BACTERIAL COMPOSITION OF CONSTRUCTED WETLAND'S BIOFILM: THE INFLUENCE OF BIOTIC AND A-BIOTIC PARAMETERS ON THE COMPOSITION OF THE BIOFILM

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Lilach Iasur-Kruh

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This work was carried out under the supervision of:

Dr. Dror Minz at the Institute of Soil, Water and Environmental Sciences, Agricultural Research Organization of Israel, Volcani Center, Bet Dagan

And of

Prof. Yitzhak Hadar at the Department of Plant Pathology and Microbiology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot.

Abstract

In recent years water availability has become an issue of global concern. Overpopulation and industrial development are causing a substantial shortfall in availability of potable water. This shortage becomes of greater importance in countries like Israel that have limited resources of drinking water. Developing efficient wastewater treatment (WWT) systems which produce high-quality effluent water for reuse in agriculture is significant for both reducing the usage of potable water reservoirs, and for controlling environmental pollution. Constructed wetlands (CW) are becoming increasingly popular as economical and environmentally friendly solutions for WWT. Similar to conventional WWT systems, microbial biofilms, attached to the CW matrix, are responsible for most of the essential transformations and decomposition of contaminants in the wastewater. Still, relatively little is known about the bacterial populations involved in the formation and activity of this biofilms.

The focus of this thesis was microbial aspects of biofilm formed during advanced treatment of purified wastewater in CW.

The wetland is a very dynamic and variable system with respect to its physical and chemical properties. This variability is a result of different gradients of redox potential, substrate availability, and environmental conditions, such as pH and temperature. The different gradients create variable niches throughout the CW ponds, in which different biochemical processes take place. These diverse conditions at the niche level may affect the composition and activity of the microbial community in wetlands biofilm.

The first aim of this work was to investigate the microbial population composition and activity of CW biofilm with respect to the influence of vegetation, depth, and CW matrix type.

Dominant bacterial assemblage in biofilm developed on gravel of different layers of wetland microcosms constructed with or without vegetation was examined. Highly complex bacterial diversity was observed in the biofilm, including representatives of the α, β and δ subdivisions of *Proteobacteria, Acidobacteria* and *Bacteroidetes*. Cluster analysis of denaturing gradient gel electrophoresis (DGGE) patterns demonstrated that depth within the wetland microcosm had a stronger effect on microbial community composition of the biofilm formed on wetland matrix than vegetation. Measurements of fluorescein diacetate hydrolysis activity and nitrification potential revealed that hydrolytic activity was affected by both microcosm depth and vegetation presence, whereas nitrification potential was mostly influenced by depth. These results suggest that depth is the main criteria affecting the microbial community composition and function of the studied CW biofilm.

Biofilm initiation is the first step of biofilm assemblage. As such, this process is affecting the composition and function of the mature biofilm. Biofilm development is dependent on various conditions, including water bacterial species, flow parameters, surface characteristics and nutrient availability. Nutrient concentration and organic load have a strong effect on biofilm initiation in terms of microbial biomass and diversity.

The second aim of this study was to evaluate the influence of organic load on microbial biomass productivity and diversity in developed biofilms formed on CW matrix under controlled conditions.

Seven bacterial isolates, different in their colony morphology, were obtained from wetland biofilm. These isolates, when inoculated separately under laboratory conditions, formed a single-species biofilm (SSB) on the surface of CW matrix (sterile basalt gravel). Multiple-species biofilm (MSB) was initiated by inoculating a mixture of the seven isolates. The biomass productivity of SSB and MSB increased with the level of organic matter in the medium. MSB microbial community composition varied according to the treatment, and evidence suggested that increased organic load resulted in decreased diversity. In addition, biofilm initiation by two water types, being the early and advanced influent water, was investigated. The treated effluent water from the early treatment was the influent water for the advanced treatment. Hence, the influent of the early treatment contained more organic matter than the influent of the advanced stage. The biofilm initiated by the early influent water was less diverse than the biofilm initiated by the advanced influent. Thus, in all experiments of biofilm initiation in this study high organic load resulted in low biofilm diversity.

CW is considered an efficient WWT system. However, some contaminants remain in ecologically relevant quantities in CW effluent. One group of these pollutants, which is known to harm the balance of natural ecological system, is estrogenic residues. These contaminants interfere with endocrine processes and are potentially harmful to aquatic biota and to public health. Natural estrogens such as 17-β-estradiol (E2) remain in

ecologically relevant quantities in the CW effluent. Reduction of a specific pollutant, such as E2, may be achieved by bioaugmentation (adding bacteria with specific degradation abilities into a polluted area). The efficiency of bioaugmentation is determined by the survival of the added bacteria and the contaminant degradation rate. Several pollutants had been previously shown to be degraded efficiently as a result of successful bioaugmentation and therefore we decided to study the use this technique in estradiol degradation.

Therefore, the third aim of this study was to isolate an estradiol degrading bacterium from CW biofilm and examine the potential of integrating it into wetland biofilm (bioaugmentation).

An estradiol degrading bacterium was isolated from wetland biofilm. This isolate, EDB-LI1, was efficient in transforming E2 to estrone (E1). In addition, it had the ability to grow on and remove E1, which is one of the environmentally stable byproducts of E2 metabolism. EDB-LI1 showed a 98% match (by comparison of 16S rRNA sequence) to *Novosphingobium JEM-1*, another E2 degrading isolate from wastewater treatment plant in Japan. The effects of bioaugmentation of EDB-LI1 on structure and function of wetland biofilm were examined under laboratory conditions. EDB-LI1 was inoculated into wetland biofilm originated from two wetland ponds representing two consecutive water treatment stages (early and advanced). EDB-LI1 bioaugmentation into the biofilm resulted in three significant observations: (i) although isolated from CW biofilm, EDB-LI1 presence was detected (by qPCR analysis) only in augmented biofilms (ii) the augmented biofilms acquired the ability to removed E2 and E1 and (iii) the bacterial community composition (analyzed by DGGE) of the augmented biofilm was different

from that of control biofilm. Furthermore, EDB-LI1 bioaugmentation showed higher removal of E1 and E2 in biofilm originated from the advanced-treatment-stage wetland pond than from the early-treatment-stage pond. Hence, bioaugmentation efficiency of EDB-LI1 likely depends on both the quality of the feed water and the microbial community composition in the pond.

To summarize, we investigated the microbial assembly, composition and activity of CW biofilm as well as the possibility for integration of E2 degrading bacteria into the biofilm. We suggest that organic matter is one of the main criteria which influenced CW biofilm initiation, maturation and bioaugmentation.

Further, long term investigation of bioaugmentation in-situ (in the CW site) is the next step, which will indicate whether EDB-LI1 bioaugmentation can be a valid solution for E2 remains in CW effluent.

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1. Introduction

Constructed wetlands (CW) are low cost natural solutions which combine efficient wastewater cleanup and environmental conservation with landscape development. Biofilms of CW are of interest for both ecological and applied disciplines. The complex interactions of bacteria inside these biofilms are influencing the biofilm function and, as a result, the efficiency of the wetland system. Meaning, biofilms affect the CW's effluents quality (Larsen and Greenway, 2004, Faulwetter *et al*., 2009). The assembly of microbial populations in the biofilm is spontaneous and therefore considered a 'black box' (Baptista *et al*., 2008). In the last decade only a few studies had tried to resolve the bacterial assemblage of CW biofilm (Ahn *et al*. 2007, Ibekwe *et al*., 2007, Osem *et al*., 2006, Silyn-Roberts and Lewis, 2004, Truu *et al*., 2005). The influence of different wetland conditions on microbial community composition and function were investigated (for review: Truu *et al*., 2009). However, still a large part of this 'black box' remains unraveled (Truu *et al*., 2009). For example; biofilm from CW aimed for post treatment of effluent wastewater was hardly explored (Toet *et al*., 2005, Ibekwe *et al*., 2007). A profound understanding of the microbial community composition, function and microbial interactions within the CW and the ability to manipulate the bacterial composition of the wetland's biofilm can help to optimize the efficiency of water treatment in the constructed wetland system.

1.1 Biofilm development

"A biofilm is a complex community of microorganisms that are embedded in a matrix of extracellular polymeric substances they have produced, and they are attached to either an inert or living surface and formed by one or more microbial species" (Carpentier and Cerf, 1993, Costerton *et al.*, 1999, Davey and O'Toole, 2000, Kraigsley *et al.*, 2002). This very long and specified definition still does not explain the complexity and importance of biofilm in different environments and disciplines. It is now widely recognized that most bacteria found in natural, clinical, and industrial settings persist in association with surfaces (Donlan, 2002).

Biofilm initiation has three major steps: (1) Initial attachment- using polar flagella, planktonic bacteria randomly attach to fixed surfaces. Attachment is initially reversible, but later becomes permanent. (2) Replication- early colonizers replicate to form monolayer microcolonies on the fixed surfaces, and (3) Maturation- a diverse array of bacterial species adhere to the original biofilm monolayer to form a three-dimensional biofilm. As the biofilm develops exopolysaccharides (EPS) are produced by the different bacteria strengthening and stabilizing the biofilm structure (Watnick and Kolter, 2000).

1.1.1 Biofilm development as affected by different parameters (organic load)

Biofilm development is dependent on various conditions, including bacterial species, flow parameters, surface characteristics and nutrient availability (Watnick and Kolter, 2000). The ability of single-species cultures to form biofilms has been studied in detail in a variety of bacterial species, including *Escherichia coli* (Pratt and Kolter, 1998, Lee *et al*.,2007), *Vibrio cholerae* (Watnick *et al*., 1999), *Pseudomonas aeruginosa* (see review

Harmsen *et al*., 2010) and *Staphylococcus aureus* (Tormo *et al*., 2007). Those studies focused on biofilm morphology, formation mechanisms and gene expression. It was shown that bacteria in biofilm form exhibiting an altered phenotype from their planktonic counterpart with respect to growth rate and gene transcription (Donlan, 2002).

Different investigators have studied the effect of nutrients and organic load on single-species biofilms (SSB). For instance: *Citrobacter* sp. biofilm formation and cell phenotype were shown to be influenced by P, N and glucose limitation (Allan *et al*., 2002). The biofilm morphology of *Serratia marcescens* was affected by concentrations of glucose and casamino acids; under reduced carbon or nitrogen conditions, *S. marcescens* formed a biofilm consisting of microcolonies, but when the nutrient composition was increased a filamentous biofilm was formed (Rice *et al*., 2005).

Organic load also affects multiple species biofilm (MSB): previous experiments have shown that increased organic load results in enhanced growth rate and biomass in complex waste water treatment (WWT) biofilms (Tal *et al*., 2003, Yung-pin, 2005). Furthermore, several studies have shown that organic and nutrient load have an effect on microbial diversity (Hewson *et al*., 2003, Barton *et al*., 2004). For example, Li *et al*. (2008) showed that changes in organic load lead to differences in biofilm morphology, structural properties and bacterial community composition in granules of sequenced batch reactors. Therefore, nutrient concentration and organic load have a strong effect on both SSB and MSB.

1.1.2 Multiple species biofilm- community composition and interactions

Although SSB experiments are important for understanding basic concepts in biofilm development, they do not portray the complex dynamics characteristic of the MSB found in nature. Interspecies relationships including competition (Omil *et al*., 1998, Tsuno *et al*., 2002), quorum sensing (Dobretsov *et al*., 2009) and the production of different compounds by other bacteria (Rao *et al*., 2005) may affect the biofilm community composition and function. Indeed, dual-species biofilm formation assays illustrated that combinations of two bacterial species can cause either complementation or antagonism in biofilm biomass (Mengying *et al*., 2007 and Rao *et al*., 2005). Bench-scale experiments using mixed cultures of isolated bacterial species can give a better understanding of multi-species bacterial interactions in complex biofilms (Rao *et al*., 2005, Burmølle *et al*., 2006, Jamal *et al*., 2006, Hansen *et al*., 2007, Andersson *et al*., 2008). Also, further investigation of naturally occurring biofilms in terms of bacterial community composition and function may lead to comprehension of environmental biofilms.

1.2 The role of biofilms in wastewater treatment (WWT) systems

Most functional wastewater treatment (WWT) systems depend on naturally occurring microorganisms that are responsible for organic carbon degradation and nutrient cycling (Daims *et al*., 2006). Although microorganisms involved in WWT processes are traditionally assayed in liquid media, it is now clear that most of the active microorganisms in these systems are located in attached or flocculent biofilms (Wuertz *et al.,* 2003). Investigation of these biofilms has obvious applied significance since wastewater cleanup is important to public health and to the ecological system as a whole.

The damage caused by releasing treated wastewater into different water bodies can be reduced by advance wastewater treatments producing high quality effluent. Also, the quality of the treated wastewater is essential in countries like Israel, that have limited water resources and use treated wastewater for irrigation (Smakhtin, 2004, Navon *et al*., 2011).

1.2.1 Biofilm in wetland system for WWT

Constructed wetlands are becoming increasingly popular as economical and environmentally friendly solutions for WWT. Although natural wetlands exist around the world, the use of constructed or artificial ones, built for the improvement of water quality, is a concept that has only been adopted in the last five decades (Vymazal, 2011). As the water flows through the wetland, it is treated by physical (sedimentation, filtration, UV exposure), chemical (precipitation, adsorption, volatilization), and biological (microbial degradation, microbial nutrient transformations, uptake from water column and root zone, microbial competition, and bacterial die-off) processes (Vymazal, 2011). Microbial biofilms attached to the wetland matrix (solid particles and/or plant roots), are responsible for most of the essential transformations and decomposition of contaminants in the wastewater (Larsen and Greenway, 2004, Faulwetter *et al*., 2009). Still, relatively little is known about the bacterial populations involved in the formation and activity of this biofilm (Truu *et al*., 2009). Bacteria originating from different phyla were found in a variety of wetland systems; Ahn *et al*. (2007) characterized microbial communities in planted and unplanted constructed wetland microcosms with different phosphorus loading. The microbial community of that wetland microcosm sediment was dominated

by *α- Proteobacteria* (48–60% of clones) and second in abundance were bacteria related to *Actinobacteria* and *Firmicutes* (Ahn *et al*. 2007). Ibekwe *et al*. (2007) examined sediments and rhizosphere from surface flow constructed wetland system and showed that the majority of obtained sequences belonged to unclassified taxa, while the second dominant group consisted of members of the *Proteobacteria*.

The wetland is a very dynamic and variable system with respect to its physical and chemical properties. This variability is a result of different gradients of redox, substrate availability and environmental conditions, such as pH and temperature. The different gradients create variable niches throughout the wetland ponds, in which different biochemical processes take place (Reddy and Dangelo, 1997, Scholz and Lee 2005). Several studies investigated the effect of different wetland conditions on the microbial community composition of the wetland biofilm. Truu *et al* (2005) have successfully assessed microbial community structure in different layers of planted soil wetland for domestic wastewater treatment. In that study, depth affected the microbial community structure of the wetland biofilm with respect to communities of bacteria (among them, ammonia oxidizing bacteria) and Archaea. In contrast, presence of different substrate materials or vegetation did not significantly affect wetland microbial community composition (Osem *et al*., 2006, Silyn-Roberts and Lewis, 2004). Also, biofilm biomass and activity of different wetland systems were influenced by organic matter (Nguyen, 2000), surface properties (Ladd *et al*., 2004) and depth (Tietz *et al*., 2007). Hence, physical and chemical properties of the wetland may influence biofilm assembly and function.

1.2.1.1 Different types of constructed wetlands

Constructed wetlands may be categorized according to the various design parameters, but the three most important criteria are hydrology (open water-surface flow and subsurface flow), type of macrophytic growth (emergent, submerged, free-floating) and flow path (horizontal and vertical) (Kadlec and Knight, 1996).

Constructed wetlands with surface flow (FWS CWs)- contain areas of open water and floating, submerged, and emergent plants. The shallow water depth, low flow velocity, and presence of the plant stalks and litter regulate water flow and, especially in long, narrow channels, ensure plug-flow conditions. The FWS CWs are very effective in removal of organics through microbial degradation and removal of suspended solids through filtration and sedimentation. Removal of nitrogen is variable and sustainable removal of phosphorus is also performed at relatively slow rates (Vymazal, 2011).

Horizontal subsurface flow constructed wetlands (HF CWs)- the wastewater is fed in at the inlet and flows slowly through the porous medium under the surface of the bed planted with emergent vegetation to the outlet where it is collected before leaving via a water level control structure. During passage the wastewater comes into contact with a network of aerobic, anoxic, and anaerobic zones. Most of the bed is anoxic/ anaerobic due to permanent saturation of the beds. Therefore, Removal of ammonia-N, hence nitrification, is limited by the lack of oxygen in the filtration media. HF CWs, however, provide suitable conditions for denitrification. Macrophytes planted in HF CWs have several properties that make them an essential component of the design; the most important properties are filtration bed insulation during the winter, substrate for growth of attached bacteria, oxygen release to the rhizosphere, nutrient uptake and storage, and

root exudates with antimicrobial properties. HF CWs are very effective in removal of organics, suspended solids, microbial pollution, and heavy metals. Removal of phosphorus is low unless special media with high sorption capacity are used (Vymazal, 2009).

Vertical flow constructed wetlands (VF CWs) - comprise a flat bed of graded gravel topped with sand planted with macrophytes. Contrary to HF CWS, VF CWs are fed intermittently with large batches, thus flooding the surface. Wastewater then percolates down through the bed and is collected by a drainage network at the bottom. The bed drains completely which allows air to refill the bed. Thus, VF CWs provide greater oxygen transfer into the bed, thus producing a nitrified (high NO3 -) effluent. On the other hand, VFCWs do not provide suitable conditions for denitrification to complete conversion to gaseous nitrogen forms which escape to the atmosphere. Removal of organics and suspended solids is high. As compared to HF CWs, vertical flow systems require less land. One of the major problems with efficient performance of VF CWs is clogging of the filtration substrate. Therefore, it is necessary to select the filtration material carefully, distribute the wastewater evenly across the wetland surface, and also select the optimum hydraulic loading rate (Vymazal, 2011). In the current study we used VF CWs since it is an experimental setup which requires less land area than other CW systems.

1.2.1.2 Wetlands aimed at water polishing

A wide range of wastewater qualities are being treated by wetland systems (Kadlec and Knight, 1996), the most common being municipal wastewater with only limited pretreatment. 'Water polishing', a post-treatment of effluents from activated sludge plants,

is recently gaining more attention as a means to further improve effluent quality (Toet *et al*., 2005). This effluent is characterized by relatively low chemical and biological oxygen demand values and moderate N and P concentrations. In such cases, the purpose of the purification system may not be primarily the reduction of nutrients, but rather a reduction in the impact of periods of high discharge (peaks of pathogen and contaminants which were not properly reduced in the sewage-treatment plants (STPs)) and reduction of micro-pollutants which does not degrade well in WWT systems (Toet *et al*., 2005, Matamoros *et al*., 2008).

1.3 Endocrine disrupting chemicals (EDCs) residues in the environment

In recent years, there has been increasing concern over estrogenic effects on aquatic fauna due to endocrine disrupting chemicals (EDCs) residues in treated wastewater. EDCs residues are found in STPs effluent due to insufficient removal in WWT systems (Servos *et al*., 2005, Stumpe and Marschner, 2007, Liu *et al*., 2009, Pacáková *et al*., 2009). These residues are reaching different water bodies (Auriol *et al*., 2006) and also may contaminate soils as a result of irrigation with effluent of STPs (Stumpe, 2007). For example, Kinney *et al*. (2006) found that measurable but low concentrations of pharmaceuticals can be detected in soil irrigated with reclaimed water from STPs. In Israel, which is a semi arid country, over 50% of the water used for agriculture irrigation is reclaimed wastewater (Navon *et al*., 2011) and therefore EDCs residues might be found in these effluents- irrigated soils (Stumpe, 2007).

Several studies showed that effluent of STPs contain sufficient amount of EDCs to induce harmful effects on the endocrine system of different organisms (Pacáková *et al*.,

2009). The major contribution to the estrogenicity in these effluents has been shown to be related to the presence of natural and synthetic estrogens (Desbrow *et al*., 1998, Routledge *et al*., 1998).

1.3.1 Estradiol and estrone

The natural sex hormone 17β-estradiol (E2) (Fig. 1), which has a critical impact on reproductive and sexual functioning (Jobling *et al*., 2002), is the most potent estrogen pollutant and is ubiquitous in many aquatic systems (D'Ascenzo *et al*., 2002, Pacáková *et al*., 2009). E2 has been shown to be partly or fully metabolized in different WWT systems (for review see Auriol *et al*., 2006). However, estrone (E1) (Fig. 1), one of the byproducts of E2 metabolism which retains estrogenic potency, is accumulated (D'Ascenzo *et al*., 2002, Auriol *et al*., 2006). Therefore, mere transformation of E2 to E1 is not sufficient for environmental purposes, and the removal of these two estrogenic pollutants (E2 and E1) is preferable. Degradation pathways of estrogens, including E1 and E2 in mammalian cells are well documented (for review see Ting Zhu, 1998). Different enzymes involved in estrogen degradation were identified, including hydroxysteroid dehydrogenases (Kitawaki *et al*., 2000, Mindnich *et al*., 2004) and cytochrome p450 (Ohe *et al*, 2000, Thompson and Ambersone, 2000, Cribb *et al*., 2006). Also, some intestinal microorganisms are able of anaerobic degradation of steroids (see review Groh *et al*., 1993). The enzyme 17β hydroxysteroid dehydrogenase, which transforms E2 to E1 (Mindnich *et al*., 2004), was isolated from different bacterial species such as *Pseudomonas sp.* (Talalay and Dobson, 1953, Schultz *et al*., 1977, Nakamura *et al*, 2006), *Alcaligenes sp.* (Payne and talalay, 1985), *Mycobacterium sp.* (Egorova *et al*.,

2002) *and Cochlilobolus lunatus* (Brannon *et al*., 1967). Furthermore, numerous phylogenetically diverse bacteria with the ability to transform E2 to E1 by 17β reduction were isolated from different environments (Fujii *et al*., 2003, Yoshimoto *et al*., 2004, Shi *et al*., 2004, Donova *et al*., 2005, Lucas and Jones, 2006, Hashimoto *et al*., 2010). Some of these bacteria were also able to degrade E1 (Yoshimoto *et al*., 2004, Shi *et al*., 2004., Fujii *et al*., 2003, Hashimoto *et al*., 2010). However, little is known about the mechanism of E1 degradation by bacteria in the environment (Rowley et al., 2003, Sang *et al*., 2011).

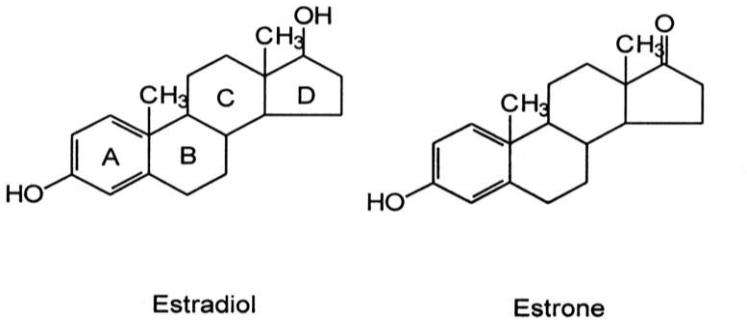


Fig. 1: Chemical structures of 17β-estradiol and estrone.

