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ASSESSMENT OF CARRIER MATERIALS FOR BIOFILM FORMATION AND DENITRIFICATION

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Abstract

The capacity for biofilm attachment and activity of 20 low cost materials with little environmental impact (leftovers, byproducts or reusable waste materials) were investigated using two denitrifying biomarker organisms, *Comamonas denitrificans* 110 and *Brachymonas denitrificans* B79, and the non-denitrifying strain *E. coli* K12. The amount of attached biofilm was indirectly measured by analyzing the denitrification activity. Four materials; LECA, Pumice, Wood chips and Kaldnes K1, performed best and were therefore subjected to further investigation. The result from the second phase showed that wood chips gave the highest average denitrification activity over time while Kaldnes K1 gave the highest peak values. However, considering mechanical properties, cost and energy requirements for production in addition to denitrification activity over time, pumice was considered to be the most promising material.

Key words – Biofilm formation; *Brachymonas denitrificans*; *Comamonas denitrificans*; carrier material, denitrification, Kaldnes K1, LECA, pumice, wood chips

Introduction

We all know that the discharge of nutrients and organic material into the environment leads to eutrophication and oxygen depletion. In Sweden this problem has been recognized and attended to since early 20th century by treatment of wastewaters. The treatment methods used have constantly been improved. Since the early eighties, innovative designs using biofilm techniques have been developed. Biofilm systems permit enhanced control of reaction rates, biofilm growth, biomass age and population dynamics (Lazarova & Manem 2000).

Biofilms are typically described as heterogeneous, highly structured dense clusters of cells embedded in a hydrated matrix composed of extracellular polymeric substances (EPS), i.e. polysaccharides, protein and nucleic acids. The gene expression patterns in biofilm bacteria differ from those in planktonic bacteria (Stewart & Franklin, 2008). This can be seen as reduced growth rate as well as increased and sometimes altered production of EPS (Denkhaus *et al.,* 2006). Bacteria in biofilms are protected by the matrix against desiccation, toxicity and shock loads. When a biofilm is initially formed, a chain of events is usually followed (Fig. 1) beginning with an accumulation of nutrients and particles at the biofilm substratum surface. Bacteria thus move towards the surface by chemotaxis or twitching motility. At the surface, the bacterial cells adhere through weak reversible van der Waal forces. This is followed by irreversible attachment due to production of EPS. The subsequent maturation process involves formation of micro and macro colonies that are eventually developed to a complex three dimensional architecture with pores and channels for transport of substrate. During the maturation process, bacteria from the bulk fluid can be integrated into the matrix and detachment of cells or pieces of the biofilm occurs (Stoodley *et al.,* 2002; Qureshi *et al.*, 2005; Denkhaus *et al.,* 2006). Biofilms can be studied either as a mixed consortia of microorganisms as they occur in nature or in controlled systems with mono or dual cultures (Anders-

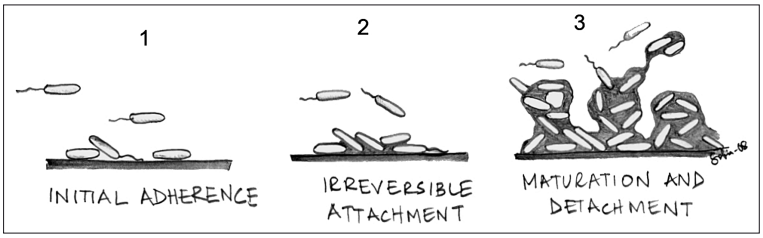

Figure 1. *The development process for biofilm formation. 1) Bacteria adhere to the surface through weak van der Waal forces. 2) Production of EPS is initiated, gluing the cells together, causing irreversible attachment. 3) Complex three dimensional structures are formed. Cells from the bulk fluid are integrated and single cell or cluster detachment occur.*

son *et al.*, 2008). Mono species biofilms formed under laboratory conditions have been reported to exhibit similar overall structural features as naturally grown mixed species biofilms (Davey & O'Toole, 2000).

