interface but not in the splash area above it. *D. geothermalis* that favours air-liquid interfaces thus formed new biofilm, in the splash area, even during the polarization (Paper IV Fig 7A).

Therefore, biocides (Paper IV Fig. 6) were tested in combination with cathodically weighted pulsed polarization. It was found that the tested halogen containing biocides (bromochloro-5,5-dimethylhydantoin, 2,2-dibromo-2-cyanodiacetamide) and peracetic acid increased the biofilm removal from the stainless steel surfaces. The additive value of biocides was that the accumulation of *D. geothermalis* cells to the air-liquid interfaces was prevented (Paper IV Fig 7C). The tested reductive biocide (methylene bisthiocyanate) did not prevent such attachment when combined with the pulsed, cathodic programme but rather looked like neutralizing the antifouling action of pulsed cathodic polarization.
To conclude, cathodically weighted pulsed polarization could be used to remove biofilms in wet industry. For problematic biofilms at the air-liquid interface, polarization can be combined with a low dose of oxidative biocides to keep the air-liquid interfaces clean. Alternatively, the water level could be purposely raised for effective polarization treatment.

4.6.2 Detection of reactive oxygen species generated during polarization: the Radbox instrument

The RadBox instrument was designed to measure the ROS that may be generated during electrochemical polarization of stainless steel. This instrument consisted of a cuvette (Fig 10), polarization control unit and fluorescence reader (Paper IV). The RadBox instrument allowed inspecting the ROS produced by the polarization by the same settings used in the DBA. ROS monitoring was done in SPW medium at room temperature.

![RadBox](image)

**Figure 10.** RadBox. The cuvette (volume 4 ml) is lined with red. The polarizable (working) electrode is inserted in the center, and the reference electrode underneath the working electrode. The stainless steel walls of the cuvette worked as the counter electrodes.

The challenge in detecting ROS is that the radicals are very short-lived (nanoseconds). It was first searched for a sensitive method for detecting also small output of ROS. Those in mind a method was developed based on the ROS sensitive fluorogenic dye, Tempo-9-Ac, and online detection with a microplate reader of the fluorescence emitted from the cuvette during the polarization. To map the ROS generation in the cuvette, microplate reader scanned the whole cuvette area continuously (from 72 measurement locations) during the whole period (300 s) of polarization (Paper IV Fig 4).

Previous studies of electrochemical treatment with low currents have given no evidence of ROS being involved in the antifouling effect. We detected ROS with a pulsed, cathodically...
stressed polarization programme around the working electrode. Fig 4 in Paper IV shows kinetics of the ROS generation. The RadBox instrument was able to record the ROS emissions in the same rapid tempo in which polarization was pulsed. Interesting was that ROS production was detectable in the SPW medium which was rich in organic matter (> 500 mg L\(^{-1}\)).

It was hard to distinguish whether the ROS production linked to the anodic or to the cathodic pulse. Based on the cathodic dominance of the current that was effective for antifouling I estimated that ROS most likely generated at the cathode. In other studies where nobler metal surfaces than steel, were used as the substratum ROS was generated with high anodic voltage (1 to 4 V) to concentrations that effectively disinfected planktic cells (Jeong et al., 2006; Jeong et al., 2009). Oxygen reduction at the cathode can indirectly generate superoxide anion and hydroxyl radicals (Zhang et al., 2008). In the recent study of Perez-Roa et al. (2009) fluorescent dye specific for ROS was used to show that hydroxyl radicals were formed at the cathodic electrode when pulsed a voltage sufficiently negative to reduce oxygen to hydrogen peroxide.

Even though *D. geothermalis* possesses a high tolerance to oxidative stressors and uses multiple tools to adhere to the abiotic surfaces and forms a web-like network connecting to other cells, we managed to crack its defence so that the biofilms were detached from stainless steel surface. Continuous production of oxygen radicals may overwhelm the protection capacity of the cells.