1.4 Bioaugmentation for estrogen bioremediation

Pollutant removal can be enhanced by the introduction of specific strains or consortia of degrading microorganisms to a contaminated site, termed bioaugmentation (Morikawa, 2006). Bioaugmentation is used as an *in-situ* bioremediation technique (Alexander, 1999) and is considered a green technology. Crucial criteria for the efficiency of bioaugmentation are survival of the added bacteria and the *in-situ* degradation rate of the contaminant (El Fantroussi and Agathos, 2005, Götz *et al*., 2006). Studies have shown successful degradation of pollutants by bioaugmentation, such as petroleum hydrocarbon (Mishra *et al*., 2001), dehydroabietic acid (Yu *et al*., 2002), phenol (Selvaratnam *et al*.,

1997), atrazine (Kim, 2003, Runes *et al*., 2001) and recently, successful bioaugmentation of estradiol degrading bacteria into conventional activated sludge (Hashimoto *et al*., 2010). Therefore, it might be feasible to reduce estrogenic pollutants by bioaugmentation in other WWT systems and even in soil. Bioaugmentation can alter the potential activity as well as the composition of naturally occurring microbial biota during bioremediation of a contaminated site. Introducing a new bacterial strain into native microbial communities can cause changes in the complex interactions of different bacterial groups (Niu *et al*., 2009).

Objectives

The general purpose of this study was to examine the bacterial composition and activity of constructed wetland's biofilm. We investigated the influence of a-biotic (depth, wetland matrix and organic matter) and biotic parameters (vegetation presence and augmentation of E2 degrading bacterium) on the microbial composition of the biofilm. In addition, we investigated the effect of bioaugmentation on bacterial composition and activity in an additional system (soil column).

Specific aims:

- Defining the microbial composition and activity of an existing wetland-microcosm's biofilm which developed under wetland conditions.
- Studying the initiation and development of biofilm under laboratory conditions as affected by wetland organic load (water quality).
- Isolating and characterizing estradiol degrading bacteria.

- Studying the integration of estradiol degrading bacteria into biofilm under laboratory conditions. Examining the effect of these bacteria on biofilm community composition and function.
- Studying the integration of estradiol degrading bacteria into soil column. Examining the effect of these bacteria on soil microbial community composition and function.

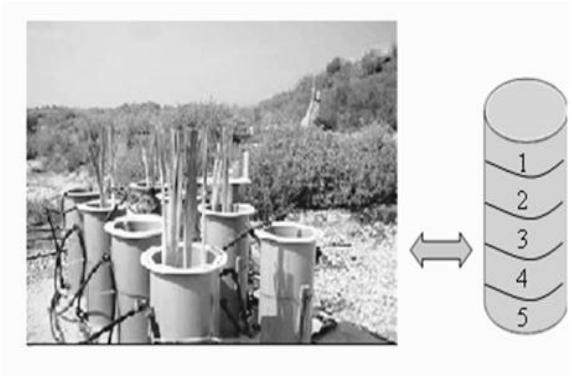
2. Methodology

2.1 Wetland and microcosm design

An experimental wetland, designed for post treatment of effluent wastewater, was constructed at the Tel-Aviv metropolitan SHAFDAN wastewater-treatment plant (Israel). A set of seven wetland ponds of about $30m^2$ diameters each were designed with vertical sub-surface flow treatment, two of these ponds were un-planted, two ponds were planted with *Typha angustifolia* and three other with a mixture of *Cyperus papyrus*, *Canna sp.*, *Iris pseudoacorus, Phragmites australis* and *Juncus ensifolius*. Another set of seven ponds with the same characteristics were constructed and connected vertically to the first set of ponds, resulting in two treatment stages: the treated effluent water from the first set of ponds (early treatment) is the influent water for the second set of ponds (advanced treatment). Each pond was constructed of five layers made from different sizes and types of gravel, of which the upper (layer 1, basalt gravel size 20-30 mm) and lower (layer 5, dolomite gravel size 50-60 mm) were designed for stabilization and clogging prevention. Layers 2-4 were made of smaller gravel size (basalt 2-3 mm, dolomite 8-10 mm and basalt 8-10 mm, respectively). The variability of the wetland layers, as designed by constructed wetland engineers, is detailed in Fig. 2.

Since the wetland is gravel bedded it was not possible to test core samples of the different ponds layers. For this reason, acrylic cylinder microcosms (80 cm high with 33 cm diameter) were constructed and stationed near the wetland ponds, receiving influent at identical rates from the same reservoir. The microcosms were designed with identical conditions and operation parameters as the first set of wetland ponds (type of gravel,

depths of each layer and presence versus absence of vegetation (*Typha angustifolia*, Fig. 2)).



Gravel type	Porosity	Gravel size (mm)	Depth in column (cm)
Basalt	0.47	20-30	10
Basalt	0.43	2-3	25
Dolomite	0.47	8-10	35
Basalt	0.46	8-10	45
Dolomite	0.48	50-60	55

Fig. 2: Structure of the wetland microcosm. Column system corresponding to the wetland ponds at the SHAFDAN experimental site. The gravel layers are arranged within the microcosm in identical layering to that of the wetland ponds.

Both wetland ponds and microcosm systems were kept unsaturated and fed for 15 min in intermittent mode every four hours (hydraulic load of 36 l/day per microcosm or 3000 l/day per pond).

2.2 Sampling procedure

2.2.1 Samples of biofilm originating from wetland microcosms

The microcosms were disassembled after three months of operation and triplicate samples of biofilm developed on gravel were taken from the center of each layer (layers 1-5). Gravel samples from the vegetated microcosm were manually separated from the roots. 0.5 g of gravel with developed biofilm from layers 2-4 was sampled for DNA extraction (see 2.5). Also, biological material was scraped off the gravel from layers 1

and 5 for DNA extraction. The gravel was vigorously scraped with a plastic brush and sterile double distilled water (DDW), resulting in biofilm removal (and parts of the upper layers of the gravel). Due to the gravel size, microbial abundance and activity were only measured for layers 2-4 (see 2.10).

2.2.2 Samples of biofilm originating from wetland ponds for bioaugmentation investigation

In order to investigate bioaugmentation process, biofilm samples were obtained from two wetland ponds. Basalt gravel with developed biofilm was sampled from the second layer of pond 1 (un- planted pond from the first set of ponds) and 2 pond (un- planted pond from the second set of ponds). These two ponds representing two treatment stages: the treated effluent water from pond 1 (early treatment) is the influent water for pond 2 (advanced treatment). The influent being treated in pond 1 and 2 had average BOD of 5.5 and 1.9 mg L^{-1}, respectively.

2.2.3 Water samples

Influent and effluent samples were collected in sterile 50-ml tubes and analyzed in the field (collaboration with Avital Gasith's laboratory, Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel, Milstein, 2010) for salinity, using YSI 85 Multiparameter meter (Geo Scientific Inc., Vancouver, Canada), and pH, using an IQ Scientific pH meter (Carlsbad, CA). Also, influent and effluent samples were collected in sterile 50-ml tubes and stored in a dark, cool environment untill analyzed in the laboratory (no longer than three hours) for ammonium (NH_4-N), nitrate (NO_3-N) and phosphate (PO_4-P) by LASA 100 kit (HACH LANGE

Ltd., Manchester, UK). Total nitrogen, biological oxygen demand (BOD) and total suspended solids (TSS) were measured according to standard methods of the American Public Health Association (methods #4500-NorgD, #5210B and #2540D, respectively) (Eaton *et al*., 2005).

Coliforms concentration was measured as colony forming units per milliliter (cfu mL^{-1}) by plating the influent and effluent water from the different microcosms and wetland ponds on mEndo Agar LES (Voigt Global Distribution Inc., Lawrence, KS) and incubation in $37^{O}C$ over night. These results gave us an indication of the CW ability to remove bacteria. 2 ml of water samples were taken for DNA extraction in order to examine bacterial community composition.

All samples were collected three times in April and May 2008 (after 3 years operation of the ponds and three months operation of the columns).

2.3 Bacteria isolation from wetland biofilm

2.3.1 Isolates for investigation of biofilm initiation

In order to investigate biofilm development under laboratory conditions different species of bacteria, which differed in colony morphology, were isolated on 1/2 Luria Broth (LB) plates directly from biofilm obtained from the study site and incubated in $30\ ^{O}C$ for a 1-3 days until visualizing of different colonies morphology was possible. These conditions were used in order to isolate relatively fast growing bacteria with different morphologies which enable a simple experimental system.

2.3.2 Estradiol degrading bacteria (EDB) isolation

In order to isolate EDB, an enrichment culture using E2 as a sole carbon source was used. EDB enrichment was performed by inoculating biofilm samples into minimal medium with and without (control) 50 mg L^{-1} E2 as sole carbon source and incubated in 30 ^{O}C with shaking at 200 rpm. The enriched culture was transferred (at 1:10 dilution) into new medium every 3 weeks. DNA was extracted from samples taken from each transfer and the bacterial community composition was analyzed by denaturing gradient gel electrophoresis (DGGE) of the amplified fragment of the 16S rRNA gene.

Potential EDB were isolated by plating aliquots from the 5th transfer of the enrichment culture on minimal medium agar containing E2 above its soluble concentration (50 mg L^{-1}), resulting in an opaque agar plate. Colonies forming a clearing zone on the agar plates were inoculated into fresh liquid minimal medium containing 50 mg L^{-1} E2 in order to examine their E2 degradation ability. Selected isolates, which degrade E2, were further characterized for their phylogenetic affiliation by sequencing the 16S rRNA gene. The isolate EDB-LI1, exhibiting the most efficient removal of E2 and E1, was selected for further study.

2.3.3 Hydrophobicity test for isolated bacteria

The bacterial adhesion to hydrocarbons (BATH) test (Rosenberg *et al*., 1980) was performed for each of the isolates to determine their cell hydrophobicity. Liquid culture of each isolate was grown in 1/2 LB, with shaking, at 30^{o}C overnight. The culture was centrifuged at 5700 X g for 5 min, and the pellet was washed once with phosphate buffered saline (PBS: 130 mM NaCl, 10 mM Na_3PO_4, pH 7.2) and resuspended in PBS to

an A_{600} of 0.25 ± 0.05 in order to standardize the number of bacteria (10^7-10^8 cfu/ml); then an equal volume of hexadecane (Sigma, St. Louis, MO) was added. The two-phase system was vigorously vortexed for 5 min. The aqueous phase was removed after 1 h of incubation at room temperature and its A_{600} was measured. Affinity to hydrocarbons (hydrophobicity) was calculated according to the formula: $[(A_0- A)/A_0] *100$, where A_0 and A are the absorbance before and after extraction with hexadecane, respectively.

2.4 Preparation of growth media

2.4.1 Mineral medium

Modified minimal medium according to Vader *et al*. (2000): 1.5 g L^{-1} $(NH_4)_2SO_4$, 0.3 g L^{-1} NaCl, 0.3 g L^{-1} K_2HPO_4 and 0.05 g L^{-1} $MgSO_4*7H_2O$, with and without (control) 50 mg L^{-1} E2 as sole carbon source.

2.4.2 Different organic load media

Different organic loads media were used as a foundation for biofilm development: high (0.5 g L^{-1} tryptone, 0.25 g L^{-1} yeast extract and 0.25 g L^{-1} NaCl), medium (0.05 g L^{-1} tryptone, 0.025 g L^{-1} l yeast extract and 0.25 g L^{-1} NaCl), low (0.01 g L^{-1} tryptone, 0.005 g L^{-1} yeast extract and 0.25 g L^{-1} NaCl) and no organic load (tap water with 0.25 g L^{-1} NaCl).

Bioaugmentation examination was done in Luria Broth (LB) medium (2.5 g L^{-1} NaCl, 2.5 g L^{-1} yeast extract and 5 g L^{-1} tryptone), which was diluted to 1/100 or 1/500 in order to create artificial effluent and adjust the organic matter to similar BOD levels found in the influent of pond 1 and 2, respectively, as described by Liu *et al*., (2000).

2.5 DNA extraction

Total DNA was extracted from samples of 0.5 g of gravel/soil. Extraction of DNA was carried out using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) as described by the manufacturer. DNA concentrations were determined using the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was suspended in 60 µl TE (Tris-EDTA pH 8.0 buffer, Amresco Inc., Solon, USA) and stored at -20°C until further processing.

2.6 Primers and Polymerase chain-reactions

All polymerase-chain reactions were conducted on a T-Gradient thermocycler (Biometra, Goettingen, Germany) and performed in sterile 0.2 mL polypropylene tubes. PCR amplifications for DGGE analysis of the gene coding for the 16S rRNA were performed with the primer pair Eub341 (with a GC clamp at its 5' end) and Univ907 (Table 1). Each 50-µl reaction mixture contained the following: 1.5 U Taq DNA polymerase (Red Taq; Sigma, St. Louis, MO), Taq buffer containing a final magnesium concentration of 2.5 mM, dNTPs (20 nmol each), 12.5 µg bovine serum albumin, 25 pmol of each primer, and 1.2 µl DNA template. The PCR program was carried out with an initial denaturation step of 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The program was completed with a final elongation step at 72°C for 10 min. PCR products were resolved by agarose gel electrophoresis (1%).

The different primers used in this study are specified in Table 1. All primers were synthesized by Integrated DNA Technologies, Inc. (Iowa, USA).

Table 1. List of primers used in this study.

Gene/ region	Primer	Direction	Sequence	Reaction	Reference
16S	Eub341	forward	CGCCCGCCGCGCCCCGCGCCC GTCCCGCCGCCCCCGCCCGCC TACGGGAGGCAGCAG	PCR-DGGE	Muyzer *et al.*, 1998
16S	Univ907	reverse	CCGTCAATTCMTTTGAGTTT	PCR-DGGE and qPCR	Muyzer *et al.*, 1998
16S	Eub519	forward	CAGCMGCCGCGGTAANWC	qPCR	Lane, 1991
16S	EDB632	forward	GATAGCTAGAATCCTGGAGAGGC	qPCR	This study
16S	EDB1200	reverse	CCTCGCGAGGTTGCTGCC	qPCR	This study

The EDB-LI1-specific primers were designed by aligning (using clustalW program) the EDB-LI1 16S rRNA gene sequence with the hundred closest *Sphingomonadaceae* 16S rRNA sequences, found in GenBank using Blastn. The most variable area of the sequence was chosen to design specific primers for EDB-LI1, and verified for specificity by BLAST. PCR amplification of DNA obtained from several bacterial species (EDB-LI1, *Escherichia coli Dhα-5* (laboratory strain), *Erythrobacter* clone A6T_UNP4_B1 (GU319422) and *Sphingomonas* clone A4T_UNP2_A2 (GU319237) (both clones originated from the gulf of Eilat)) was attempted with these primers. Only DNA extracted from EDB-LI1 was amplified at an annealing temperature of 64^{o}C. Therefore, 64^{o}C was chosen as the annealing temperature for qPCR.

2.7 Denaturing gradient gel-electrophoresis (DGGE)

DGGE was performed in 6% (w/v) acrylamide gels containing a linear urea-formamide gradient ranging from 30 to 60% denaturant (with 100% defined as 7 M urea and 40% (v/v) formamide). Gels were run for 17 h at 100 V in the Dcode Universal Mutation System (Bio-Rad Laboratories, Hercules, CA). DNA was visualized after staining with Gelstar (Invitrogen Corporation, Carlsbad, CA) by UV transillumination (302 nm) and photographed with Kodak KDS digital camera (Kodak Co., New Haven, CT).

2.7.1 Cluster analysis of DGGE community fingerprints

DNA fingerprints obtained from the 16S rRNA gene banding patterns on the DGGE gels were digitized by using Fingerprint®II Informatix software (Bio-Rad Laboratories). The lanes were normalized to contain equal amounts of total signal after background subtraction. The cross comparison was done using a marker designed in our laboratory by combining PCR products of selected fragments with a broad migration range. A neighbor-joining tree based on UPGMA (unweighted pair-group method using arithmetic average) dendrogram using the Dice coefficients was produced. Dominant bands were excised and purified (see below) for the identification of bacterial species (see 2.8). In order to further analyze associations between the bacterial community patterns, non-metric multidimensional scaling (NMDS) analysis was performed using pair-wise 1- Pearson r correlations of densitometric curves obtained from the DGGE patterns. This analysis allows grouping of individual patterns avoiding the misrepresentation of distances inherent to hierarchical cluster analysis methodologies. The low stress value given for the NMDS

analysis (0.014) indicated that the reproduced distances reliably represent the original pair wise distances.

2.7.2 Comparisons of bacterial community compositions

Bacterial community PCR-DGGE patterns were aligned using the Fingerprint®II software. Densitometric curves were then imported to STATISTICA®, and Pearson r correlation matrices were calculated. Matrices were resolved in two methods: 1) Hierarchical clustering based on neighbor-joining and UPGMA algorithms, and 2) Non-metric multidimensional scaling (nMDS) analysis.

2.8 Phylogenetic analysis of sequences obtained from selected DGGE bands

Excised DGGE bands were placed into sterilized vials with 20 µl sterile distilled water and 2 mm diameter glass beads. The vials were shaken in a bead beater (Bio 101, Irvine, CA) at 4.5 S for 20 s and then incubated for 30 min at 37°C to allow the DNA to diffuse out of the homogenized gel strips. One microliter of eluted DNA was used as the DNA template for PCR amplification with the bacterial primers. The PCR product was ligated into the pCR2.1 TOPO TA plasmid (Invitrogen, Carlsbad, CA) and cloned into *Escherichia coli* DHα-5. The inserts were re-amplified by PCR and compared to the environmental samples by DGGE to verify their correct position in the pattern. Plasmids were purified from *E. coli* using a HiYield plasmid mini kit (Real Biotech Corporation, Taipei, Taiwan) and the cloned fragments were sequenced by Macrogen Inc. (Seoul, Korea). The sequences were aligned to the 16S rRNA prokMSA database using the

NAST server (DeSantis *et al*., 2006). The alignment was used to construct a neighbor joining phylogenetic tree with Mega 3.1 software (Kumar *et al*., 2004).

2.9 Quantitative real-Time PCR

qPCR was performed to quantify the bacterial population in the biofilm. Both a general bacterial primer set (Eub519F and Univ907) and a specific primer set designed here for EDB-LI1 (EDB632F and EDB1200R) were employed (Table 1). qPCR reaction mix (20 µl) contained 10 µl Absolute Blue SYBR green ROX mix (Thermo Fisher Scientific Inc., Wilmington, MA), 0.5 pmol µl-1 of each primer, 1 µl DNA and 7.5 µl ddH2O. Three pseudo-replicates were used for each sample. Amplicon length was approximately 570 bp. Negative controls (containing all reagents except target DNA) and 10-fold serial dilutions of positive control DNA (plasmid containing the whole 16S rRNA gene of EDB-LI1) were included in each run. DNA amplification and quantification were performed using an MX 3000 Real Time PCR system (Stratagene, La Jolla, CA). Each qPCR consisted of the following steps: 15 min initial denaturation at 95°C to activate the polymerase, followed by 45 cycles of 30 s denaturation at 95°C, 30 s annealing at 64°C, and 30 s elongation at 72°C. Efficiency of qPCR of EDB-LI1 and total bacteria were 105.1% and 96.4% and slope (dR) was 3.206 and 3.416, respectively. No-template control (NTC) background was equal to 200 copies.

2.10 Microbial biomass and activity of biofilm obtained from microcosms

Total biomass was estimated by measuring protein concentration using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL), as previously described (Ragusa *et al*., 2004). General microbial hydrolytic activity was measured by hydrolysis of fluorescein diacetate (FDA) (Schnürer and Rosswall, 1982). Briefly, 5 g of gravel with biofilm was incubated at 30^{o} C with phosphate buffer (0.5 mM, pH= 7.6) and 100 µl of FDA (2 mg/ml). After three hours of incubation, the samples were centrifuged (1 min, 13000 g) and the supernatant was analyzed in a spectrophotometer (494nm). Nitrification potential was estimated by potential ammonium oxidation (Weaver *et al*., 1994).Twenty g of biofilm and gravel samples were incubated at 30^{o}C with shaking in 90 ml of sterile phosphate buffer (0.5 mM, pH= 7.6), 0.2 ml of $(NH_4)_2SO_4$ (0.25 M) and 0.1 ml of $KClO_3$ (1 M). Aliquots of 0.5 ml were taken every two hours and 0.05 ml of merthiolate (1% w/v) was added in order to stop the reaction. Determination of the nitrite concentration was done by a Quikchem 8000 flow injection analysis system (Lachat instrument, Loveland, CO, USA).

2.11 Estrogen analysis by HPLC

To study removal of E2 and E1, 1 g of biofilm formed on gravel or 5 µl liquid culture from EDB-LI1 over-night starter was incubated at 30^{o}C with 1 ml of minimal medium (described above-2.4.1), containing 50 or 100 mg L^{-1} E2. E2 was extracted by adding 1 ml acetonitrile and incubating for 2 h at 30^{o}C with shaking. The extract was filtered (0.45-µm cut off, Durapore membrane, Millipore, Dublin, Ireland) and analyzed by

HPLC (Shimadzu, Columbia, MD) in an Apollo C18 column (Alltech, Deerfield, IL). The HPLC protocol was modified according to Weber *et al*. (2005) (mobile phase: 60% acetonitrile and 40% double-distilled water (Millipore), flow rate: 0.5 ml min^{-1}. Extraction efficiency was calculated by comparing values of known E2 and E1 concentrations of the extracts to the same concentrations of hormones directly dissolved in acetonitrile. Extraction efficiency was 90%.

2.12 Experiments and experimental designs

In order to allow for detailed examination of biofilm assemblage, a simple model of static incubation was used. 24-h-old biofilm development was tested under laboratory conditions with bacteria isolated from CW biofilm.

2.12.1 Biofilm colonization assay

24-h-old biofilm development was tested under laboratory conditions. The biofilm was constructed by seven bacterial isolates (differed in colony morphology), obtained from wetland biofilm. These isolates were grown separately in 1/2 LB, with shaking, at 30^{o}C overnight. Cultures were adjusted to equal quantities by determining their protein concentration with a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL). Correlation between protein measurements and CFU was checked and found to be constant in all isolates. Single (each isolate separately) - and multiple (all seven isolates together) - species cultures were suspended in sterile media with one of several different organic loads: high, medium, low and no organic load (see 2.4.2). In addition, single and multiple-species cultures were incubated with sterile (0.22μm filtrated) influent and effluent from the CW. Each of these media, containing the single- or multiple-species

cultures, were incubated with sterile basalt gravel (8 mm), identical to that used in the constructed wetland, in sterile tubes at 30^{o}C. After 4 h of incubation, the tubes were drained, and the gravel was gently washed twice with sterile saline (Distilled water with 4 g/l NaCl) and incubated for 20 h at 30^{o}C (forming 24-h-old biofilm) (appendix, Fig. 4). Sterile saline was added to the 24-h-old biofilm and the tubes were vortexed for 5 min. The community composition of the 24-h-old biofilm was studied by plating the liquid samples on 1/2 LB plates and counting the colony-forming units for each isolate based on colony morphology. The number of bacteria assembling the biofilm was determined by enumeration of the total and isolate-specific colony-forming units after their removal from the gravel.