To obtain a high active biomass concentration in a biofilm wastewater treatment reactor, carrier materials with a high specific surface area are being used. A large active biofilm area enhances the capacity to treat large volumetric and hydraulic loads. However, several other aspects have to be taken into consideration when selecting carrier material for biofilm formation, such as economic aspects (initial capital costs, operating costs), environmental aspects (impact of production and disposal of carriers) and process functionality (attrition resistance, biodegradability, backwash). Some characteristics of biofilm carrier materials are claimed to be of particular importance; size, porosity, density, attrition resistance and capacity for biofilm attachment and activity (Lazarova & Manem, 2000). Small size (>10mm), large protected surface area e.g. high porosity, a low density close to the one of water (1g/cm^3), high resistance to attrition and high capacity to develop active biofilm are all desirable carrier qualities. The most widely used carrier materials today are specifically manufactured for wastewater treatment purposes. The main types of materials used are mineral particles such as sand and expanded clays or low density plastic materials like polystyrene, polyethylene and polyurethane (Lazarova & Manem, 2000). The specifically designed carriers convey a high initial cost that is not always feasible in a global perspective. It is therefore important to find low cost material, preferably locally available and environmental friendly that can be used as a substitute for the commercial carriers.

Two denitrifying organisms previously isolated from wastewater treatment sludge at the Department of Environmental Microbiology, KTH, Sweden, *Comamonas denitrificans* 110 and *Brachymonas denitrificans* B79, along with the well-known lab strain *E. coli* K12, were used to assess 20 different carrier materials on their suitability for biofilm formation. The aim was to find a suitable low cost carrier with low environmental impact for denitrification of municipal wastewater.

Methods

Bacterial strains and culture media

Two well documented denitrification biomarker organisms previously isolated at the Department of Environmental Microbiology, KTH, Sweden, were used in the study; *Comamonas denitrificans* 110 (ATCC 900937) and *Brachymonas denitrificans* B79 (CCUG 45880) (Gumaelius et al., 2001; Leta et al., 2004). *Escherichia coli* K-12 (ATCC 10798) was used as a non-denitrifying reference strain. Sterile filtered (0.2µm, Millipore) municipal wastewater (WW) from Henriksdal wastewater treatment plant in Stockholm was used as culture medium.

Screening of carrier materials

Twenty potential carrier materials with low production cost and low environmental impact were selected (Table 1). The carriers were subjected to vigorous shaking (250rpm, 30ºC, 48h) and autoclaving (121°C, 1bar, 20min) to ensure resilient mechanical qualities. The accessibility to biofilm formation was then assessed using sterile material units (15mL) incubated aerobically with an inoculum of either *C. denitrificans*, *B. denitrificans* or *E. coli* in 100 ml Erlenmeyer flasks with 20 ml WW (100 rpm, 30ºC) for two weeks. The culture medium was replaced every second day and inoculum was added weekly. Denitrification activity was measured once a week.

The materials which displayed best denitrification activity were subjected to a five weeks biofilm growth test under the same conditions as described above. Biofilm composition and density was determined using fluorescent *in situ* hybridization (FISH) at the end of the five weeks.

Denitrification activity test

Duplicates of 5mL of biofilm carriers were placed in capped sterile glass tubes after thorough rinsing with sterile Milli-Q water. Five mL WW with 8mg/L $NaNO_2$-N was added to the tubes. The nitrite concentration was

Table 1. *Potential biofilm substratum materials and results from the primary screening.* (–) signify no, (+) low, (+ +) moderate and (+ + +) high relative denitrification activity. NI = Not Included, failed mechanical test.

Material	Specificity	Origin	Primary screening
Natural materials			
BF-stone	Melted limestone, Ø3–6mm	BP from mining, Merox	–
Limestone	Limestone gravel, Ø2–6mm	BP, Nordkalk	–
Pumice	Vulcanic lava stone	Natural material, Eritrea	+ + +
Wood chips	Birch, 1 cm^3	LO, pulp industry, KTH	+ + +
Processed materials			
Cell rubber Nitto 1686	Porous EPDM rubber, 1cm^3	LO, production of rubber items*	–
Cotton gauze	Cotton fabric, in Ø1cm frame	LO, cotton gauze production	+ +
Insulation trim	Rubber foam, 2cm^3	RP	–
LECA	Light expanded clay aggregates	RP, construction, AB Svensk Leca	+ + +
Mineral wool	Glass fiber $(SiO_2)_n$, 2cm^3	RP, insulation material	+
Ribbed rubber	Rubber, 3 × 20 × 20 mm	RP, rubber carpet	–
Synsafe G3	Synthetic organic fibers, 1cm^3	LO from production of air filters*	+ +
Plastic materials			
Bulpren FCT280	PU foam (polyester), 1cm^3	LO, production of plastic items*	–
Filtren TM 23220	PU foam (polyether), 1cm^3	LO, production of plastic items*	+
Kaldnes K1	HD-PE, Ø 9.1mm	Commercial carrier, AnoxKaldnes	+ + +
Nylon belt	Nylon (PA), Ø1cm	RP, nylon belt	+
Packing peanuts	Recycled PS, 1cm^3, 99.6% air	RP, protects items during transport	NI
Plastic tube	PP, grooved, Ø1cm	RP, part of coat hanger	+
Plastic wheels	Tube with internal cross, 1cm^3	RP	NI
Lamiflex 301DX	PU foam (polyether), 1cm^3	LO, production of plastic items*	–
Screw cap	HD-PE, Ø1cm	RP, Sarstedt	–