The antifouling effect of pulsed polarization towards *D. geothermalis* may result from a sum of repulsive forces between the bacterial cell and the steel, generation of oxidative ROS and the membrane disturbance by the pulsed polarization. Our results show that these effects could be promoted by combining the polarization with a suitable oxidative biocide.

In practice the electrochemical antifouling in paper mills is applicable. It can be used during the paper machine operation. The benefit of electrochemical antifouling would be that downtime needed for cleaning could be less frequently and that the usage of biocides could be diminished. The electrochemical antifouling program has to be individually designed for each machine since the conditions in each paper machine are different. More research on the effectivity of the electrochemical antifouling towards mixed biofilms and primary biofoulers
other than *Deinococcus*, such as *Pseudoxanthomonas taiwanensis* or *Meiothermus*. It would be interesting to study how spores are affected by the electrochemical treatment.
5 Conclusions

1. We found using fluorescently tagged lectin stainings and CLSM that *D. geothermalis* possessed five different spatial arrangements of EPS 1) the glycoconjugates surrounding each cell individually, 2) the intercellular matrix, 3) the skeleton structures inside the biofilm, 4) biofilm interfaces with the nonliving substratum and 5) adhesion threads connecting the cells to the abiotic surface and to each other.

2. We revealed that the glycoconjugates located between the abiotic substratum and the deinococcal cell surface reacted with *Phaseolus vulgaris* lectin. This study opens the door for studying the glycoconjugates involved in the attachment and gliding motility of *D. geothermalis* cells on a glass or steel substratum.

3. Microcolonies of *D. geothermalis* contained numerous adhesion threads connecting the cells to the abiotic surface and to one another. The adhesion threads were covered with gal(β1,3)galNAc glycoconjugates.

4. The adhesion threads of *D. geothermalis* contained components of the type IV pilin. These threads were not expressed by planktic cells indicating they were needed for adhesion and/or biofilm formation.

5. A culture independent method, real-time QPCR, was developed for quantifying *D. geothermalis* in biofilms and deposits of paper mills.

6. Based on QPCR less than 10 % of the 120 collected coloured biofilms, deposits and paper products contained measurable amount of *D. geothermalis* DNA. The contribution of *D. geothermalis* ranged from 0.0001 to 1.3 % of total bacterial content (2 × 10^7 – 3 × 10^10 g^(-1) wet wt). Although the biomass of *D. geothermalis* in paper mill deposits was minor, its DNA was found in colour defective paper products indicating that this species could spoil paper product quality.

7. We showed that cathodically weighted pulsed polarization effectively detached *D. geothermalis* from stainless steel surface. This treatment was advantageously in combined with oxidative biocides to remove biofilms and to prevent the further attachment of the biomass on to the steel surface.

8. We detected generation of ROS on steel surface by cathodically weighted pulsed polarization that was effective in antifouling. This indicates that ROS has a role in the antifouling effect.
9. We suggest that the antifouling effect of pulsed cathodic polarization results from multiple factors, generation of ROS and of the membrane disturbing effects of the electric pulses with alternating polarities.

10. To investigate the efficiency of electrochemical antifouling on deinococcal biofilms under conditions simulating paper machine environment we designed two novel tools: the double biofilm analyzer (DBA) and the Radbox. DBA was used to identify the antifouling effective polarization program and the combining of biocides and polarization to optimize the antifouling potency. Radbox was used to demonstrate the generation of reactive oxygen species in real-time.

11. *Deinococcus geothermalis* E50051 grew under low oxygen conditions as pale pigmented colonies, implying that red pigment is a part of the cellular machinery method for combating the oxidative stress by this bacterium.

12. Electrochemical polarization combined with biocides was effective towards *D. geothermalis* that is tenacious biofouler and possesses a high tolerance to oxidative stressors. This method could be effective also towards other persistent biofoulers and applicable in wet industrial processes elsewhere.
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