The colonization assay was also used to investigate the process occurring during biofilm initiation in the presence of treated wastewater with its native bacterial community. Sterile basalt gravel (8 mm) was incubated with influent or effluent of SHAFDAN wetland ponds in sterile tubes at 30^{o}C for 4 h. At the end of the incubation period, the tubes were drained, and the gravel was gently washed twice with sterile saline and incubated for 20 h at 30^{o}C to allow the first colonizers, which established in the first four hours, to settle. DNA samples were extracted from the gravel for community composition analysis of bacterial colonizers.

2.12.2 Bioaugmentation assay to biofilm covered CW gravel

Bioaugmentation of the EDB-LI1 isolate into wetland biofilm samples originated from wetland ponds 1 and 2 was examined under laboratory conditions. LB diluted to 1/100 and LB diluted to 1/500, containing EDB-LI1 were incubated for 4 h at 30^{o}C in sterile

tubes with samples of basalt gravel with biofilm, originating from pond 1 or pond 2, respectively. At the end of the incubation, tubes were drained and the gravel was gently washed with sterile saline. The moist samples were then incubated for 20 h at 30^{o}C to allow the augmented colonizers to integrate into the CW biofilm. At the end of the incubation, 0.5 g of gravel with biofilm was collected from each tube for DNA extraction, and 1 g gravel with developed biofilm was examined for E2 removal by HPLC.

2.13 Soil column construction

Aluminum columns were used (30 cm in length, 3.1cm inside diameter) with aluminum screw caps, Teflon® tape for sealing and stainless steel fitting for connection to an HPLC pump with an injection loop of 1 mL as inlet, and to a 0.16 cm diameter stainless steel tube as outlet to a fraction collector (Spectra/Chrom fraction collector CF-2, Spectrum Labs) with silanised 10 mL glass tubes. Two stainless steel meshes with an opening size of 0.12 mm at the column inlet and outlet ensured minimum soil loss and optimum inlet and outlet flow conditions.

The column packing was started by introducing ~10 mL of background solution into the vertical column from the bottom. Air-dry Bet Dagan soil was then poured from the top into the solution in ~1 cm increments while being stirred and tapped to ensure uniform packing and to avoid air entrapment. The mean bulk density of the soil was 1.58 ± 0.02 g cm^{-3}. Bet Dagan soil is sandy clay with a clay content of 25.3%, 14% silt, 48.6% sand, 12.1% coarse sand, 0.5% organic matter and specific surface area 68 m²/g (Yaron *et al*.,

1989, Solel et al., 1979). The soil was sieved (1mm mesh) and air-dried at room temperature (22°C). The flow rate was kept constant at 1 mL min-1. Prior to the experiment, approximately 30 pore volumes (which correspond roughly to a total volume of 3 L) of background solution were introduced into the column to equilibrate the soil. E2 was injected at three different concentrations along with sodium fluorescein (concentration:1 mg L-1) at the bottom of the column as a 1 mL pulse, and time was set zero when the pulse reached the bottom of the soil column (appendix, Fig. 5). 1 ml was sampled every 10 min, which reflects 10% of the pore volume. These samples were used for estrogen measurement by LC-MS. All experiments were conducted at room temperature (20 °C ± 1 °C). Column de-packing was done by opening the aluminum screw caps and pushing the soil out of the column. Each 6 cm of soil, pushed out from the column, were mixed and determined as a layer, resulting in 5 layers representing different depths in the column (layer No. 5 is in the bottom of the column, which means it is closest to the inlet). 0.5 g of each layer was taken for DNA extraction (see 2.5).

2.14 Statistical analysis

The presented results are of representative experiments of three similar ones for each treatment. Each experiment was run in triplicates. Two - way ANOVA was used for comparison of the mean parameters of microbial activities and biomass of biofilm obtain from microcosms. T test was used for comparing the mean chemical parameters of influent versus effluent water and the differences in EDB-LI1 activity and biomass. For all tests, the significance level was set at 0.05.

3. Results

3.1 Efficiency of wetland ponds and microcosms constructed for polishing secondary effluent

An experimental constructed wetland (CW), designed for post treatment of effluent wastewater, was constructed at the Tel-Aviv metropolitan SHAFDAN wastewater-treatment plant (Israel). Since the CW is gravel bedded, it was not possible to test core samples of the different ponds layers. For this reason, acrylic cylinder microcosms were constructed identically to the CW ponds construction. Water samples from the influent and effluent of both the wetland microcosms and the experimental wetland ponds were analyzed for different water parameters (Tables 2 and 3, respectively) (Milstein, 2010).

Table 2. Water quality analysis of the influent and effluent of microcosms with and without vegetation

	Influent (reservoir water) mean ± S.D.	Effluent (gravel only) mean ± S.D.	Effluent (gravel and vegetation) mean ± S.D.
PO_4-P (mg L^{-1})	1.33 ± 0.25	1.59 ± 0.15	1.43 ± 0.17
NH_4-N (mg L^{-1})	3.17 ± 0.86	0.54 ± 0.08	0.48 ± 0.06
NO_3-N (mg L^{-1})	1.27 ± 0.26	4.08 ± 0.59	3.05 ± 0.54
Total N(mg L^{-1})	7.98 ± 0.88	7.30 ± 0.84	5.61 ± 0.81
BOD (mg L^{-1})	7.00 ± 1.28	3.46 ± 0.34	3.83 ± 0.66
TSS (mg L^{-1})	9.71 ± 1.90	1.75 ± 0.15	1.56 ± 0.19
pH	7.50 ± 0.06	7.48 ± 0.06	7.47 ± 0.06
Salinity(mg L^{-1})	0.84 ± 0.01	0.83 ± 0.01	0.83 ± 0.01
Coliforms (cfu mL^{-1})	1833± 441	310± 127	371± 100

Values are averages of three replicated microcosms sampled three times in the month before microcosm disassambly.

Several significant changes occurred in the water during infiltration through the microcosms, namely a 50% reduction in BOD and ca. 80% reduction in ammonia, TSS and coliforms. The nitrate concentration was increased by 280.

Table 3. Water quality analysis of the influent and effluent of wetland ponds after 3 years of operation with and without vegetation

	Influent (reservoir water) mean ± S.D.	Effluent (gravel only) mean ± S.D.	Effluent (gravel and vegetation) mean ± S.D.
PO_4-P (mg L^{-1})	1.37 ± 0.35	1.31 ± 0.26	1.36 ± 0.08
NH_4-N (mg L^{-1})	2.19 ± 0.37	0.09 ± 0.08	0.1 ± 0.15
NO_3-N (mg L^{-1})	0.93 ± 0.14	2.41 ± 1	3.18 ± 0.9
Total N(mg L^{-1})	4.4 ± 1.8	5.1 ± 2.3	4.9 ± 3
BOD (mg L^{-1})	5.55 ± 3.33	1.9 ± 0.75	2.03 ± 1.59
TSS (mg L^{-1})	7.9 ± 1.6	2 ± 0.22	1.8 ± 0.16
Coliforms (cfu mL^{-1})	850 ± 81	150 ± 41	141 ± 35

Values are averages of two or three replicated ponds sampled three times during one month after the CW was operational for 3 years.

No significant changes were found in total N values. Also, the microcosms did not significantly reduce PO_4-P, in agreement with reports on performance of other wetland systems (Greenway and Woolley, 1999; Jenssen *et al*., 1993; Watson *et al*., 1989). pH values of influent and effluent of the wetland microcosms remained constant, probably due to the system being well buffered (Table 2). Examination of the same parameters in effluents of the CW ponds revealed 73, 94, 83 and 51% removal of TSS, ammonia, coliform level and BOD, respectively (Table 3), similarly to microcosm performance.

Also, vegetation presence had no significant effect on the measured characteristics of effluents in both microcosms and wetlands ponds. Hence, it can be concluded that the microcosms functioned in the same way to the three year old wetland system, thus they can be considered as a valid wetland model.

3.2 Microbial population and activity in wetland microcosms

This part of the study focused on biofilm obtained from wetland microcosms after three months of operation. Microcosm disassembly allowed a thorough investigation of bacterial assembly and function in the different depths of the wetland microcosms.

3.2.1 Potential microbial activity of CW microcosms' biofilm

Layers 2-4 of the microcosms were examined for microbial fluorescein diacetate (FDA) hydrolysis activity and nitrification potential. The results are presented in figures 3 and 4, respectively.

Microbial FDA hydrolysis activity was significantly affected by depth of the microcosm and vegetation presence, but without interaction between these two parameters. The highest hydrolytic activity was measured in layer 2, which was ca. 3 fold higher than in layer 3, which exhibited the lowest FDA activity (Fig. 3). In addition, hydrolytic activity was lower in the presence of vegetation in all layers.

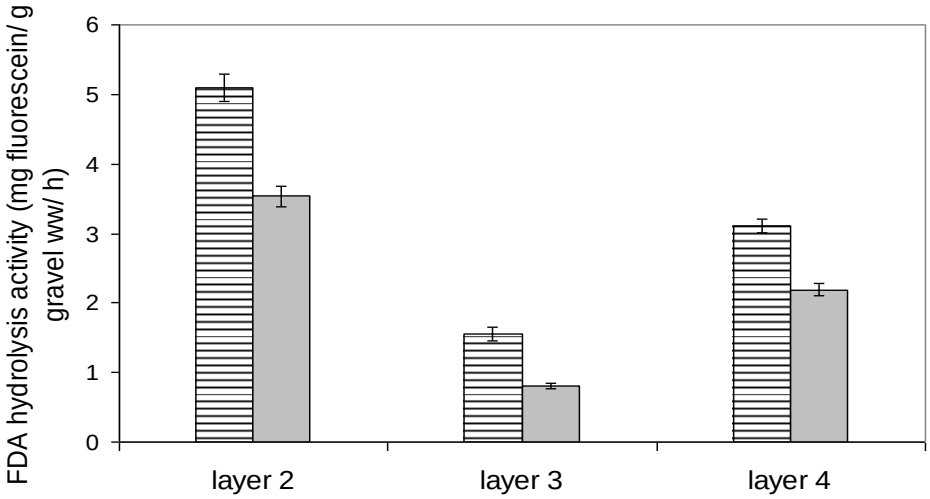


Fig. 3: Microbial hydrolysis activity in different layers of the microcosm with (gray) and without (streaks) vegetation. Values are averages of three replicated samples. Error bar represent standard deviation.

Nitrification potential was significantly higher in layers 2 and 3 in comparison to layer 4, with no influence of vegetation (Fig. 4). Layer 2 was composed of basalt gravel while layer 3 was composed of dolomite gravel, but no significant differences in nitrification activity were observed between these two layers. Hence, nitrification potential was found to be most affected by the depth of the layer and not by gravel type or presence of vegetation (Fig. 4).

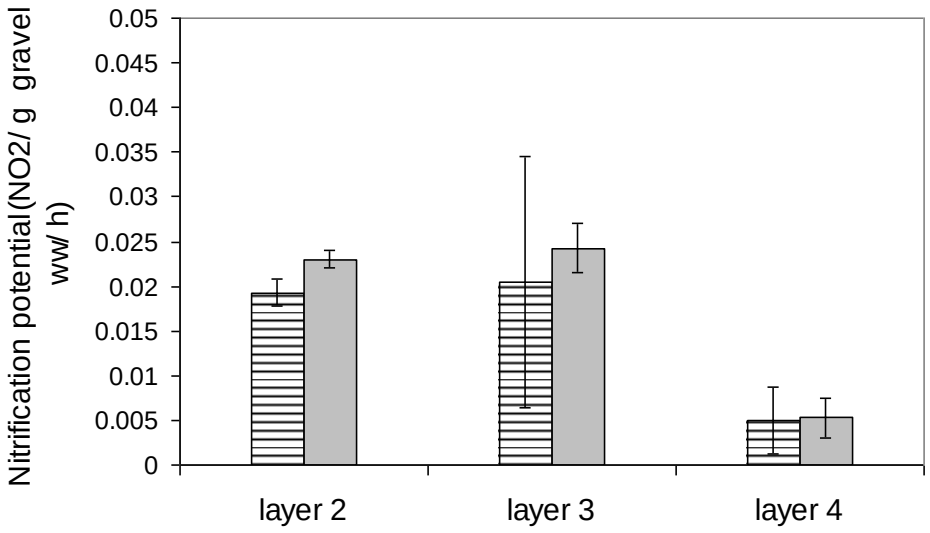


Fig. 4: Microbial Nitrification potential in different layers of the microcosm with (gray) and without (streaks) vegetation. Values are averages of three replicated samples. Error bar represent standard deviation.

3.2.2 Microbial abundance and community composition

As was explained in the Material and Methods section, layers 1-5 were examined for microbial community composition while layers 2-4 were examined for microbial biomass of biofilm obtained from wetland microcosms.

Microbial abundance of biofilm obtained from the microcosms was examined by quantification of protein content. Although the different surface areas and characteristics of the gravel types were different, significant differences were not found in biofilm biomass among any of the layers (Fig. 5).

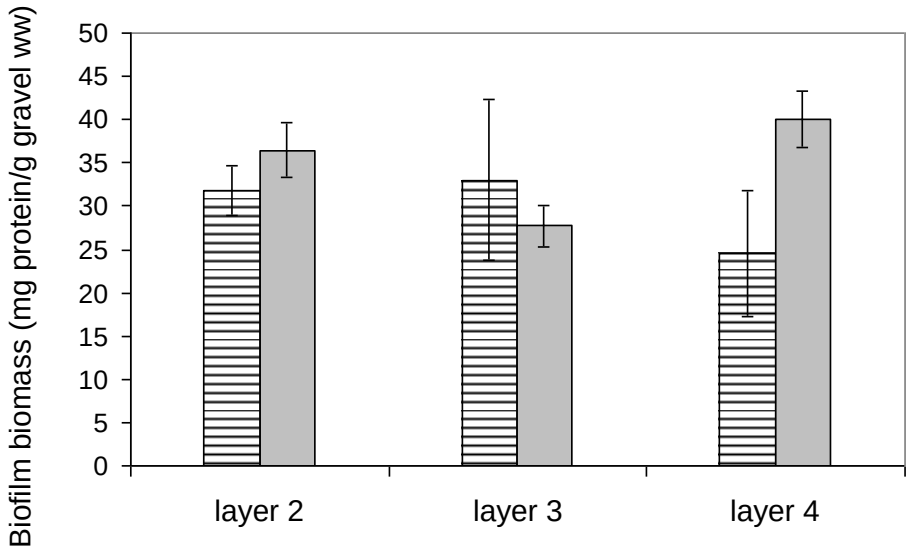


Fig. 5: Microbial biomass (measured by Protein content) in different layers of the microcosm with (gray) and without (streaks) vegetation. Values are averages of three replicated samples. Error bar represent standard deviation.

DGGE using a bacterial primer set was employed in order to examine community fingerprinting of biofilm obtained from the different layers of the microcosms. Cluster analysis performed on the different DGGE patterns (Fig. 6), demonstrated a strong effect of depth on community composition. The two upper layers (1 and 2) clustered together with ca. 70 percent resemblance and the lower three layers (3, 4 and 5) clustered in a separate branch, also with ca. 70 percent resemblance. Within the second cluster, the similarity of the two lower layers (layers 4 and 5) was even higher (ca. 75%). The bacterial community composition that developed on layer 4, which was composed of basalt gravel, was more similar to the dolomite layers above and below it than to the two upper basalt layers (Fig. 6).

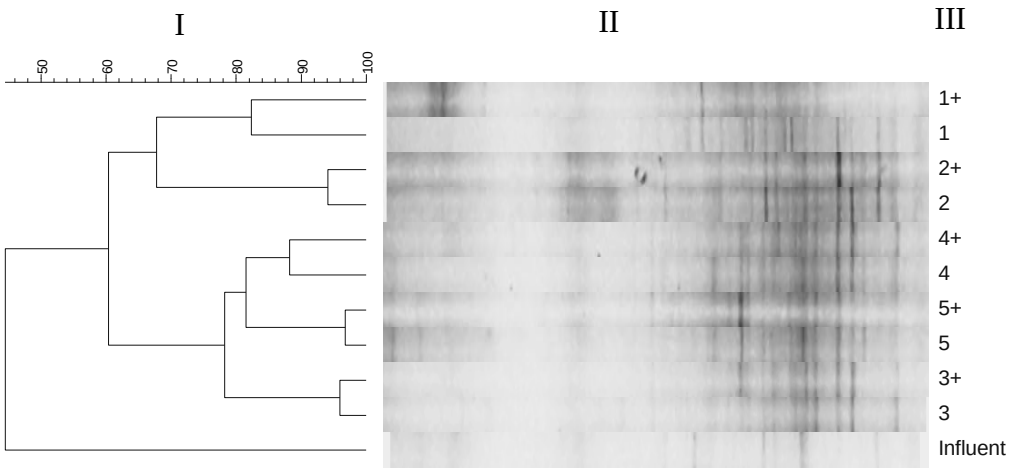


Fig. 6: Cluster analysis of the wetland biofilm bacterial community as affected by vegetation and depth in the microcosm. 16S rRNA gene fragments were amplified and used for DGGE analysis. The layers (1-5) and presence or absence of vegetation in the microcosm (+/-) are indicated. Cluster analysis (I) was performed using Fingeprint II software. UPGMA tree based on Jaccard similarity matrix between the different DGGE patterns (II) of different samples (III).

In the current study, vegetation did not significantly change the bacterial population of the microcosm biofilm formed on gravel. The community patterns obtained from identical layers of microcosms with and without vegetation were highly similar, excluding band no. 14 which will be discussed in below. Each two parallel depth profiles, with and without vegetation, were clustered together with 82-95% resemblance (Fig. 6). Dominant DGGE bands, and bands which were unique for a specific sample, were selected for sequencing (bands 1-19, Fig. 7).

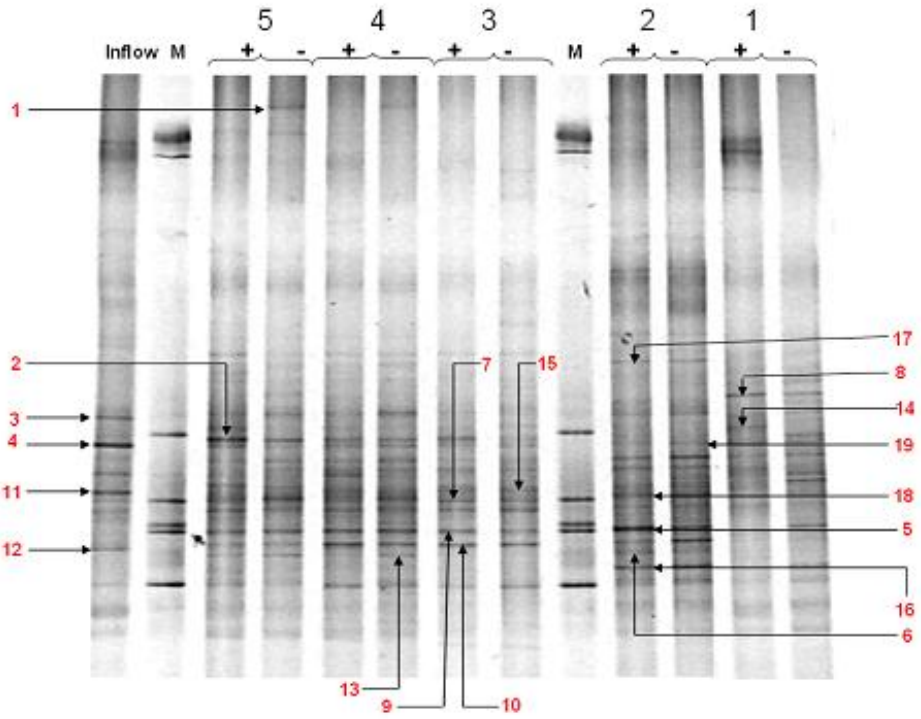


Fig. 7: Composition of the wetland biofilm bacterial community as affected by vegetation and depth in the microcosm. The layers (1-5) and presence or absence of vegetation in the microcosm (+/-) are indicated. Bands 1-19 were excised from the gel, cloned and sequenced.

The 16S rRNA gene sequences were analyzed using GreenGene software (DeSantis *et al*., 2006) and a phylogenetic tree was constructed (Fig. 8). The analysis revealed a highly diverse biofilm community, including dominant representatives of the *Acidobacteria*, *Bacteroidetes*, α, β and δ subdivisions of the *Proteobacteria*. Sequence analyses against the GenBank revealed similarity to bacteria performing specific activities expected to be relevant in a wetland environment. For example; band no. 17, whose closest match was *Nitrosomonas* sp. JL21 (with 98% DNA identity of the fragment sequenced according to BLAST) and band no. 6, whose closest match was *Nitrospira* sp. (98% DNA identity), appeared only in layer 2 (25 cm depth). This corresponds well with the relatively high

nitrification potential found in this layer (Fig. 4). Even though the microbial community composition of microcosm layers was similar in vegetated and non- vegetated microcosm, there was one clear difference in the DGGE pattern. Band no. 14, whose closest match was *Janthinobacterium* (96% DNA identity), is a member of the *Oxalobacteraceae*. Bacteria from this family are known to associate with plant roots (Green *et al*., 2007, Ofek, 2010) and indeed, this band appeared in layer 1 only in the presence of vegetation. Despite this "vegetation related band", the DGGE pattern of layer 1 with and without vegetation was highly similar (82%).

Band no. 7, the sequence of which was closest to *Bdellovibrio* CA-2E (91% DNA identity), appeared in all layers. Although the similarity of this sequence to its closest match was not high, it appeared in the middle of the *Bdellovibrio*-and-like organisms (BALOs) branch in the phylogenetic tree. Members of the BALOs are characterized as ubiquitous predatory bacteria (Pineiro *et al*., 2004, Jurkevitch, 2007) and thus are not expected to be specific to any microcosm layer.

Screening of the 16S rRNA sequences against the GenBank database using BLASTN (version 2.3.08) (Altschul *et al*., 1997) showed that 10 out of the 19 sequences obtained from the DGGE bands matched sequences of uncultured bacteria (below 97% similarity to cultivated bacteria).