BP=Byproduct, LO=Leftover, RP=Reusable product, EPDM=Ethylene Propylene Diene M-class rubber, PU=Polyurethane, HD-PE=High density polyethylene, PA=Polyamide, PS=Polystyrene, PP=Polypropylene
* National Gummi AB

analyzed spectrophotometrically (Merck spectroquant reagent no. 1.14776.0001) every 30–60 minutes and the denitrification activity [mg NO_2-N/100mL carriers, h] was determined. The carriers were returned to the Erlenmeyer flask after the test.

Fluorescent *in situ* hybridisation (FISH)

Two milliliters of carriers were fixed with 4% paraformaldehyde for 6h according to a previously described procedure (Amann, 1995). Biofilms were detached using ultrasonication (1 min, Branson Sonifier 250). Subsequent hybridization followed Manz's protocol and was performed at 46°C for 60 minutes (Manz, et al., 1992). The oligonucleotide probes EUB338 (Amann, *et al.,* 1990) targeting all EU bacteria, COM1424 (Amann *et al.*, 1996) targeting *Comamonas sp.*, OTU6-178 (Juretschko *et al.*, 2002) targeting *Brachymonas denitrificans* and ECO645 (Neef *et al.*, 1995) targeting *E. coli* were used.

Results

The 20 potential carrier materials listed in Table 1 were screened for their suitability to serve as substratum for mono species biofilm formation by the two denitrifying bacteria *B. denitrificans* and *C. denitrificans* as well as the reference organism *E. coli*. Most of the materials were leftovers or byproducts from manufacturing processes or products that could be reused as carrier material instead of being discarded. All materials except two, the packing peanuts and the plastic wheels, withstood the mechanical treatment. The BF-stone, limestone and pumice showed indications of abrasion after shaking for 48h but to such a small extent that they were still subjected to the next step of the screening process. In the subsequent biofilm formation test the amount of biofilm formed on the carriers was indirectly estimated by measuring the denitrification rate. A high denitrification activity, corresponding to a high biofilm formation, was found in four of the carrier materials (LECA, pumice, wood chips and Kaldnes K1). Moderate or low denitrification rates

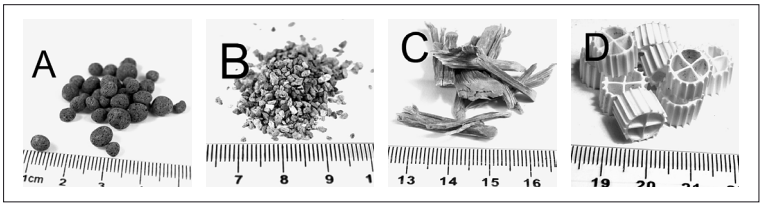

Figure 2. *The four carrier materials that performed best in this study. A) LECA, B) pumice, C) wood chips and D) Kaldnes K1. The scale bars are in cm.*

were found in two and four materials respectively while the remaining eight materials did not give rise to denitrification (Table 1). Comparable results with a clear correlation between denitrification and biofilm formation were obtained in the *C. denitrificans* and *B. denitrificans* monocultures whereas *E. coli* produced biofilms (confirmed by FISH) without any denitrification ability.

The four materials with highest denitrification rate, LECA, pumice, wood chips and Kaldnes K1, were selected for the second phase (Fig. 2). Some characteristics of these materials are listed in Table 2. The biofilms were allowed to grow for 5 weeks to obtain a picture of the long-term biofilm development and denitrification performance. Fig. 3 shows the results from the denitrification activity tests performed on the four materials inoculated with *B. denitrificans* and *C. denitrificans*. The highest denitrification activity, 1 mgN/L material, h, was found in *B. denitrificans* biofilm on Kaldnes K1 after three weeks. In general, Kaldnes K1 showed great variations in the measured denitrification activity over time. The overall best performance was observed for the wood chips, which showed a stable performance after the first (*C. denitrificans*) or second (*B. denitrificans*) week of biofilm establishment. LECA and pumice also gave quite stable denitrification rates from week 3 onward. Microscopic investigation of the biofilms, using FISH, showed that dense cell clusters of each species colonized the surfaces and that no visible contamination occurred. Fig. 4 shows that low intensity ultra sonication resulted in detachment of pieces of the biofilm matrix rather than single cells and that the bacteria maintained their morphology and was not damaged by the treatment.