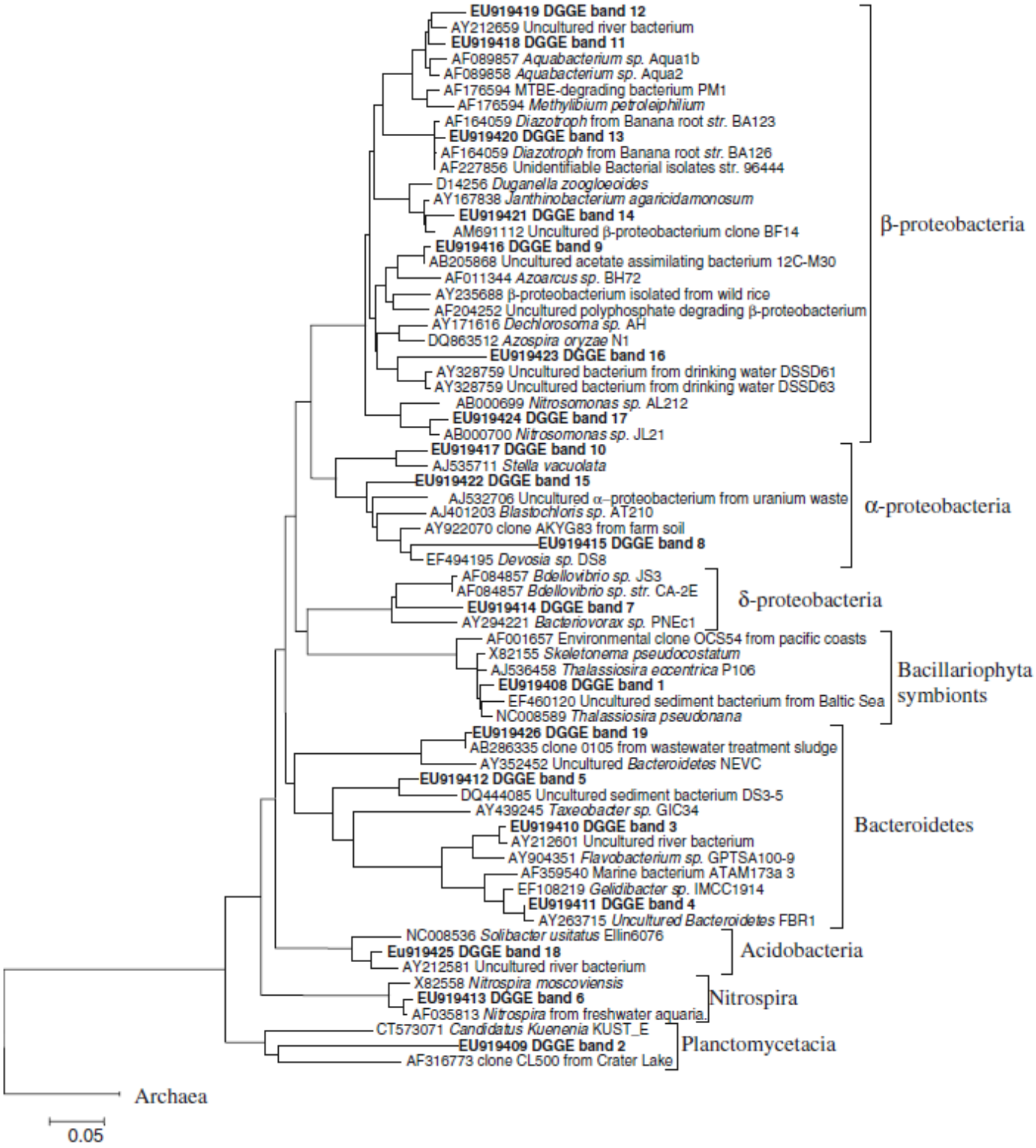


Fig. 8: Phylogenetic tree associations of microbial populations within the biofilm. Phylogenetic tree showing the relations of excised band sequences (550 bp) of the different species from the gravel biofilm, the influent and the effluent water relative to closely related reference species. The tree was inferred with the neighbor-joining method using a 70% similarity cutoff filter and Olsen correction method. Scale bar = 5% estimated difference in nucleotide sequence position.

3.3 biofilm development after 24-h assembly

24-h-biofilm assemblage on basalt gravel was studied by static incubation in single and multiple species biofilm. Isolated bacteria in different organic media as well as wastewater with its native bacteria were examined under laboratory conditions.

3.3.1 Colonization assay of single species biofilm

In the course of this research, sterile basalt gravel, identical to the one used in the constructed wetland site, was incubated with solutions containing bacterial cultures as a laboratory model for biofilm initiation in a constructed wetland (see Materials and Methods- 2.12.1).

Seven different species of bacteria (bearing different colony) were isolated from biofilm originating from CW system (Fig. 9). Characteristics of these bacterial species are summarized in Table 4.

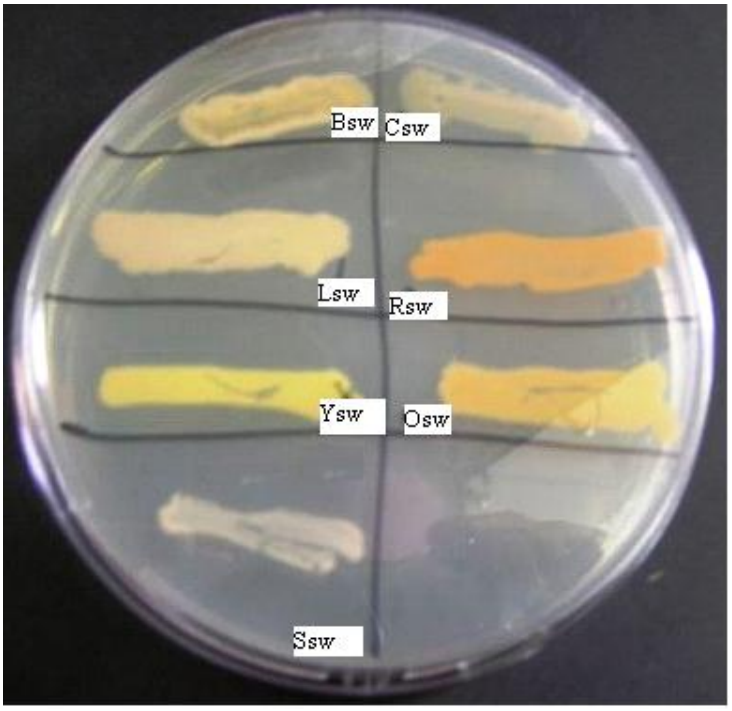


Fig. 9: Different bacterial strains isolated from the CW biofilm. Colony forming units of the different isolates on LB plate exhibiting different morphologies.

Phylogenetic analysis of 16S rRNA gene sequences showed that these bacteria are closely related to members of the *Actinobacteridae*, *Bacillaceae*, *Paenibacillaceae*, *Rhodobacteraceae* and *Staphylococcaceae* lineages (Table 4).

Colonization assay of these isolates showed that the medium with highest levels of organic load supported the highest single species biofilm (SSB) biomass levels (represented as the number of colony-forming units) in all of the isolates tested excluding isolate Ssw (Fig. 10).

Table 4. Biofilm characteristics of isolates originated from wetland

Isolate	Closest relative by NCBI blast	Similarity (%)	Colony morphology	Hydrophobicity (%)	Biofilm formation (cfu/g gravel)
Bsw GU458408	*Streptomyces cinereoruber*	89	Black	6.5	$3.8*10^4$
Ysw GU458412	*Rhodobacteracea bacterium D1-6*	97	Yellow	19	$1*10^5$
Rsw GU458411	*Micrococcus sp. 100H40-1*	97	Red	28	$1*10^3$
Lsw GU458410	*Brevibacillus sp. B2*	94	Large, white	33	$1.1*10^5$
Ssw GU458414	*Staphylococcus sp. Ca8-4M04*	99	Small, white	59	$2.2*10^4$
Csw GU458409	*Bacillus sp. AM20*	99	Cream	85	$3.7*10^5$
Osw GU458413	*Brevibacterium sp. SC9*	99	Orange	93	$6*10^5$

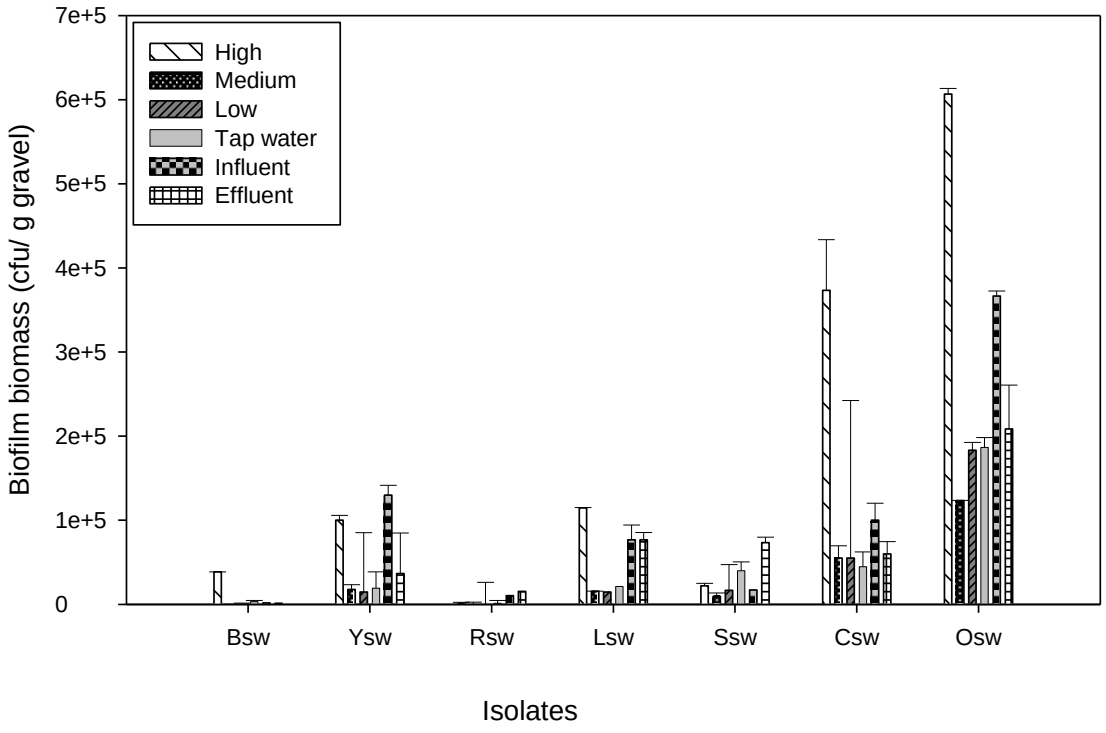


Fig. 10: Single-species biofilm biomass (cfu/g gravel) of each isolate incubated with different organic loads: high, medium, low and tap water. Results are representative of two similar experiments, each performed in triplicate. Bars represent standard deviation of triplicate samples.

3.3.2 Biofilm richness and diversity as affected by organic load

Organic load affected the biomass and diversity of the multiple species biofilm (MSB- all seven isolates together). An increase in the organic load of the medium resulted in reduced species diversity in the MSB (Fig. 11). Biofilm developed under high organic load contained only two of the seven inoculated species (Lsw, Csw), whereas biofilm that developed under medium organic load contained four species. Both the low organic load biofilm and the tap water (no organic load) - initiated biofilm contained five species (Fig. 11). Examination of MSB developed in sterile influent and effluent water, containing

organic loads similar to the medium and low media, showed the same phenomenon (4 and 5 species, respectively).

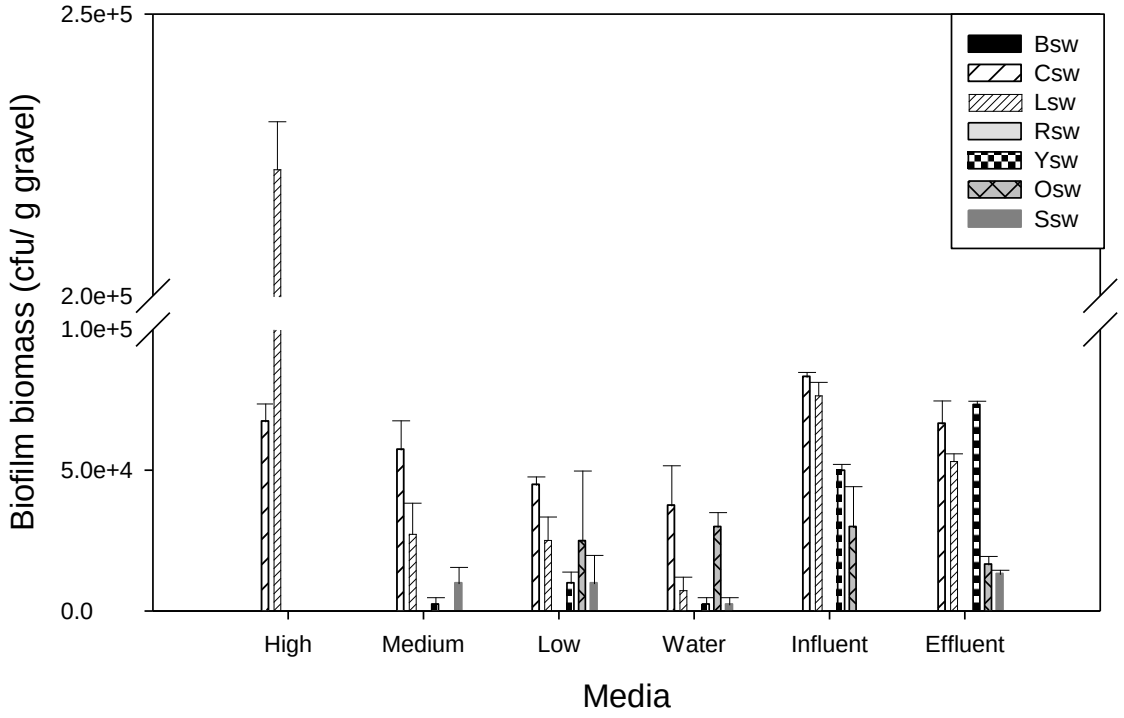


Fig. 11: Bacterial species biomass (cfu/g gravel) of multiple-species biofilm formed under different organic loads: high, medium, low, water, influent and effluent. Results are representative of two similar experiments, each performed in triplicate.

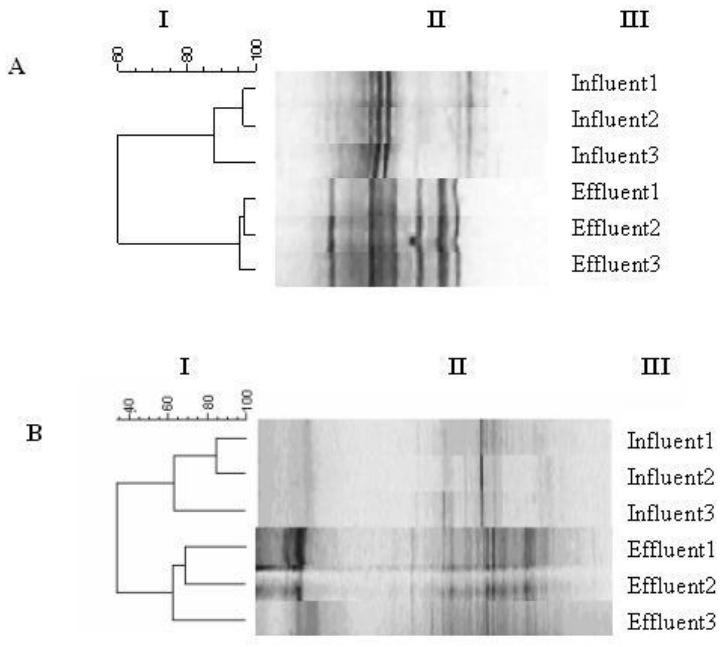


Fig. 12: Cluster analysis of microbial community composition of biofilm initiated by influent and effluent water. The dendrogram was generated by 16S rRNA gene DGGE patterns representing the genetic similarity of the microbial community profiles obtained from 24-h-old biofilm generated in CW influent versus effluent water in triplicate. The water samples were collected from wetland ponds on 30 May 2006 (A) and 27 Oct 2008 (B). Cluster analysis was performed using Fingerprinting II software. UPGMA tree based on Dice similarity matrix (I) between the different DGGE patterns (II) of the different samples (III).

Examination of the different isolates constructing MSB in the presence of different organic media showed that isolates Ssw, Rsw and Bsw, which produced nominal amount of biofilm in single-species culture, retained their inability to form high biomass in MSB. However, the other isolates showed substantial differences in their ability to form SSB versus MSB. SSB results showed that four isolates had the ability to form high and medium biofilm biomass (Osw, Csw and Lsw, Ysw, respectively). Only two of these isolates (Lsw, Csw) were significant community members of MSB with high organic load. Isolate Osw, which produced the highest SSB biomass of all seven isolates on

gravel (Fig. 10), was not present in a detectable level in high organic-load MSB. Moreover, Osw represented only a limited proportion of the low organic-load MSB (Fig. 11). The same trend occurred with isolate Ysw.

The high organic-load MSB was dominated by isolate Lsw, which produced medium SSB biomass (Figs. 10 and 11). However, the abundance of Lsw in the biofilm decreased with the reduction of organic load. In contrast, Isolate Csw, which produced the second highest SSB biomass, was characterized by relatively constant levels of biomass in all MSB. Furthermore, Csw represented the highest portion of the medium-, low- and water media-MSBs (Fig. 11).

We also investigated 24-h-biofilm formed by incubating sterile basalt gravel with influent and effluent water with its native bacterial communities. Partial 16S rRNA gene fragments were amplified, and the bacterial community composition was studied by DGGE analysis. High reproducibility was found in the DGGE patterns of triplicate samples for each of the two treatments. Clear differences in DGGE patterns were found between biofilms initiated with influent versus effluent water (Fig. 12). Biofilm initiated by incubation of gravel with influent water showed fewer dominant DGGE ribotypes than that initiated by incubation of gravel with the effluent water (Fig. 12). Two similar experiments with influent and effluent sampled in different dates (Fig. 12 A and B) showed the same phenomenon.

3.4 Isolation of estradiol degrading bacteria

Estradiol was chosen as a model pollutant to examine the early events occurring during bioaugmentation. The aim of the first part of this study was to isolate different estradiol degrading bacteria (EDB).

3.4.1. Enrichment, isolation and characterization of EDBs

Enrichment culture of EDB, originating from CW biofilm, was repeatedly transferred to a fresh sterile medium containing E2 as a sole carbon source. As expected, enriched cultures from each consecutive transfer varied in their DGGE patterns (Fig. 13). Some of the bands from the original enrichment culture (1st transfer) disappeared, while new dominant bands appeared with subsequent transfers. More dominant bands were detected in the enriched culture containing E2 than in the control culture without E2. Clear differences between the DGGE patterns of these two cultures were detectable at the 4th and 5th transfers, suggesting that the bacterial community composition of the enrichment cultures was affected by the presence of E2 as sole carbon source.

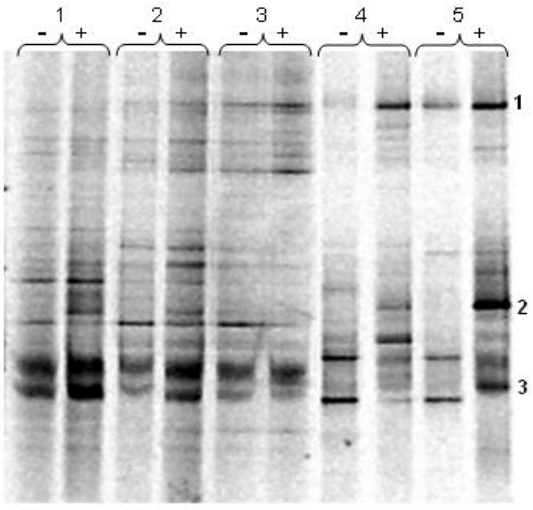


Fig. 13: Bacterial community composition of the enrichment culture through five consecutive transfers in the presence (+) or absence (-) of estradiol as sole carbon source. DGGE analysis of 16S rRNA gene fragments was performed. The marked bands appearing in the presence of estradiol were sequenced and exhibited similarity to 16S rRNA genes of: 1) 97% *uncultured Bacteroidetes* (FJ517119); 2) 98% *Novosphingobium JEM-1* (AB219359) and 3) 99% *Hydrogenophaga intermedia* (FJ009392).

The three most dominant DGGE bands from the 5th transfer containing E2 were cloned and sequenced (bands 1-3, Fig. 13). These bands, representing different species in the enrichment culture, had a similarity of 97, 98 and 99% (by 16S rRNA sequence comparison) to uncultured *Bacteroidetes*, *Novosphingobium sp. strain JEM-1* and *Hydrogenophaga intermedia*, respectively.

Eight phylogenetically diverse bacteria were isolated from the 5th transfer of the enrichment culture, based on their ability to form a clearing zone on E2 agar plates. These isolates showed the ability to transform E2 to E1 (Table 5).

Table 5. Bacteria isolated from E2 enrichment culture, their taxonomy (by comparison of 16S rRNA sequence to GenBank data) and their estradiol-degrading ability.

Bacteria	Estradiol transformation to estrone (ppm) after 48 h	Closest isolate (accession number) % similarity
EDB-LI1	29	*Novosphingobium* JEM-1 (AB219359) 98%
EDB-LI2	24	*Pimelobacter simplex* strain S151 (AY509240) 89%
EDB-LI3	19.6	*Staphylococcus saprophyticus* BMSZ711 (GQ869683) 99%
EDB-LI4	6.8	*Pediococcus acidilactici* strain GL20 (GQ421480) 81%
EDB-LI5	34	*Enterococcus durans* strain IMAU10167 (FJ915822) 88%
EDB-LI6	25.3	*Brevibacillus* sp. DA2 (AB507254) 87%
EDB-LI7	16.3	*Pseudomonas* sp. BXWY1 (DQ839555) 89%
EDB-LI8	16.8	*Achromobacter xylosoxidans* strain LHB21 (GQ359326) 98%

However, only one of these isolates (EDB-LI1) removed E1 as well. The 16S rRNA sequence of isolate EDB-LI1 was found to be 98% similar to that of *Novosphingobium* JEM-1, originated from wastewater treatment plant in Japan (accession no. AB219359) (Hashimoto *et al.*, 2010), and identical to the sequence of DGGE band no. 2 from the 5th culture transfer with E2 (Fig. 13).

Isolate EDB-LI1 (Table 5 and Fig. 14) was selected for furthered investigation.

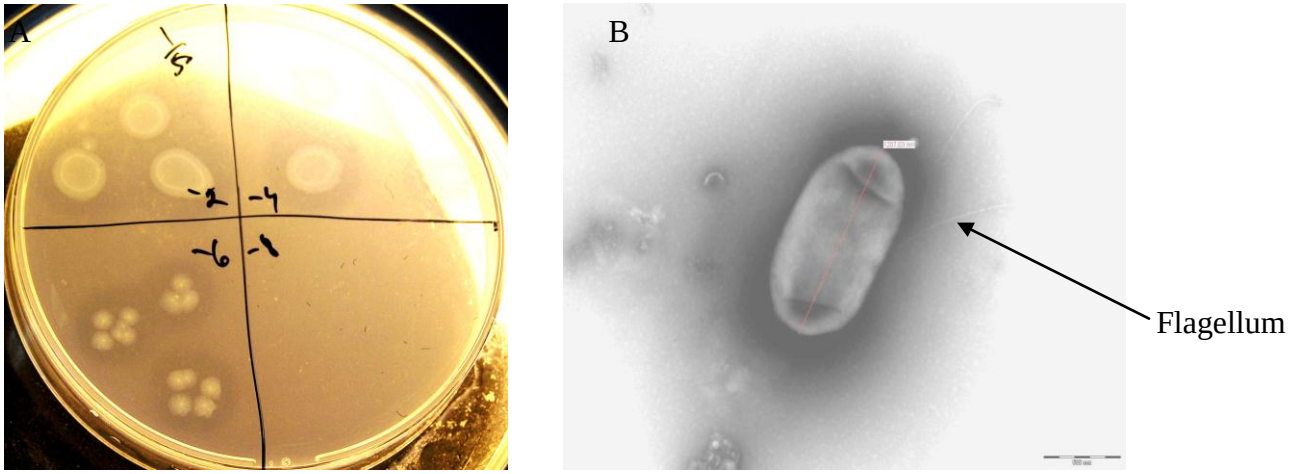


Fig. 14: EDB-LI1 isolate. A- Colony forming units of EDB-LI1 on agar plate containing E2 as a sole carbon source. Clearing zone surrounding the colonies is suggesting E2 degradation. B- Transmission electron microscope snapshot of EDB-LI1. Bar representing 500nm.

3.4.2 Estrogen degradation and EDB-LI1 growth in liquid culture and in biofilm form

The E2-degradation ability of a pure culture of EDB-LI1 was examined in both biofilm and liquid culture. Under both conditions, transformation of E2 to E1 and removal of E1

occurred in parallel, resulting in degradation of E2. However, the removal rates differed: full removal of 80 mg L^{-1} E2 by EDB-LI1 biofilm required 2 days whereas removal of the same amount of E2 by liquid culture required 6 days (Fig. 15).

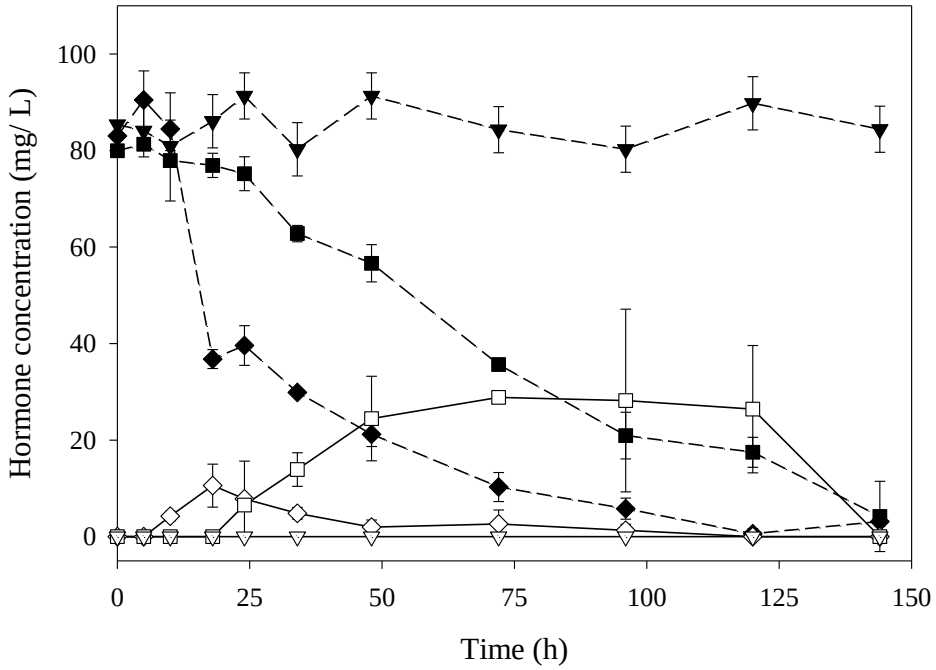


Fig. 15: Rate of estradiol (E2) and estrone (E1) degradation by EDB- LI1. Changes in E2 (filled symbols) and E1 (open symbols) concentrations with time in the presence of EDB-LI1 liquid culture (square), EDB-LI1 biofilm (diamond) or without EDB-LI1 (triangle). Error bar represent standard deviation.

Calculation of E2 removal per EDB-LI1 cell showed that the plaktonic cells were capable of removing E2 at a maximum daily rate of ca. 90 picomole per cell in mineral media where E2 was the sole carbon source whereas EDB-LI1 biofilm was more efficient, degrading E2 at a maximum daily rate of ca. 300 picomole per biofilm cell. Organic matter had an effect on the degradation of E2: transformation of E2 to E1 and E1 removal

by EDB-LI1 in liquid culture was more efficient when the organic matter concentration in the medium was lower (Fig. 16). Estriol and ethinylestradiol were not degraded by this isolate under the examined conditions (data not shown).

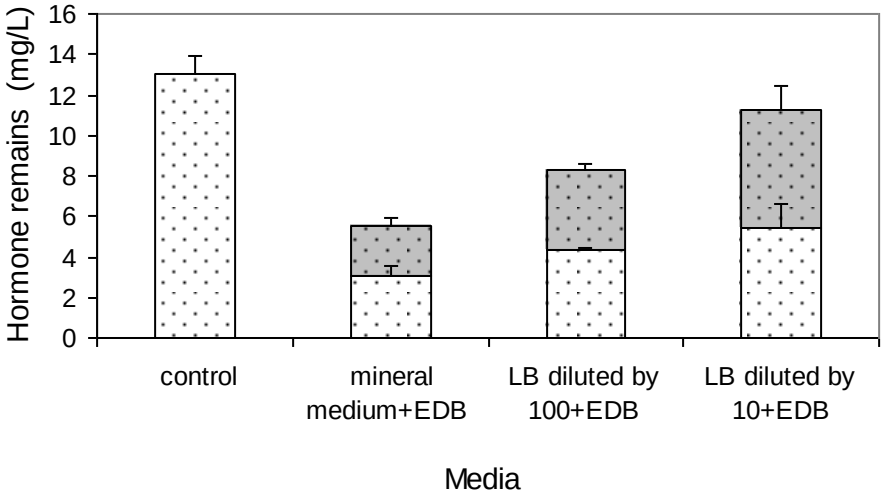


Fig. 16: Effect of organic matter concentration on E2 degradation by EDB-LI1 (transformation of estradiol to estrone and removal of estrone). E2 (dots) and E1 (gray) remains after incubation of 12 mg/L of E2 with EDB-LI1 for 24h.

The total number of EDB-LI1 16S rRNA gene copies, representing EDB-LI1 cells, was higher at the first 48 h in the biofilm than in the liquid culture, as determined by qPCR (Fig. 17). Higher growth rate constant (μ) was found in the biofilm form of EDB-LI1 than in its liquid culture form (0.082 h^{-1} and 0.053 h^{-1}, respectively).

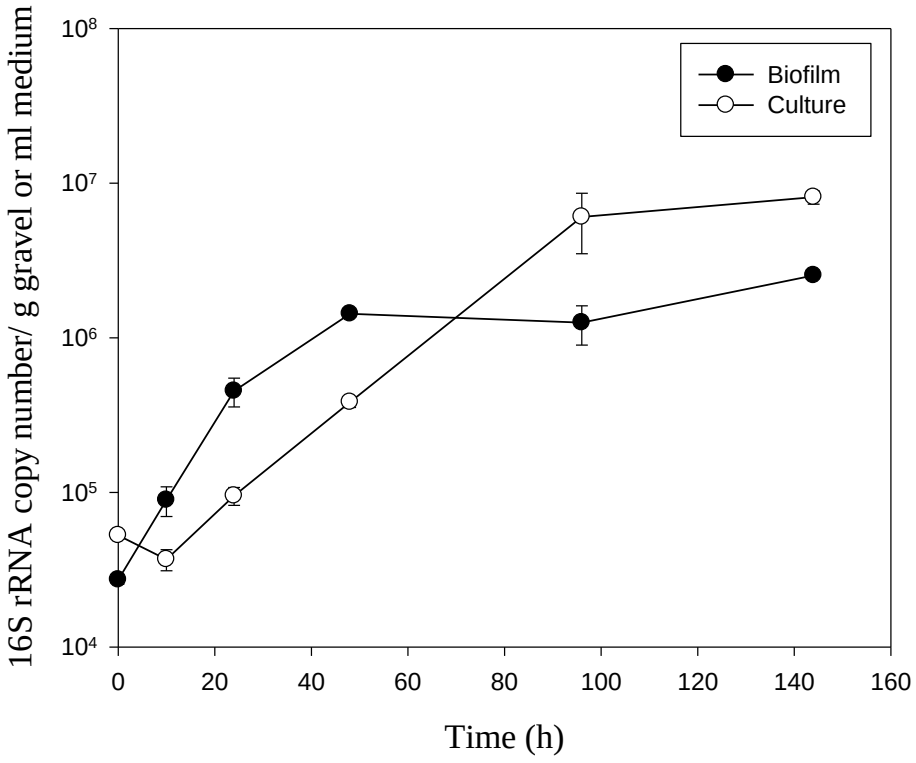


Fig. 17: Growth of EDB-LI1 in liquid culture and as biofilm, using estradiol as sole carbon source. Changes in EDB-LI1's 16S rRNA gene copy number with time in the presence of estradiol as sole carbon source. EDB-LI1 biomass of liquid culture (○) and biofilm (●) as measured by qPCR with specific primers designed to amplify the 16S rRNA gene of EDB-LI1. Error bar represent standard deviation.

3.5 Bioaugmentation of EDB-LI1 into biofilm originated from CW

Early events occurring during bioaugmentation of EDB-LI1 into wetland biofilm were examined under laboratory conditions. The influence of augmented EDB-LI1 on biofilm community composition and activity were investigated.

Community fingerprints, represented by DGGE patterns, of augmented and un-augmented biofilm samples, obtained from ponds 1 and 2, separated into two clusters according to their original wetland pond. Augmentation of EDB-LI1 had no significant

effect on the bacterial composition of biofilms originating from pond 1. However, augmentation of EDB-LI1 in biofilms originated from pond 2 caused a division of DGGE patterns into two sub-clusters: augmented biofilm and non-augmented biofilm (Fig. 18).

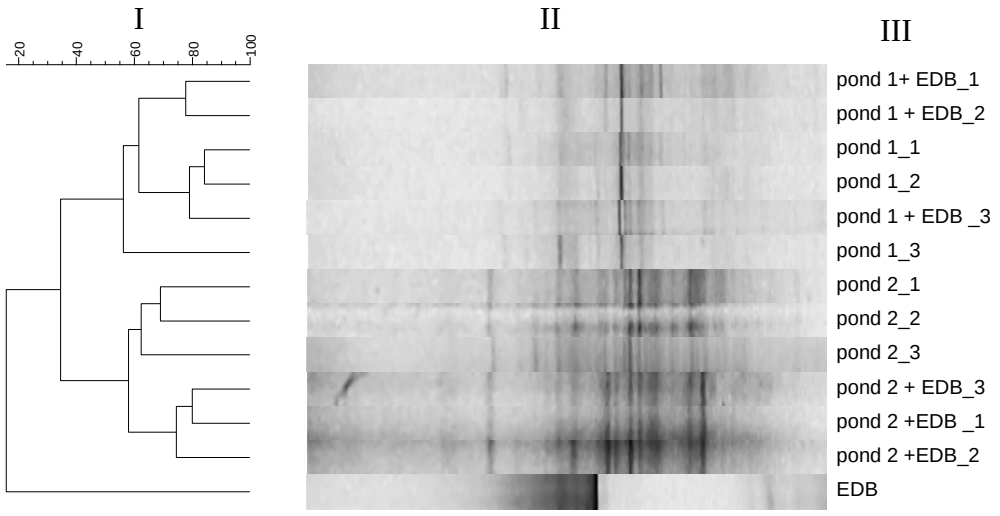


Fig. 18: Cluster analysis of bacterial community composition in wetland biofilm. Cluster analysis of DGGE patterns was performed using Fingerprint II software. The UPGMA tree (I) was calculated according to Dice similarity matrix between the different DGGE patterns (II). (III) Sample names: pond 1, biofilm originating from pond 1; pond 1+EDB, biofilm originating from pond 1 augmented with EDB-LI1; pond 2, biofilm originating from pond 2; pond 2+EDB, biofilm originating from pond 2 augmented with EDB-LI1. Each treatment was conducted in triplicates.

Quantification of the total biofilm microflora by qPCR with general bacterial primers did not show any significant difference in total bacterial abundance among the samples (Fig. 19). However, there was a significantly ($p \leq 0.05$) higher presence of EDB-LI1 in augmented biofilm originating from pond 2 (ca. 45,000 copies of EDB-LI1 16S rRNA gene per gram of gravel) in comparison to that in pond 1 (ca. 30,000 copies g^{-1}). The

EDB-LI1 population was below the detection limit (<200 copies) in the non-augmented biofilm samples (Fig. 19).

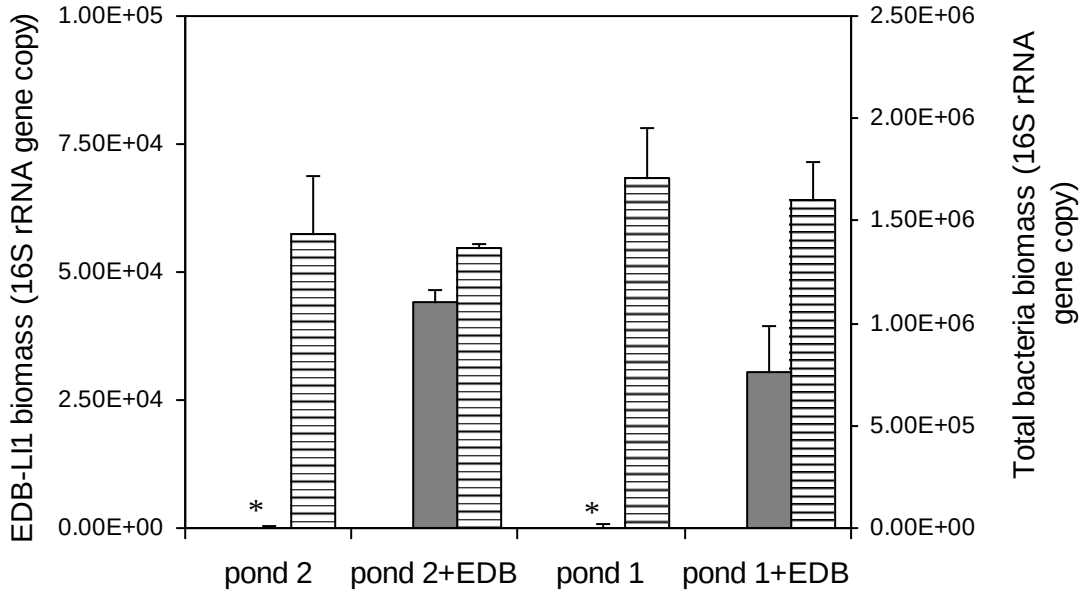


Fig. 19: EDB-LI1 bioaugmentation in wetland pond biofilm. Total bacteria 16S rRNA gene copy number (striped column) and EDB-LI1 16S rRNA gene copy number (gray column) as measured by qPCR analysis in different biofilm samples: pond 1, biofilm originating from pond 1; pond 1+EDB, biofilm originating from pond 1, augmented with EDB-LI1; pond 2, biofilm originating from pond 2; pond 2+EDB, biofilm originating from pond 2, augmented with EDB-LI1. *, below detection limit. Each treatment was conducted in triplicates. Error bar represent standard deviation.

E2 removal by augmented and non-augmented biofilm was monitored by incubating the biofilms in E2 solution for 24 h, in order to evaluate the contribution of EDB-LI1 to biofilm function in degradation of estrogens. No degradation of E2 and E1 was observed in the controls (non-augmented biofilm originating from both wetland ponds) (Fig. 20).

E2 transformation to E1 by the augmented biofilm originating from pond 1 was significantly ($p \leq 0.05$) lower than that by the augmented biofilm originating from pond 2 (9.1 and 17.6 mg L^{-1} g $gravel^{-1}$ day^{-1}, respectively).

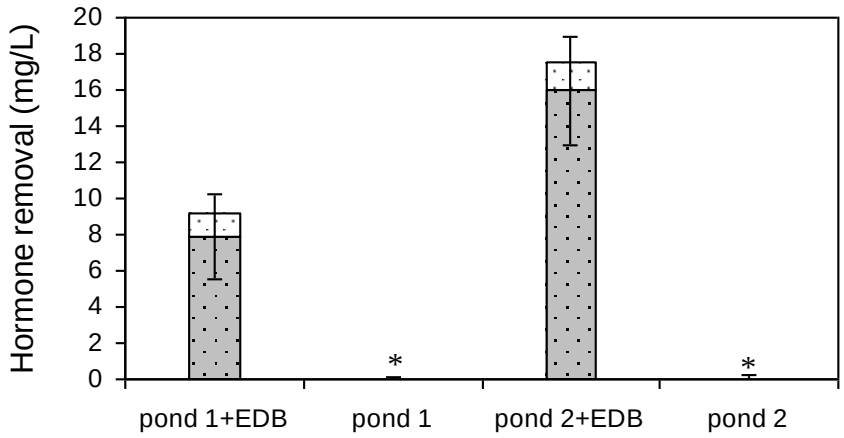


Fig. 20: EDB-LI1 bioaugmentation in wetland pond biofilm. E2 (dots) and E1 removal (gray) (mg/L) during 24 h of incubation, as measured by HPLC in different biofilm samples: pond 1, biofilm originating from pond 1; pond 1+EDB, biofilm originating from pond 1 augmented with EDB-LI1; pond 2, biofilm originating from pond 2; pond 2+EDB, biofilm originating from pond 2 augmented with EDB-LI1. Each treatment was conducted in triplicates. Initial estradiol concentration was 40 mg L-1. *, below detection limit. Error bar represent standard deviation.

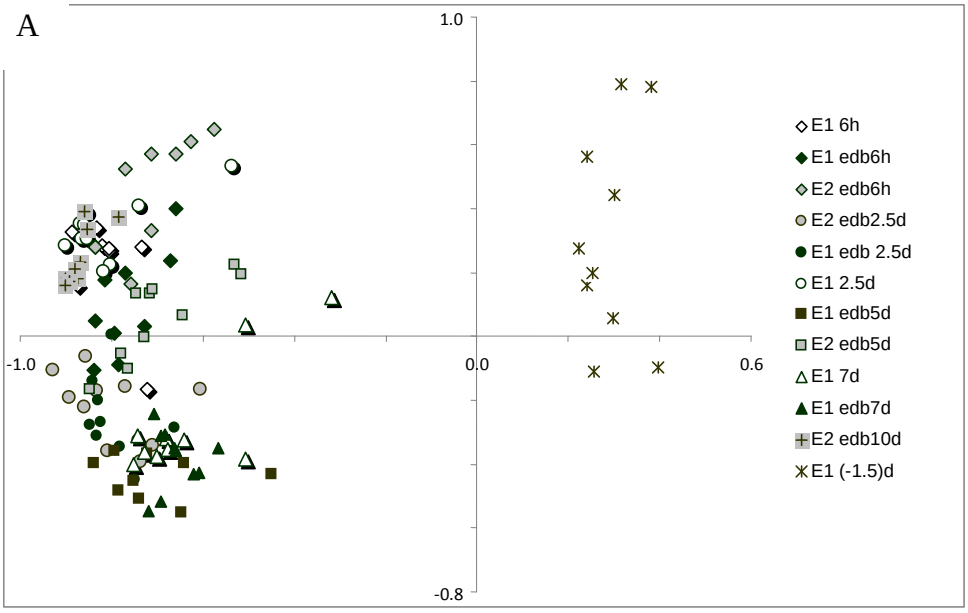
In addition, E1 removal by the augmented biofilm originating from pond 1 was significantly ($p \leq 0.05$) lower than that of the augmented biofilm originating from pond 2 (7.9 and 16 mg L^{-1} g $gravel^{-1}$ day^{-1}, respectively). Hence, the full combined removal of E2 and E1 was higher by augmented biofilm originated from pond 2 in comparison to augmented biofilm originated from pond 1 (Fig 20).

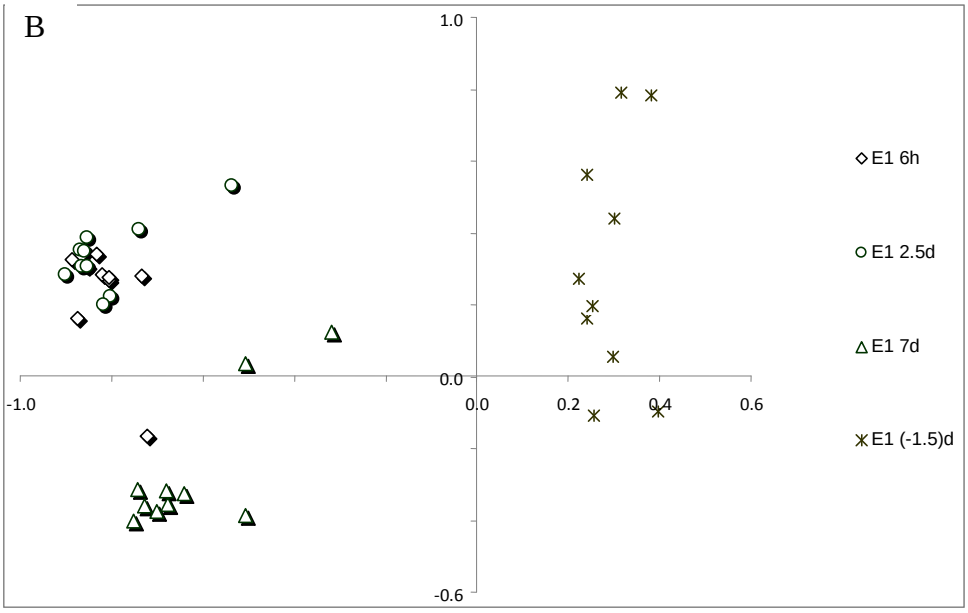
3.6 Bioaugmentation of EDB into soil columns

As was mentioned in the introduction, EDCs residues, like E2, are found in sewage treatment plants (STPs) effluent due to insufficient removal in WWT systems (Servos *et al*., 2005, Stumpe and Marschner, 2007, Liu *et al*., 2009, Pacáková *et al*., 2009). These residues may contaminate soils as a result of irrigation with effluent of STPs (Stumpe, 2007). Since 50% of the irrigation water in Israel is treated wastewater (Navon *et al*., 2011), we decided to investigate the effect of EDB-LI1 bioaugmentation on bacterial community composition and function in soil columns system.

The augmentation of EDB-LI1 into the soil column had an effect on the microbial community composition of the soil as presented by Multidimensional scaling analysis (MDSA) (Fig.21A-D) of DGGE patterns (appendix, Fig.1). The microbial community composition of soil obtained from column one and a half days after the construction of the column (approximately one and a half days **before** the estrogen spiking) was significantly different from the microbial community composition of all the other samples (Fig. 21A and 21B). Furthermore, 6h after the estrogen spike the community composition of all samples clustered together. However, after 2.5 days the augmented columns (both spiked with E1 and spiked with E2) were distinguished from that of un-augmented column (Fig. 21A).

MDSA of DGGE pattern of soil obtained from column spiked with E1 without augmentation of EDB-LI1 showed no significant differences in the microbial community composition between 6h and 2.5 days after the spiking. Clear differences were found after 7 days (Fig. 21B).


A
1.0
-1.0
0.0
0.6
-0.8
E1 6h
E1 edb6h
E2 edb6h
E2 edb2.5d
E1 edb 2.5d
E1 2.5d
E1 edb5d
E2 edb5d
E1 7d
E1 edb7d
E2 edb10d
E1 (-1.5)d


B
1.0
0.0
-1.0
0.0
0.2
0.4
0.6
-0.6
E1 6h
E1 2.5d
E1 7d
E1 (-1.5)d

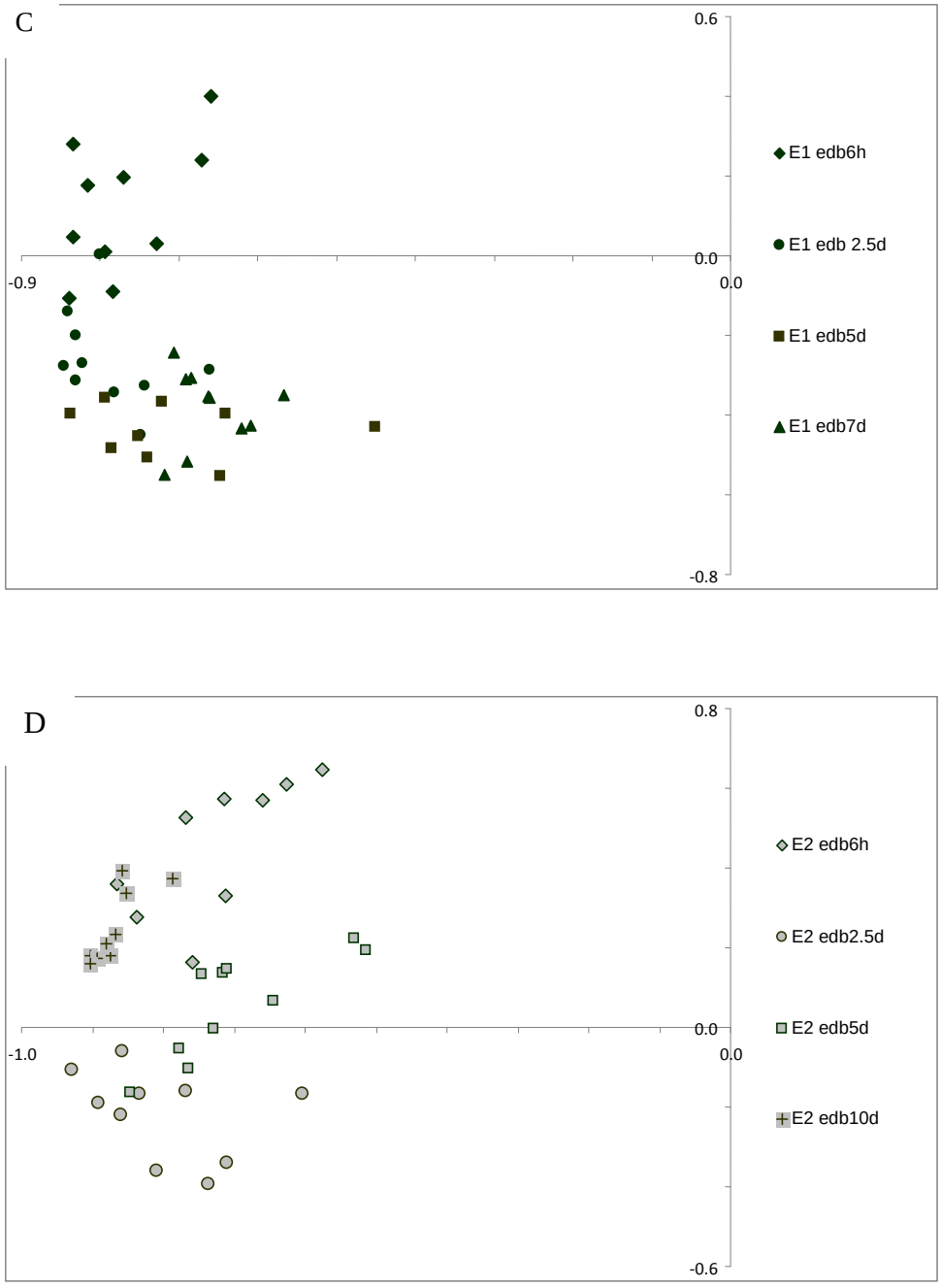


Fig. 21: Microbial community composition of different soil column. Multidimensional scaling analysis (MDSA) of DGGE patterns densitometric curves of DGGE patterns of different treatments with time: (A)- all samples together. (B)- column spiked with E1. (C)- column augmented with EDB-LI1 and spiked with E1. (D)- column augmented with EDB-LI1 and spiked with E2. Each column contained 5 layers, representing different depths in the column (see 2.13), in duplicates.

The microbial community composition of soil obtained from column augmented with EDB-LI1 and spiked with E1 formed two separate clusters: microbial community composition of the soil 6 hours after E1 spike differed from that of later time points (5 and 7 days) of the same treatment (Fig. 21C). The MDSA of time point 2.5 days is connecting these two clusters together.

MDSA of DGGE pattern of soil obtained from column augmented with EDB-LI1 and spiked with E2 showed differences in the community composition between 6h and 2.5 days after E2 spiking. These changes gradually faded and the microbial community composition of the soil 10 days after E2 spike was similar to that of 6h after the E2 spike (Fig. 21D). As was mentioned above, in the column that was spiked with E1 without EDB-LI1 augmentation, the differences in the microbial community composition were detected in a latter time point then in the augmentation treatments (Fig. 21B).

The microbial community compositions of different depth of the same column clustered together. Hence, bacterial community composition of the soil column was not significantly affected by the depth of the column.

EDB-LI1 was detected in the outflow water of the augmented columns as was measured by plating the column's effluent on a specific agar medium. Meaning, EDB-LI1 was able to pass through the whole column. Furthermore, the total amount of EDB-LI1 in the inflow (average: $6.8*10^8 \pm 5.9*10^3$ CFU) and the accumulated number in the outflow (average: $5.8*10^8 \pm 4*10^3$ CFU) were similar. Meaning, almost all the augmented EDB-LI1 had washed though and cross the column. However, a low portion (approximately $5*10^3$ EDB-LI1 copy number per g soil, as measured by qPCR of the 16S rRNA gene) of EDB-LI1, which augmented into the column, remained in the inlet the column (layer 5-

the soil closest to the bottom entry of the column) and settled there for a few days (Fig. 22). EDB-LI1 was detected only in the layer 5 and was not found in the other depths of the column. EDB-LI1 in the layer 5 survived and even slowly multiplied for 6 days reaching up to $5*10^4$ CFU per g soil. However, seven days after the estrogen spiking EDB-LI1 community collapsed and its quantity was under detection limit. Even though a similar number of EDB-LI1 was augmented into both columns (spiked with E1 and spiked with E2), the amount of EDB-LI1 found in them was slightly different. However, the same trend of establishment in the inlet (layer 5), slow growth and rapid disappearance was observed in both treatments (Fig. 22). The number of EDB-LI1 found in the un-augmented column was below detection limit (<200 16S rRNA gene copies).

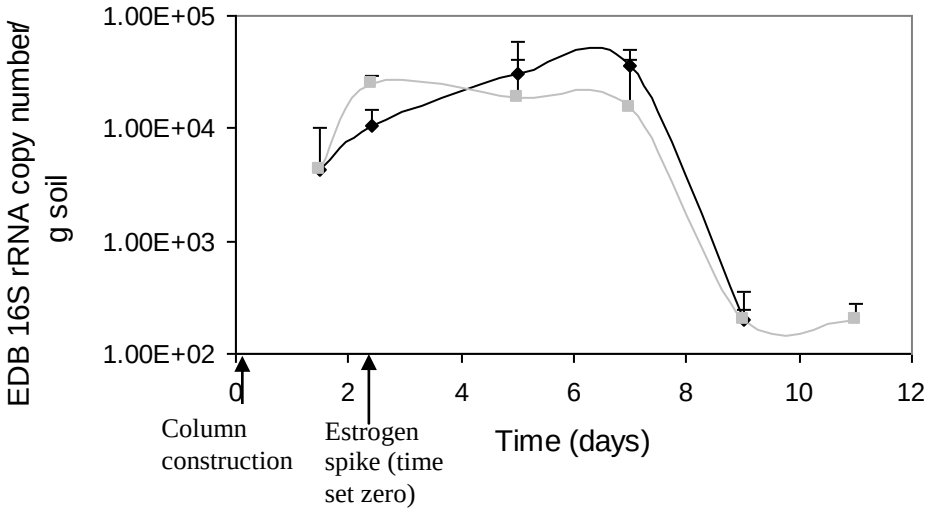


Fig. 22: Changes in 16S rRNA gene copy number of EDB-LI1 with time as affected by E1 and E2 spike. Growth of EDB-LI1 as was measured by qPCR with specific primers in two soil columns: black– the inlet layer of column augmented with EDB-LI1 and spiked with E1. Gray ray- the inlet layer of column augmented with EDB-LI1 and spiked with E2. The amount of EDB-LI1 in the other layers was under detection limit.

The quantity of 16S rRNA gene of total soil bacteria was also monitored; significant differences in microbial biomass at different time points were observed (Fig. 23). Total bacteria abundance in all treatments was characterized by three stages: (i) 1.5 days after the construction of the column (approximately half a day before the estrogen spiking); the total bacterial number (as measured by qPCR) was around $1*10^6$ bacteria per g soil. (ii) 6h after the estrogen spiking, the quantity of the total bacteria increased to $1*10^8$ bacteria per g soil and remained constant for five days. (iii) seven days after the estrogen spiking, the amount of total bacteria decrease to $3*10^6$ bacteria per g soil. The quantity of the total bacteria in the soil column was not significantly affected by the different treatments or the depths of the column (Fig. 23).

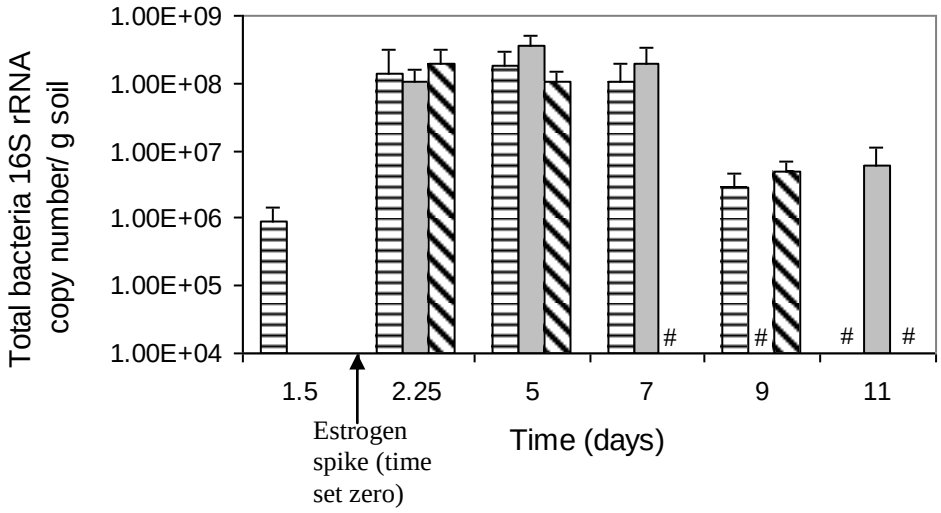


Fig. 23: Changes in 16S rRNA gene copy number of total soil bacteria with time in the different treatments. Growth of total bacteria as was measured by qPCR with general bacteria primers in different soil columns: striped - column augmented with EDB-LI1 and spiked with E1. Gray- column augmented with EDB-LI1 and spiked with E2. oblique lines - column spiked with E1. Each column is an average of 5 layers in duplicates. # - data not tested.

Degradation of E1 and E2 during infiltration through the soil column was followed by LC-MS. Higher degradation rates of estrogens were detected in columns augmented with EDB-LI1 in comparison to un- augmented columns (Dr. Nadine Goeppert at the laboratory of Prof. Brian Berkowitz Department of Environmental Sciences and Energy Research, Weizmann Institute of Science, Rehovot 76100, Israel)

4. Discussion

4.1 Constructed wetland biofilm

The type and size of microbial populations composing biofilms are the keys for efficient wastewater cleanup processes in a constructed wetland (CW) system (Faulwetter *et al*., 2009). The treated water, including its native bacterial species, is flowing though the wetland matrix. A portion of these bacteria attach to the CW matrix and assemble into a biofilm. Different CW biofilms vary in their microbial community composition, since the treated water in each CW is diverse in terms of chemical and microbial properties (Truu *et al*., 2009). For this reason, the community composition of these biofilms is relatively un-known and usually considered a black box. In recent years, the bacterial community composition and function of CW biofilm is gaining more attention (Truu *et al*., 2009). Better understanding of the CW biofilm development, maturation and interaction between the different species of bacteria within the biofilm as well as the influence of different CW gross parameters on biofilm formation may enable to improve the CWs' biofilm function and, as a result, the CW efficiency.

The efficiency of CW is examined by its ability to improve the treated wastewater quality. Examination of the influent and effluent chemical parameters may indicate different microbial activities, which employed in the CW during wastewater infiltration. For example: in the current study nitrate concentration in the effluent was higher by 280% from that of the influent (Table 2 and 3), suggesting conversion of ammonia to nitrate by nitrification. Ammonia and nitrite oxidizing bacteria are found in a variety of wastewater treatments systems (Dionisi *et al*., 2002, Sundberg *et al*., 2007, Harms *et al*., 2003). *Nitrosomonas* spp. are commonly found in conventional wastewater treatment plants (Harms *et al*., 2003, Rowan *et al*., 2003) and their abundance can reach up to 1.5% of total bacteria biomass in constructed wetlands (Truu *et al*., 2009). *Nitrospira* and *Nitrobacter* may reach values of 1.5% and 4.3% of biofilm biomass, respectively (Shipin *et al*., 2005). Since only abundant microbial population can be detected by PCR-DGGE of the 16S rRNA gene (Muyzer *et al*., 1998), it can be assumed that in the current study *Nitrospira* and *Nitrosomonas* are the majority of the ammonia and nitrite oxidizing bacteria in the microcosms' biofilm (Fig 7 and 8). In addition, Tables 2 and 3 show that no significant changes were found in total N values during influent infiltration through the CW. This may be due to the unsaturated state of the microcosms, which did not maintain the anaerobic conditions required for significant denitrification.

One of the variables tested in the current study, was the effect of vegetation on the wetland efficiency. Haberl *et al* (2003), reviewing a broad spectrum of studies on different constructed wetland systems, concluded that the effect of plant uptake on the effluent quality is minor compared to the microbial degradation processes. In contrast, a review by Greenway (2007) stated that since inorganic nitrogen and phosphorus are

essential for plant growth, it is possible to maximize the amount of nutrients removed from wastewater effluent by macrophytes. In the current study, vegetation presence had no significant effect on the measured characteristics of effluents in both microcosms and wetlands ponds (Table 2 and 3, respectively).

Several studies investigated the influence of vegetation and depth on hydrolysis activity in constructed wetlands. The results varied between the different experiments. Weaver *et al*. (2004) described the lack of any significant effect on FDA hydrolysis activity between excavated and shallow soil constructed wetland cells. In contrast, depth had a significant effect on FDA hydrolysis in freshwater sediments, indicating a decrease in activity as depth increases (Blondin *et al*., 2008). McHenry and Werker (2005) reported that vegetation had no significant effect on *in-situ* FDA hydrolysis in wetland mesocosms constructed by pea gravel, whereas microbial density and activity were higher in planted sand microcosms in comparison to non planted ones (Gagnon *et al*., 2007). Different plant species can affect the microbial activity in wetland systems in different manners. For example, *Myriophyllum spicatum* increased the microbial activity, while *Elodea canadensis* did not have any significant effect on FDA hydrolysis in freshwater sediments (Blondin *et al*., 2008). In the current study depth and vegetation had an effect on biofilm FDA hydrolysis activity (Fig. 3). Since the FDA hydrolysis measured was a product of the sum of bacterial activities in the biofilm, the explanation for these results may be that depth and vegetation presence induced different conditions which affected the microbial activity of a specific species and/ or the microbial composition of the tested biofilm and, as a result, the FDA hydrolysis of the biofilm.

Nitrification potential, which is another important microbial activity examined in this study, was found to be most affected by the depth of the layer in the microcosm (Fig. 4). This suggests that the conditions in the upper layers (including higher levels of oxygen and ammonium) support the nitrifying community, as described by Lorenzen *et al.* (1998). The fact that DNA sequences representing ammonia and nitrite oxidizing bacteria were detected by DGGE only in layer 2 (Fig.7 and 8) strengthens this suggestion.

Different studies investigated the effect of different CW gross parameters on microbial community composition including depth, vegetation (see review Truu *et al*., 2009) and surface properties of matrix type (Silyn-Roberts and Lewis 2004). Surface properties were shown to have an impact on microbial colonization, resulting in an effect on biofilm community composition (Watnick and Kolter, 2000). However, in our study the bacterial community composition that developed on layer 4, which was composed of basalt gravel, was more similar to the dolomite layers above and below it than to the two upper basalt layers (Fig. 6). This suggests that the location and its physicochemical characteristics have a stronger effect on bacterial community composition than the gravel type. Vegetation may affect microbial community composition as was shown in constructed wetland systems treating domestic wastewater (Vacca *et al*., 2005). In that study, macrophytes had stimulated the development of specific microbial communities. However, in the current study, vegetation did not significantly change the bacterial population of the microcosm biofilm formed on gravel (Fig. 6). These results may explain the fact that vegetation presence in the CW ponds and microcosms did not alter the physicochemical characteristics of the effluent. Similarly, Osem *et al*. (2006) found only minor differences between community metabolic profiles in biofilm from non-

vegetated and vegetated (*Typha domingensis*) microcosms, bedded with tuff stones after three months of maturation. In addition, Baptista *et al*. (2008) found that plants did not have a statistically significant effect on bacterial community structure in a laboratory-scale constructed wetland. Their results showed that the quantity of archaea, bacteria and sulfate-reducing bacteria were similar in planted and unplanted units. Ahn *et al*. (2007) also observed an absence of vegetation impact on microbial community structure in constructed wetland sediment.

Examination of sequences obtained from microcosms' biofilm showed that over 50% of the sequences obtained from the microcosms biofilm were from uncultured bacteria (Fig. 8). These results are similar to those obtained from sediment biofilm, taken from wetland treating river water containing tertiary wastewater (Ibekwe *et al*., 2007). This finding corresponds with the relatively oligotrophic nature of these habitats because conventional media do not always support oligotrophic bacteria growth (Ibekwe *et al*., 2007, Mallory *et al.*, 1977, and Phung *et al*., 2004).

4.2 Biofilm development as affected by different organic matter

Several studies showed that organic matter affects the biomass and diversity of biofilms (Allan *et al*., 2002, Chen *et al*., 2005, Rice *et al*., 2005, Xia *et al*., 2010). For this reason, we decided to examine this parameter under controlled conditions. Examination of 24 h old biofilm formed by single species culture incubated with basalt gravel showed that isolate Ssw established higher single species biofilm (SSB) biomass in the presence of low organic medium (Fig. 10). This may be explained by biofilm-formation enhancement

in response to stress conditions, as has been found in *Staphylococcus epidermidis* (Fitzpatrick *et al*., 2005) and *Escherichia coli* (Zhang *et al*., 2007). However, all six other isolates established highest SSB biomass levels (represented as the number of colony-forming units) in the presence of the highest levels of organic load (Fig. 10). These results are supported by earlier SSB studies (Allan *et al*., 2002, Chen *et al*., 2005, Rice *et al*., 2005), in which high organic load was shown to enhance biomass of biofilm-forming laboratory strains. Furthermore, highest biomass of biofilm established by a mixture of all seven isolates together (MSB) was found in the presence of the high organic load medium (Fig. 11). Xia *et al* (2010), examining the effect of organic loading on membrane biofilms, showed that membrane fouling occurred earlier and faster under high organic load conditions. In addition, enhanced growth rate and biomass were detected under high organic load conditions in WWT biofilms (Tal *et al*., 2003, Wuertz *et al*., 2003).

Previous studies demonstrated that hydrophobic bacteria generally adhere better to both hydrophobic and hydrophilic surfaces (Daffonchio *et al*., 1995, Huysman and Verstraete, 1993, Stenstrom, 1989). Therefore, cultures of each of the seven isolates were examined for hydrophobicity level (adhesion to hydrocarbons) in parallel to biofilm-formation ability on basalt gravel. Even though no significant correlation was found between the hydrophobicity characteristics of the isolates and their capacity to produce SSB on gravel, biofilm formed by the most hydrophobic isolates (Osw, Csw) formed the highest SSB biomass (Table 4). These results indicate that although hydrophobicity is important, it may not be the main force influencing the adherence to the matrix here.

The organic load concentration in the colonization assay affected the diversity of biofilm constructed by mixed culture of the isolates as well as native mixed community

originated from treated wastewater. Higher organic load media resulted in lower biofilm diversity (Fig. 11 and 12). Similar results were presented by Van der Gucht *et al*., (2005), who examined the bacterial communities in four freshwater lakes differing in nutrient load and concluded that the less diverse bacterioplankton community was related with high values of suspended matter. This trend can be described as a negative 'diversity-productivity' relationship (Smith, 2007). The rationale behind this model is that competition intensity increases as the rate of biomass production increases. An explanation for this phenomenon may be that high organic load selects for certain bacterial species that out-compete the others. In microhabitats with a modest supply of resources, groups of species can coexist at relatively low abundances, resulting in higher species richness (Guo and Berry, 1998). Similar phenomenon of reduced bacterial diversity concomitant with increased biomass has also been observed in the rhizosphere, which is characterized by high organic load (mainly due to plant exudates) relative to the bulk soil, and is described as the 'rhizosphere effect' (Gomes *et al*., 2001, Smalla *et al*., 2001, Kent and Triplett, 2002).

The ability of the different isolates to form SSB was not always consistent with their ability to form MSB; isolate Osw, which produced the highest SSB biomass of all seven isolates on gravel (Fig. 10), was not present in this high organic-load MSB. Moreover, Osw represented only a limited proportion of the low organic-load MSB (Fig.11). The same trend occurred with isolate Ysw (Fig. 10 and 11). These differences in isolates ability to form SSB versus MSB may result from interspecies relationships that affect the adherence and growth of these isolates. Such relationships may be competition (Omil *et al*., 1998, Tsuno *et al*., 2002), quorum sensing (Dobretsov *et al*., 2009) or the production

of inhibitory compounds by other bacteria (Rao *et al*., 2005). Leung *et al.* (1999) showed that the presence of one species of microorganism on a surface can promote the adhesion of another. Andersson *et al.* (2008) investigated biofilm formation of 13 different bacterial species in pure and dual mixed cultures. Their research showed that in dual-strain cultures, strains with different properties either complemented or out-competed each other, affecting biofilm biomass in either a synergistic or antagonistic way. Complementation and antagonism in dual-species biofilm formation assays were also observed by Mengying *et al*. (2007), investigating *Comamonas testosteroni A3*, and Rao *et al*. (2005), investigating *Pseudoalteromonas tunicata*.

The abundance of isolate Lsw, which dominated the high organic-load MSB, decreased with the reduction of organic load (Fig. 11). As mention above, higher organic load supporting higher biofilm biomass has been observed previously in a variety of environmental samples (Tal *et al*., 2003, Wuertz *et al*., 2003, Xia *et al* 2010). Lsw biomass reduction resulted in Csw representing the highest portion of the medium-, low- and water media-MSBs since Csw was characterized by relatively constant levels of biomass in all organic matter concentrations examined (Fig.11).

In summary; the biofilm biomass produced by the different isolates varied between SSB and MSB and organic matter had a strong effect on this system in terms of biomass and diversity. Hence, this study had revealed the affect of one a-biotic parameter on SSB and MSB of selected isolated bacteria. Further investigation is needed for other a-biotic parameters and other bacterial species under environmental conditions.

4.3 Degradation of estrogens by pure culture estradiol degrading bacterium (EDB-LI1)

In the current study, eight phylogenetically diverse estradiol degrading bacteria (EDB) were isolated from an enrichment culture originating from CWs' biofilm. All eight isolates were able to transform E2 to E1, yet only one of them was able to remove E1 (Isolate EDB-LI1). From the literature and from our results, it appears that the ability to transformation E2 to E1 is more widespread than the ability to degrade E1 (D'Ascenzo *et al*., 2002): for instance, Yu *et al*. (2007) isolated 14 phylogenetically diverse EDB from activated sludge of a wastewater-treatment plant, belonging to three different phyla: *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. All 14 isolates were capable of converting E2 to E1 by 17β-reduction, but only three strains showed the ability to degrade E1. E1 has one-half the estrogenic potency of E2, but it is not well degraded in wastewater-treatment systems. As a result, E1 appears to be an important endocrine disrupter in aquatic environments (D'Ascenzo *et al*., 2002). Therefore, we focused on the isolate EDB- LI1, found to efficiently remove both compounds and identified as a member of the *Sphingomonadaceae* family. A similar bacterium, *Novosphingobium* sp. strain JEM-1, (98% 16S rRNA gene sequence similarity to EDB-LI1), isolated from conventional activated sludge plan was also shown to be able to degrade both E1 and E2 (Hashimoto *et al*., 2010). Another E2 and E1 degrading bacterium, named CYH, isolated from an artificial sandy aquifer in Singapore, was also a member of the *Sphingomonadaceae* family (93% 16S rRNA gene sequence similarity to EDB-LI1) (Ke *et al*., 2007). It is interesting to note that bacteria of phylogenetic similarity were isolated

from different geographical regions and exhibit the ability to degrade estrogens. Other members of the *Sphingomonadaceae* family are known to degrade a variety of other pollutants (Balkwill *et al*., 2006).

EDB-LI1 was able to produce biofilm on sterile basalt gravel. It exhibited a higher growth rate in biofilm than in liquid culture (Fig. 17). E2-removal activity of EBD-LI1 was also higher in the biofilm on a per-cell basis. Even though it is generally accepted that bacteria assembled in biofilm are less active than planktonic cells (Hall-Stoodley *et al*., 2004), some studies showed that biofilm cells can be more active than planktonic cells (Griffith *et al*., 1994, Lin *et al*., 2007). The current study focused on the first few days of biofilm assembly, in which biofilm cells may be more active than planktonic cells, as was demonstrated for *Streptococcus mutans* biofilm (McNeill and Hamilton, 2004). In that study, glycolytic activity, glucose uptake and protein synthesis in the first two days were higher in biofilm cells than in planktonic cells, with overall biofilm activity 5–13 fold higher per cell than in planktonic cells (McNeill and Hamilton, 2004).

As was mentioned in the introduction of this thesis, the ability to carry out 17β-reduction of E2 to E1 was reported for a variety of microorganisms of different taxonomy (Donova et al., 2004). However, further microbial degradation of E1 is less common and very little is known about the enzymes involved in this process. Advanced molecular tools enables to search for bacterial enzyme involve in steroid degradation. For example, recent study found the enzyme carboxylesterase, which is known to be involved in steroid degradation, in a marine bacterium H5, which degrade different hormonal pollutants (Sang et al., 2011). Another recent study found steroid degradation gene hot spot in *Comamonas testosteroni* TA441 (Horinouchi et al., 2010). Also, Rowley *et al* (2003)

reported the induction of cytochrome p450BM3 in *Bacillus megaterium* by 17β-estradiol. Cytochrome P450s support the oxidative, peroxidative and reductive metabolism of steroids and a variety of other environmental pollutants (Danielson, 2002). Since cytochrome p450 is also known to be involve in estrogen metabolism in mammals (Ohe *et al*, 2000, Thompson Andambersone, 2000, Cribb *et al*., 2006), we decided to investigate the relationship between this enzyme and E1 degradation in EBD-LI1. However, preliminary experiments using 2-Methyl-1,2-di-3- pyridyl-1-propanone (metyrapone) (Guengerich and MacDonald, 1990), which is a known inhibitor for bacterial cytochrome p450, had no effect on the E1 and E2 degradation by EDB-LI1 culture (appendix, Fig. 2). In addition, preliminary experiments using 2D gel electrophoresis did not reveal any potential estrone degrading enzyme (appendix, Fig. 3). Therefore, we can not append additional information regarding bacterial pathways for E1 degradation.

4.5 Bioaugmentation of EDB-LI1

In this part of the current study we describe the events occurring during EDB-LI1 integration into two different experimental systems: biofilm originated from CW and soil columns. Bioaugmentation resulted in successful integration of EDB-LI1 into both systems and affected both bacterial community composition and E2 degradation. Success of bioaugmentation is determined by the ability of the integrated bacterium to survive in the augmented environment and its ability to degrade the target pollutant (El Fantroussi and Agathos, 2005). EDB-LI1 was able to meet both criteria within the two experimental systems: successful integration to wetland biofilm and to soil columns (Fig.

19 and 22, respectively) as well as E2 removal (Fig. 20). Several studies have shown enhancement of pollutant degradation by bioaugmentation of a specific bacterial strain or a mixture of bacteria, in biofilms aimed at treating different types of wastewater, such as petrochemical wastewater (Ma *et al*., 2009), wastewater containing 3-chloroaniline (Bathe *et al*., 2009), wastewater containing sulfate (Mohan *et al*., 2005) and recently even successful bioaugmentation of estradiol degrading bacteria into conventional activated sludge (Hashimoto *et al*., 2010). However, the effect of augmented bacteria on community composition and function of CW biofilm and soil assembled under different conditions was less explored (Niu *et al*., 2009, Wu *et al*., 2008).

We studied bioaugmentation of EDB-LI1 into a multiple-species, mature wetland biofilm with respect to microbial community composition and activity.

In the current study, bioaugmentation of EDB-LI1 was investigated in two biofilms obtained from wetland ponds representing two water-treatment stages. Bioaugmentation of EDB-LI1 into biofilm obtained from pond 2 (advanced treatment: thus the biofilm was developed in water of higher quality than that developed in pond 1) was more efficient than bioaugmentation in biofilm obtained from pond 1: (i) a higher level of EDB establishment was found in the augmented biofilm obtained from pond 2 *vs.* pond 1 (Fig. 19), (ii) higher E2 and E1 removal was found in augmented biofilm obtained from pond 2 *vs.* pond 1 (Fig. 20), (iii) changes in microbial community composition following augmentation were detected only in biofilm samples originating from pond 2 (Fig. 18). Other studies investigating the influence of bioaugmentation on microbial community composition have given varied results: Niu *et al*. (2009) showed that bioaugmentation of *Pseudomonas putida* ZWL73 into soil microcosms contaminated with 4-

chloronitrobenzene stimulates the *Actinobacteria* phyla along with a specific population of *Acidobacteria.* Also, ammonia-oxidizing bacteria, obtained from the augmented soil, were phylogenetically different from those in other treatments. However, α and *β proteobacteria* were not distinctly affected by this bioaugmentation. Wu *et al*. (2008) showed that bioaugmentation of *Monilinia* strain *W5-2* into soil microcosms contaminated with polycyclic aromatic hydrocarbons (PAHs) has only a negligible effect on the indigenous microbial community.

The observed higher efficiency of bioaugmentation in pond 2 can be explained by the negative 'diversity- productivity' relationship (Smith, 2007), which claims that in microhabitats with a modest supply of resources (pond 2 in this study), groups of species can coexist and not out-compete each other. Since pond 2 received feed water with a relatively low amount of nutrients (Materials and Methods, see 2.2.2), according to this theory it should allow coexistence of EDB-LI1 with the "native" communities. Indeed, the integration and survival of EDB-LI1 after 24 h was higher in biofilm originated from pond 2 in comparison to biofilm originated from pond 1, which received feed water with higher amount of nutrients. Furthermore, the bacterial community composition of pond 2 biofilm was more affected by the augmentation of EDB-LI1 than pond 1 biofilm.

In order to examine the integration of EDB-LI1 into a different system, characterized by different chemical and physical parameters, we also studied bioaugmentation of EDB-LI1 into soil with respect to the effect on microbial biomass, community composition and activity. The experiment was conducted in soil under constant water flow. One of the most important physicochemical factors that affect microorganisms in soil is adequate supply of available water. Bacteria are essentially aquatic organisms which require a

sufficient amount of water contents for growth (van Elsas *et al*., 1997). Bacteria within the soil flourished for a few days, utilizing the available organic matter and reaching up to 10^8 bacteria per g soil (Fig. 23). Population size in mineral soils is directly related to the organic matter content (Alexander, 1961). Since almost no organic matter was added to the column (only 14 μg estrogens to the whole column), after a few days the available natural soil organic matter was utilized. As a result, the abundance of the bacteria dropped (Fig. 23). The same phenomena occurs in soil bacteria during different seasons (Alexander, 1961, Van Elsas *et al*., 1997); microbial population burst takes place in the spring, as the soil becomes warm and organic matter from previous fall and winter becomes accessible for decay. The additional source of available carbon is enhancing microbial growth until this source has been mineralized and the activity and density of the microbiota decline to the original state. Indeed, decline occurs during the hot, dry summer months when the organic matter is utilized (Alexander, 1961, Van Elsas *et al*., 1997).

Augmentation of EDB-LI1 into the soil columns resulted in its settlement in the inlet layer. This finding may be explained by adequate environmental conditions in the inlet which supported EDB-LI1 survival (Alexander, 1999). EDB-LI1 biomass in this layer changed with time similarly to total bacterial biomass (Fig 22 and 23, respectively). Settlement and growth followed by a decrease in the EDB-LI1 abundance was detected in parallel to the increase and reduction of total bacteria biomass. Six days after being augmented, EDB-LI1 biomass dropped below detection limit. Survival of augmented microorganisms in soil or groundwater is rare (Stroo and Ward, 2010). There are numerous reasons for the failure of added bacteria to survive in the environment,

including predation, parasitism, inability to compete for nutrients or substrates and environmental condition to which they are not adapted (Goldstein *et al*., 1985). Since the reduction of EDB-LI1 biomass was parallel to the reduction of total bacterial biomass (Fig 22 and 23), we suggest that utilization of the organic matter and estrogen depletion flowing though the column was the main reason for EDB-LI1 disappearance. In the absent of estrogen, EDB-LI1 had no advantage over other naturally occurring soil bacteria and therefore it did not survive.

Augmentation of EDB-LI1 into soil column also affected the soil bacterial community composition (Fig 21). While 6h after the estrogen spike the community composition of all samples clustered together, after 2.5 days the augmented column (both spiked with E1 and spiked with E2) were distinguished from that of un-augmented column (Fig. 21A). A shift in microbial community composition as a result of EDB-LI1 augmentation was also detected in the CW biofilm (Fig. 18). Niu *et al* (2009) also showed that augmentation of *Pseudomonas putida* ZWL73 affected bacterial community composition of soil column. 5 days after E2 spike these changes in the community composition started to fade (Fig 21D). This may be explained by the disappearance of EDB-LI1 and reduction of total bacteria biomass. In contrast, after the shift in community composition of the augmented column after the E1 spike, community remained similar even after 7 days (Fig 21C). Furthermore, the bacterial community composition of this augmented column clustered together with community composition of a column spiked with E1 without EDB-LI1 augmentation (Fig.21B). Wawrik *et al*., 2005 showed that soil microbial community compositions differ in the presence of different carbon source (see review Torsvik *et al*.,

1997). This might explain the differences in bacterial community composition of columns spiked with different estrogens.

The information obtained from this part of the study may help to design an *in situ* bioaugmentation strategy for the removal of estrogenic pollution in water treated in constructed wetlands and in polluted soils.

5. Conclusions

The focus of this thesis was the microbial aspects of CW biofilm, formed during advanced treatment of purified waste water. The microbial assembly, composition and activity of the biofilm were investigated, as well as the possibility for integration of inoculated bacteria into the biofilm.

Depth had the strongest effect on microbial community composition of mature microcosm's biofilm. Since oxygen, ammonia and organic matter were consumed while the treated water was flowing through the pond, we assumed that substrate availability was a major variable affected by the depth. Therefore, further investigations on the effect of the organic matter load on biofilm formation under controlled conditions were performed. High organic load promoted higher biomass in single species biofilm of isolated bacteria, originating from CW. Examination of multiple-species biofilm of these isolates revealed that diffrences in organic matter concentrations influenced biofilm innitiation in terms of microbial community composition, diversity and biomass.

In addition, examination of two different CW treatment stages (early and advanced ponds) and their feed water revealed that the quality of the influent feeding the CW pond affected the biofilm microbial community composition, diversity and function. The bacterial community composition and diversity of 24-h-biofilm initiated in the influent water feeding pond 1 (early treatment) differ from that of 24-h-biofilm initiated in the influent water feeding pond 2 (advanced treatment). Biofilm biomass and microbial diversity are two criteria, which are essential for wastewater cleanup process by CW. The

results of this part of the study showed that high organic load promotes high biofilm biomass but low diversity. This suggests that organic load is influencing the balance of these two parameters, and as a result, possibly the wetland decontamination efficiency. Furthermore, bioaugmentation experiments revealed that mature biofilm originated from pond 2 was more affected by EDB-LI1 integration than mature biofilm originated from pond 1 in terms of microbial community composition and function. Organic matter quantity is one of the differences between the feeding water of these two ponds and could be a factor affecting bioaugmentation process and, as a result, the ability of the augmented CW to remove E2.

Hence, in the current study the effect of organic matter may be one of the main criteria which influenced CW biofilm initiation, maturation and bioaugmentation.

To summarize, the focus of this thesis was to resolve the 'black box' of CW biofilm in terms of WHO are the bacteria within the biofilm and WHAT are they doing, especially in the aspect of treating wastewater. However, new and intriguing questions had risen from the answers. For example; 50% of the sequences, representing dominate bacteria that assembled the CW biofilm, were phylogenetically close to uncultured bacteria. Who are these bacteria and what is their role in treating wastewater? What are the microbial activities that affect the quality of the CW effluent? How can we use the knowledge obtained from this study to manipulate CW biofilm in order to improve the CW efficiency? What is the long term effect of EDB-LI1 on biofilm in environmental condition in the CW site itself? Can EDB-LI1 bioaugmentation be used as a solution for E2 pollution problem in effluent of WWT systems and in irrigated soils?

Further investigation of CW biofilm will enable profound understanding of one of the techniques for biological wastewater treatment, which is an important and widespread biotechnological application.

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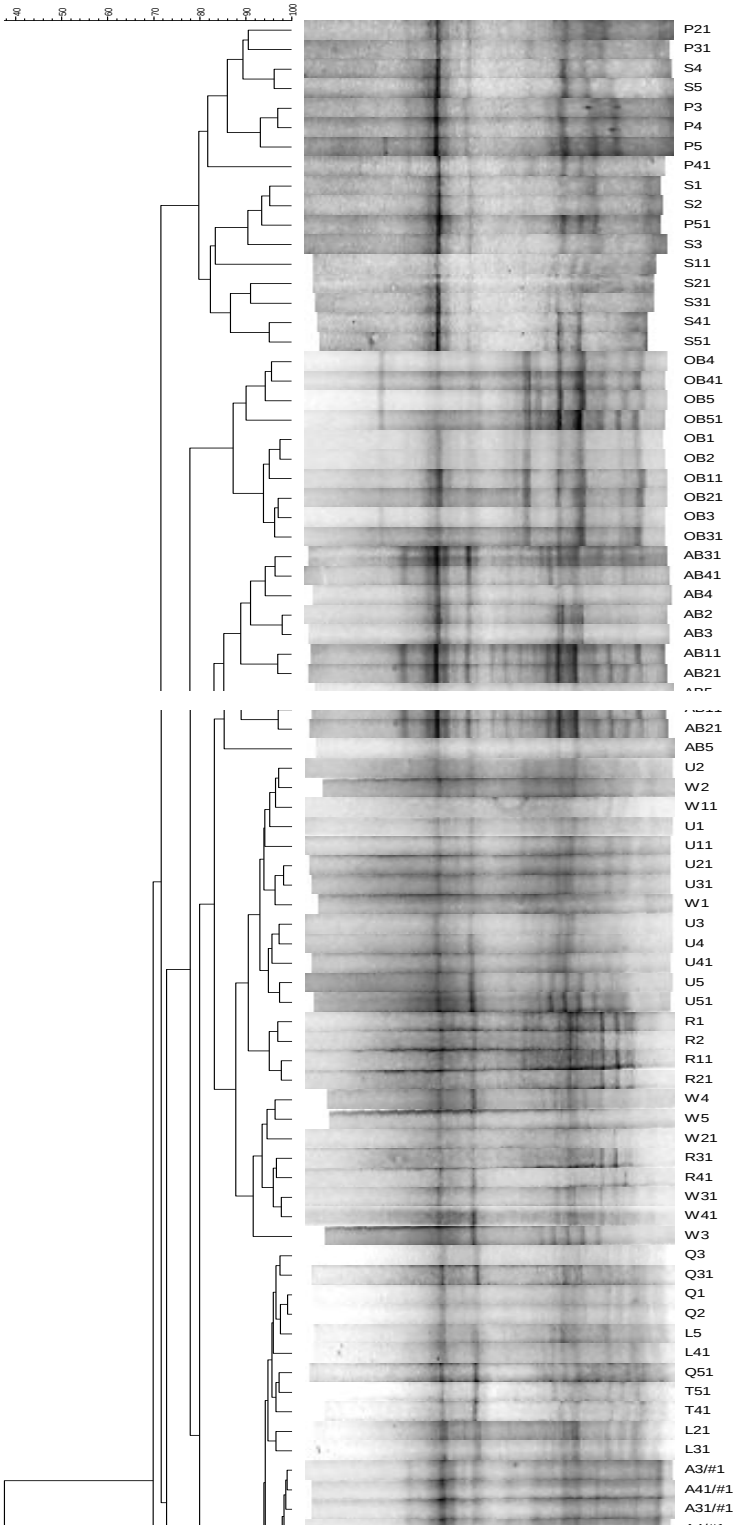
Appendix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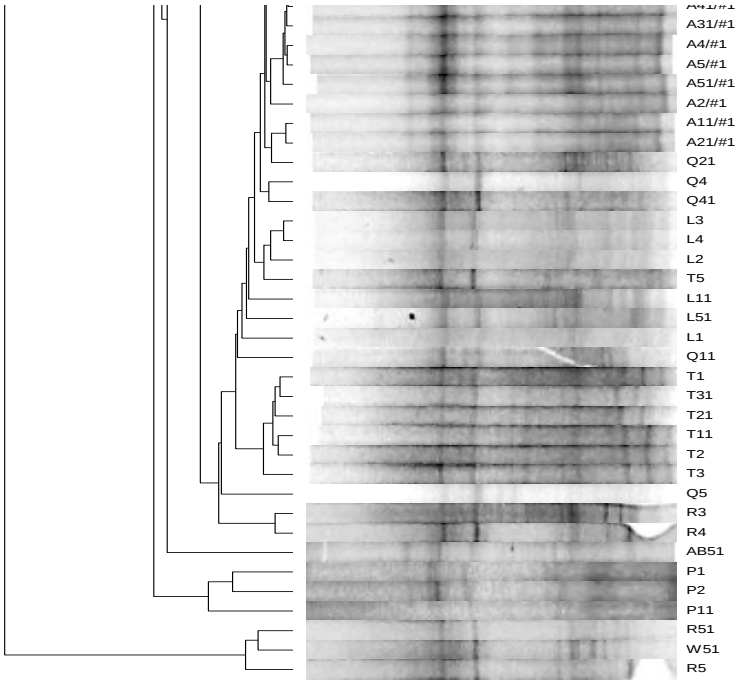


Fig. 1: Cluster analysis of DGGE patterns of the soil column bacterial community as affected by time, column depth, estrogen spike and EDB-LI1 augmentation. The layers (1-5) in the different treatments were examined in duplicates.

Principal-component analysis (PCA) analysis was preformed on this DGGE pattern (Fig. 21).

Samples name: O: 4h before the estrogen spike, R: 6h after E1 spike+EDB-LI1, T: 6h after E2 spike+EDB-LI1, L: 6h after E2 spike no EDB, Q: 2.5 days after E2 spike no EDB, W: 2.5 days after E2 spike+EDB-LI1, U: 2.5 days after E1 spike+EDB-LI1, AB: 5 days after E2 spike+EDB-LI1, OB: 5 days after E2 spike+EDB-LI1, P: 7 days after E1 spike no EDB, S: 7 days after E2 spike+EDB-LI1, A#: 10 days after the E2 spike+EDB-LI1

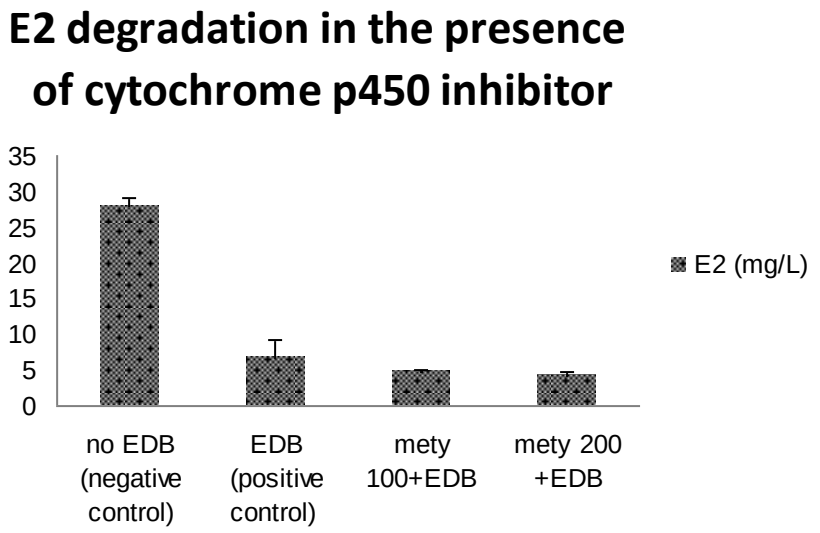
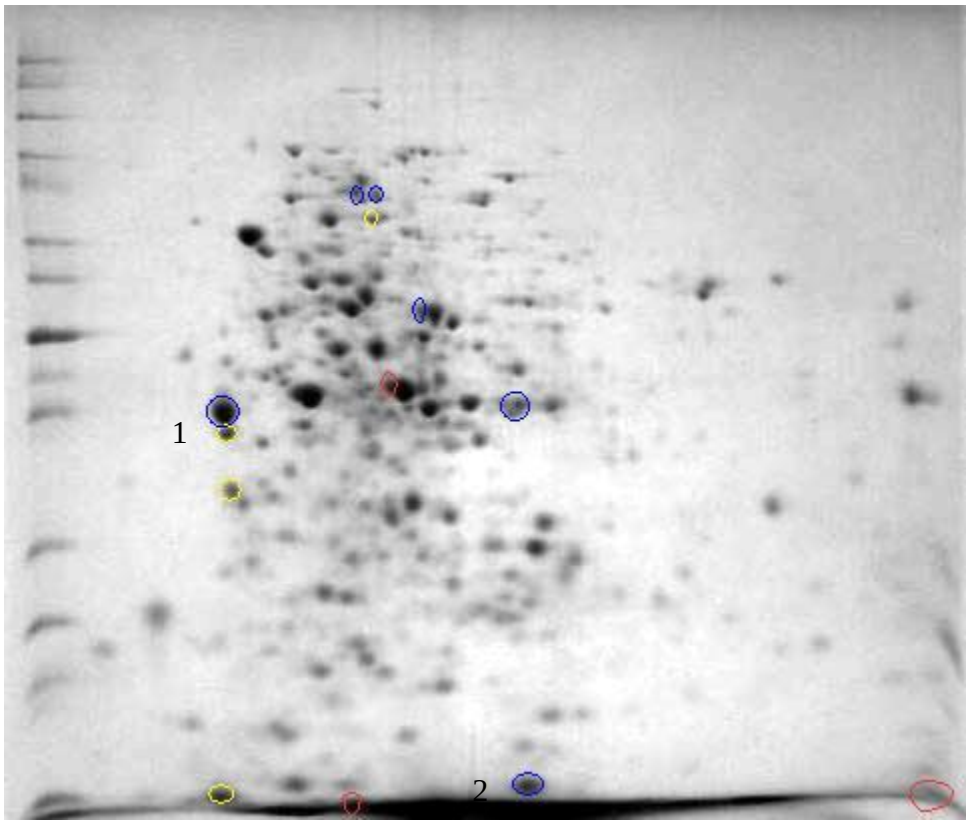


Fig. 2: Effect of the cytochrome P450 inhibitor metyrapone (mety) (concentrations; 100 and 200 mM) on E2 degradation by EDB-LI1. E2 remains after incubation of 25 mg/L of E2 with EDB-LI1 for 24h.

A



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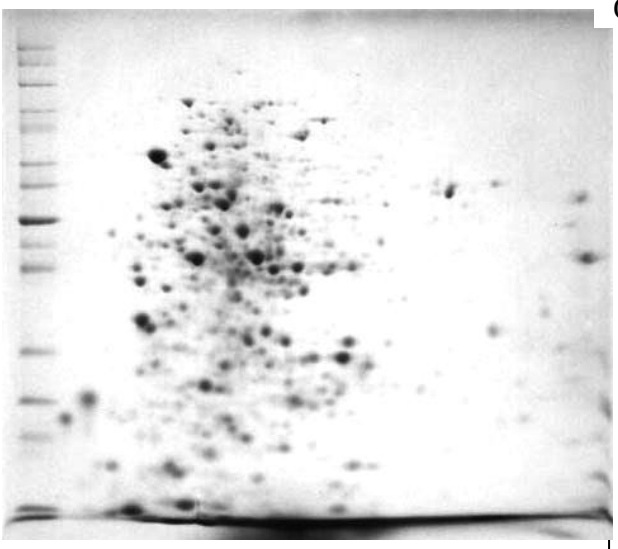

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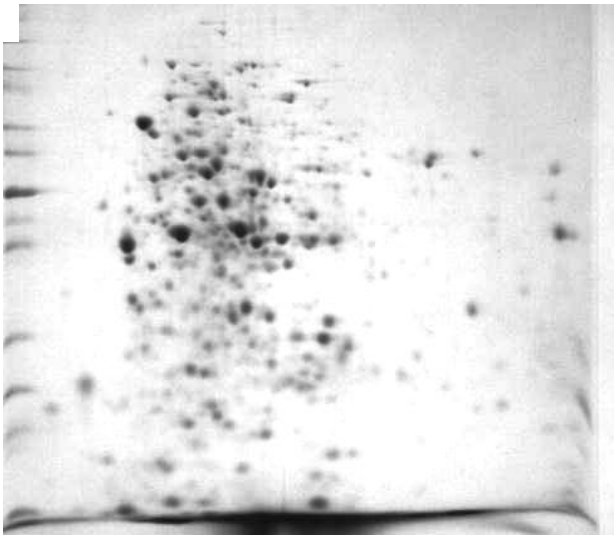

Fig. 3: Comparison of two-dimensional gel electrophoresis (A) of soluble proteins obtained from EDB-LI1 culture grown in the presence (C) and absents (B) of E1 as a sole carbon source.

Proteins marked in blue (A) are more abundance in the presence of E1. After careful examination of these two gels only two proteins seemed significantly more abundant in the presence of E1 (marked 1 and 2). The N-terminal sequence of these proteins showed similarity to: 1) Azurin- 29% coverage [Pseudomonas entomophila L48] 2) Hypothetical protein PputGB1_3141 -23.3% coverage [Pseudomonas putida GB-1]. These proteins did not appear as estrone degrading enzymes and we did not investigate this system further.

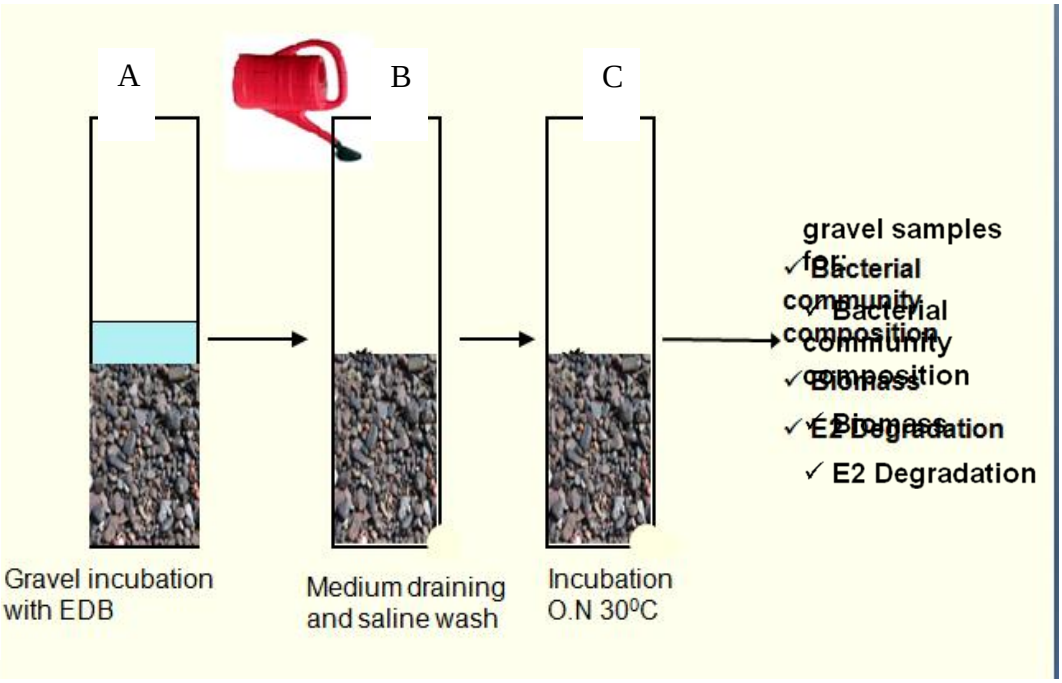

Fig. 4: Schematic drawing of biofilm colonization assay: (A) basalt gravel (8 mm), identical to that used in the constructed wetland, was incubated with bacterial cultures in sterile tubes at 30°C. (B) after 4 h of incubation, the tubes were drained by piercing and the gravel was gently washed twice with sterile saline and (C) incubated for 20 h at 30°C (forming 24-h-old biofilm). Gravel samples were collected for different analysis (plating and DNA extraction).

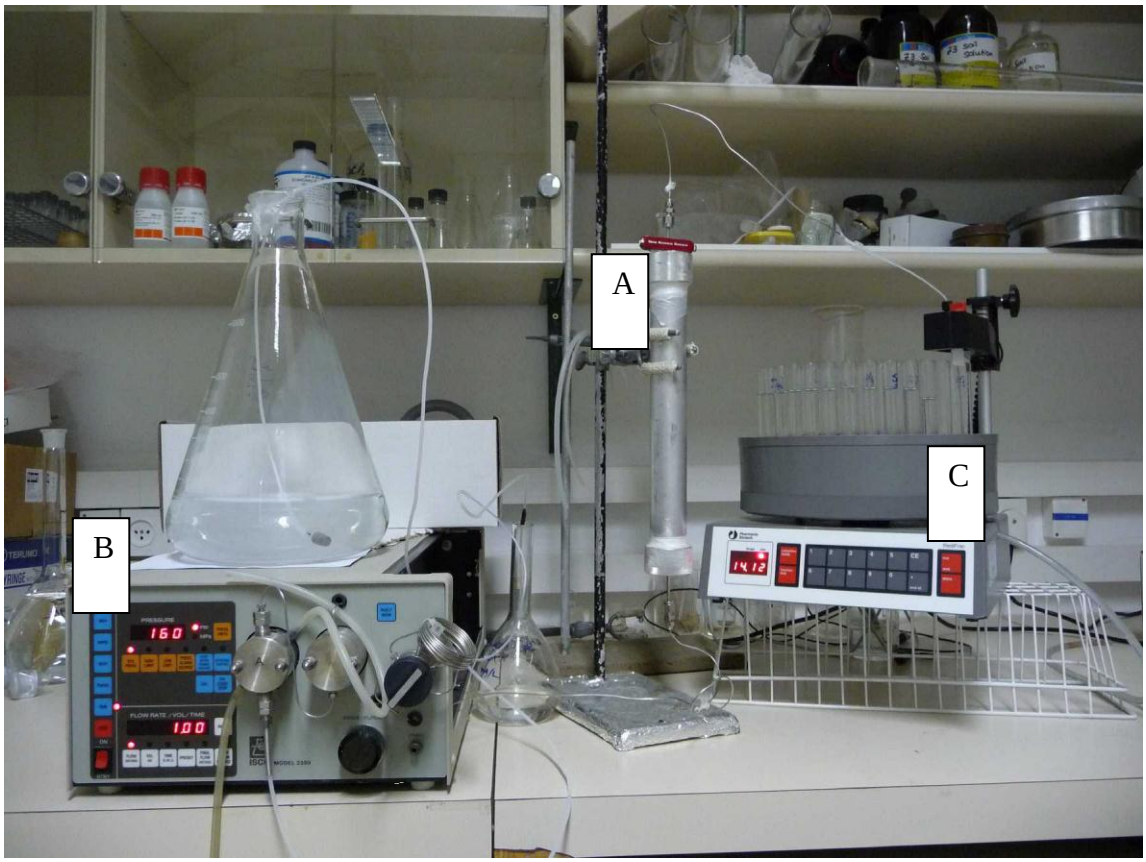

Fig. 5: Photograph of the soil column assay: (A) aluminum column filled with soil augmented/ un-augmented with EDB is connected to an (B) HPLC pump with an injection loop of 1 mL as inlet, and to a 0.16 cm diameter stainless steel tube as outlet to a (C) fraction collector with 10 mL glass tubes. Samples collected were analyzed for the presence of hormones in the outflow.

תקציר

בעשורים האחרונים, התפוצצות אוכלוסין והתפתחות התעשייה גרמו למחסור גדול במים שפירים. המחסור הזה הינו בעייתי במיוחד במדינות שבהן מקורות המים המתוקים הינם מוגבלים, כמו מדינת ישראל. פיתוח מערכות טיפול במי שופכין, שיפיקו מים באיכות גבוהה, למטרות שימוש חוזר בחקלאות הוא חשוב להפחתת שימוש במים שפירים מחד ולמניעת זיהום סביבתי מאידך במקרה של שחרור מים מושבים לנחלים. אגנים ירוקים הינם מערכות לטיפול במים המציעות פיתרון חסכוני וידידותי לסביבה. בדומה למערכות אחרות לטיפול במים, ביופילמים (מרבדים ביולוגיים), הצמודים למצע האגנים הירוקים, הם האחראים לרוב פירוק החומר האורגאני והמזהמים במי השופכין. למרות זאת, אין מספיק מידע על החיידקים המרכיבים את הביופילם הזה ועל פעילותם.

המטרה המרכזית של עבודה זו היא לחקור את האספקטים המיקרוביאליים של ביופילם הנוצר באגנים ירוקים בזמן טיפול מי קולחין שניוניים.

אגנים ירוקים הינם מערכות מאד מגוונות מבחינת התכונות הכימיות והפיסיקאליות שלהם. הגיוון הזה בא לידי ביטוי על ידי גראדינטים שונים של פוטנציאל חמצון, זמינות חומר אורגאני, ותנאי סביבה שונים כמו טמפרטורה וpH. מסיבה זו נוצרות נישות שונות בתוך בריכות האגנים הירוקים ובנישות אלו מתבצעים תהליכים ביוכימיים שונים. התנאים השונים בנישות השונות יכולים לתרום להתפתחות ביופילם בעל הרכב ופעילות מיקרוביאלית מגוונים.

מטרת המשנה הראשונה של העבודה הינה לחקור את הרכב אוכלוסיות החיידקים ופעילותם בביופילם של אגנים ירוקים בהתאמה להשפעת נוכחות צמחיה, עומק הבריכה, וסוג המצע של האגנים הירוקים.

בחלק זה של העבודה זוהו חיידקים דומיננטיים (על ידי דמיון ברצף 16S rRNA) שהרכיבו את ביופילם האגנים הירוקים על מצע חצץ עם וללא צמחיה. מגוון גדול של אוכלוסיות חיידקיות נמצא בביופילם, הכולל

נציגים מתת- מחלקות: β ,α, δ *Proteobacteria*, *Bacteroidetes* ו *Acidobacteria* . ניתוח של דפוס המקטעים באנליזת DGGE של גן הrRNA 16S הראה שלעומק בריכת האגנים הירוקים יש את ההשפעה הגדולה ביותר על הרכב אוכלוסיות החיידקים בהשוואה לנוכחות צמחיה ולסוג המצע. התוצאות האלו מצביעות על כך שעומק הינו אחד הגורמים המרכזיים המשפיעים על הרכב אוכלוסיות החיידקים ותפקודם במערכת שנחקרה.

תהליך התפתחות הביופילם הצעיר משפיע על הרכב אוכלוסיות החיידקים ופעילותם בביופילם בוגר. התפתחות ביופילם תלויה בגורמים רבים הכוללים חיידקים הנמצאים במים המזינים את המערכת, משטרי זרימה, סוג המצע וזמינות חומרי מזון (נוטריאנטים). לריכוז חומרי מזון ועומס אורגאני יש השפעה גדולה על התפתחות ביופילם מבחינת ביומאסה ומגוון חיידקי.

מטרת המשנה השנייה של עבודה זו הינה להעריך את השפעת העומס האורגאני על ביומאסה, הרכב ומגוון אוכלוסיות חיידקים בביופילם המתפתח על מצע אגנים ירוקים בתנאים מבוקרים.

שבעה חיידקים, שונים מורפולוגית, בודדו מביופילם של אגנים ירוקים. כל אחד מהתבדידים האלו בנפרד יצר ביופילם על מצע חצץ בזלת. בנוסף, כל שבעת התבדידים יחד יצרו ביופילם מעורב. הביומאסה של ביופילם מכל תבדיד בנפרד ושל הביופילם המעורב גדלה כאשר כמות העומס האורגאני במדיום עלתה. בנוסף, תוצאות המחקר מרמזות שעליה בעומס האורגאני גורמת לירידה במגוון החיידקים בביופילם המעורב. בשלב הבא בחלק זה של המחקר נבדקה התפתחות ביופילם, שנוצר ממי קולחין בשתי רמות ניקיון (מים המזינים את בריכת הטיפול המוקדם ומים המזינים את בריכת הטיפול המתקדם). המים המזינים את בריכת הטיפול המתקדם הינם המים שטופלו בבריכת הטיפול המוקדם ולכן מכילים פחות חומר אורגאני. ביופילם, שהתפתח תחת מים המזינים את בריכת הטיפול המוקדם, הכיל מגוון חיידקים קטן יותר מביופילם, שהתפתח תחת מים המזינים את בריכת הטיפול המתקדם.

משמע, בכל הניסויים שנערכו, ביופילם מגוון יותר נוצר תחת עומס אורגאני נמוך יותר.

אגנים ירוקים נחשבים למערכת יעילה לטיפול במים. אם זאת, מספר מזהמים נשארים בכמויות מזיקות במי הקולחין של אגנים ירוקים. אחת הקבוצות של מזהמים אלו היא שיירים אסטרוגנים, היכולים לפגוע באיזון של מערכת אקולוגית. מזהמים אלו משבשים את הפעילות התקינה של המערכת האנדוקרינית ויכולים להזיק לבעלי חיים ימיים ולבריאות הציבור. אסטרוגנים טבעיים, כמו אסטרדיול (E2) נשארים בכמויות היכולות להשפיע על בע"ח שונים גם לאחר טיפול באגנים ירוקים. הביואוגמנטציה (הוספת חיידק בעל יכולות פירוק ספציפיות לשטח מזוהם) הינה טכניקה היכולה לשפר סילוק מזהמים שונים, כמו E2. יעילות הביואוגמנטציה נקבעת על ידי שרידות החיידק בשטח וקצב פירוק המזהם. מחקרים שונים הראו כי מספר מזהמים מתפרקים ביעילות כתוצאה מביואוגמנטציה מוצלחת ולכן החלטנו לבחון את הטכניקה הזו על פירוק אסטרדיול.

מטרת המשנה השלישית של עבודה זו הינה לבודד חיידק בעל יכולת לפרק אסטרדיול מביופילם האגנים הירוקים ולבחון את פוטנציאל השתלבותו חזרה בביופילם (ביואוגמנטציה).

חיידק מפרק E2 בודד מביופילם האגנים הירוקים ונקרא EDB-LI1. EDB-LI1 המיר ביעילות אסטרדיול לאסטרון (E1). בנוסף, החיידק פירק וגדל על E1, אחד התוצרים היציבים מטבולית של פירוק E2. EDB-LI1 הראה 98% התאמה (על ידי השוואת רצף הגן המקודד ל 16S rRNA) ל-*Novosphingobium JEM-1*, חיידק שבודד ממפעל לטיפול בשפכים ביפן ואף הוא בעל יכולת לפרק E2. השפעת הביואוגמנטציה על הרכב ופעילות החיידקים בביופילם האגנים הירוקים נבחנה בתנאי מעבדה. EDB-LI1 הוסף לביופילמים שנלקחו משתי בריכות אגנים ירוקים המייצגים שני שלבי טיפול במים (מוקדם ומתקדם). חקר השתלבות EDB-LI1 בביופילם האגנים הירוקים העלה שלושה ממצאים עיקריים: (1) למרות העובדה שמקורו מביופילם האגנים, נוכחות EDB-LI1 נמצאה (על ידי qPCR) אך ורק בביופילם שאליו הוסף החיידק. (2) ביופילם אליו הוסף החיידק רכש את היכולת לפרק E1 ו E2. (3) הרכב אוכלוסיות החיידקים (לפי אנליזת DGGE) היה שונה בין ביופילם שאליו הוסף החיידק לבין ביופילם ללא תוספת. יתרה מכך, הוספה של EDB-LI1 לביופילם שמקורו מבריכת הטיפול המתקדם הראתה סילוק גבוה יותר של E1 ו E2 לעומת הוספה של החיידק לביופילם מהטיפול המוקדם. משמע, יעילות הביואוגמנטציה תלויה כנראה גם באיכות המים המזינים את בריכת האגנים הירוקים וגם בהרכב אוכלוסיות החיידקים בביופילם של הבריכה.

לסיכום, חקרנו את התיישבות, הרכב ופעילות החיידקים בביופילם של אגנים ירוקים וכן את האפשרות לשלב חיידק המפרק E2 לביופילם. אנו מציעים שריכוז החומר אורגאני במים המטוהרים הוא אחד הקריטריונים המשפיעים על התפתחות ביופילם, הרכב אוכלוסיות, פעילות וביואוגמנטציה במערכת האגנים הירוקים שנבדקה.

לעתיד, בדיקה יסודית לאורך זמן של ביואוגמנטציה של חיידקים לביופילם של האגנים הירוקים בתנאי שדה תברר את היתכנות ישום טכנולוגיה זו כפתרון לסילוק שאריות E2 או מזהמים נוספים בקולחין.

שלמי תודות

אני רוצה להודות למנחים שלי:

דרור מינץ, שלימד אותי איך לחשוב, לכתוב ולחיות מדע. ההנאה וההתרגשות שלו מהתוצאות עודדו אותי והלהיבו אותי כך שעבודת המחקר נעשתה בכיף ובעניין משותף.

יצחק הדר, העצות שלו תמיד חידדו את הכיוון אליו כדאי ללכת. עם זאת, תמיד אפשר לי להתנסות (ולעיתים להיכשל) בדרכי.

לחברי המעבדה הנוכחיים והקודמים: פטריסיה, שחף, משי,אייל, יעל, מקס, סמי, איגור מילנה , ואלה ובמיוחד למיה שעזרה ותמכה בי גם בשלב הטכני, המדעי, גם בכתיבה ובעיקר כי הייתה חברה אמיתית, מפרגנת ותומכת.

להילה שעזרה לי מאד בכתיבה, בה כל כך התקשיתי

ללריסה ואדי, שעזרו לי מאד במהלך העבודה אם בניסויים ואם בכתיבה.

למעבדות איתן שיתפתי פעולה: למעבדתו של פרופ' אביטל גזית באוניברסיטת תל אביב ולדר' דנה מילשטיין.

למעבדתו של פרופ' בריאן ברקוביץ, לדר' ישי דרור ולדר' נדין גרופרט.

לוועדה המלווה ד"ר אודי בנין וד"ר זאב רונן שיעצו וכיוונו אותי

למשפחה שלי: לאימא ואבא היקרים לי מכול, לגורג ודניאלה שתמיד העריכו ותמכו

לדורוני, אהובי

ולאוריה וקורן, השמש בחיי

תודה

עבודה זו נעשתה בהדרכתם של:

דוקטור דרור מינץ, המכון למדעי הקרקע, המים והסביבה, מנהל המחקר החקלאי, מרכז וולקני, בית דגן.

פרופסור יצחק הדר, המחלקה למחלות צמחים ומיקרוביולוגיה, הפקולטה למדעי החקלאות, המזון ואיכות הסביבה על שם רוברט ה. סמית, האוניברסיטה העברית, רחובות.

מבנה ופעילות חברות חיידקים בביופילם של מערכת אגנים ירוקים: השפעת פרמטרים ביוטיים וא-ביוטיים על ההרכב המיקרוביאלי של הביופילם

חיבור לשם קבלת תואר "דוקטור לפילוסופיה"

מאת

לילך יסעור- קרוח

הוגש לסנט האוניברסיטה העברית בירושלים

שבט, תשע'ב פברואר, 2012