Discussion

In this study we focused on investigating the capacity for biofilm attachment and activity of low cost material with little environmental impact. The four materials with the best results, LECA, pumice, K1 and wood chips displayed some common features; low density and high protected surface area.

Apart from the four carrier materials that underwent the second phase of this study, some of the other materials presented in Table 1 were expected to perform better. The polyurethane foams, the cell rubber and the plastic tube were materials we believed would be suitable for biofilm formation since they posses' large protected surface areas and low densities. No further studies on the reason for the poor performance were conducted; however, a reasonable speculation is that the materials were treated with some sort of antibacterial surface coating inhibiting cell attachment and growth.

Kaldnes K1 is a commercial carrier material that was specifically developed for use in wastewater treatment reactors. The patented moving bed biofilm reactor (MBBR) with K1 carriers has been implemented in full scale world-wide for treatment of municipal and industrial wastewaters (http://www.anoxkaldnes.com/Eng/c3refc3/references.htm). In the context of nitrogen removal, K1 systems have been used for both pre and post denitrification (Welander & Mattiasson, 2003; Rusten *et al.,* 2006). Our study confirm that K1 have good potential for mono species biofilm formation, however, the results showed strong weekly variations. This might be due to slow initial surface attachment. Interactions between a hydrophobic, negatively charged (at pH 6–9)

Tabel 2. *Properties of the four carrier materials in the second phase.*

Carrier Material	Dry density [g/cm^3]	Protected surface area [m^2/m^3]	Size [mm]	Chemical composition	Prod. energy req.	Attrition resistance	Rel. initial cost
Kaldnes K1	0.95	500	9	HDPE	high	high	high
LECA	0.4-0.5	700-1500	2-6	SiO_2, Al_2O_3, Fe_2O_3	high	high	low
pumice	0.5	80% porosity	1-2	SiO_2, Al_2O_3, Na_2O, K_2O	low	medium	low
wood chips	>1	unknown	<30	Carbohydrates	low	degradable	low

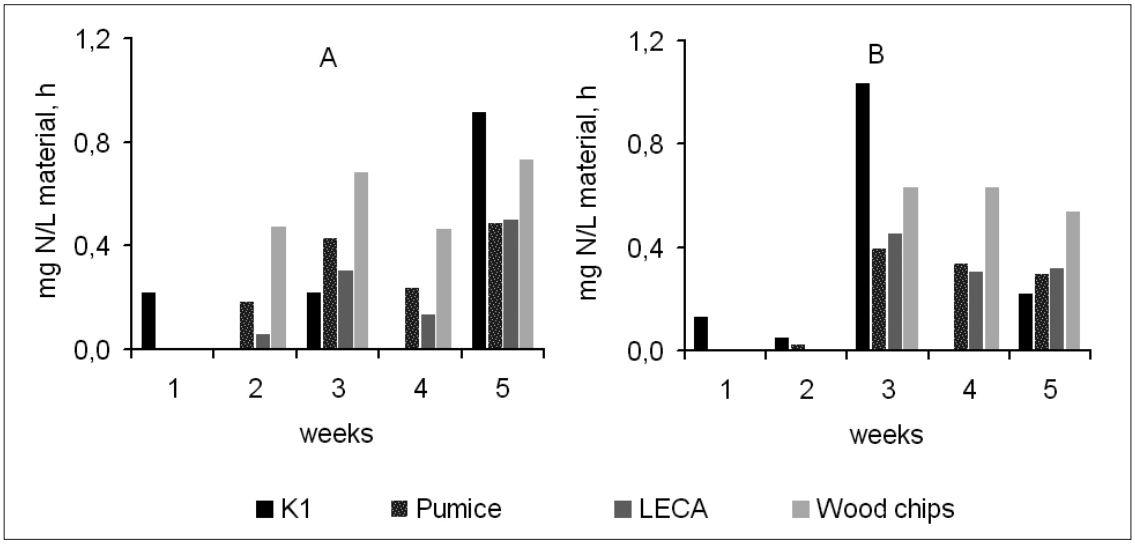

Figure 3. *Denitrification activity of biofilms of A) C. denitrificans 110 and B) B. denitrificans B79 on Kaldnes K1, pumice, LECA and wood chips, measured at the last day of week 1–5 during the second phase of the screening process.*

high density polyethylene (HDPE) surface (Oliveira el al., 2001) and negatively charged bacteria might be obstructed by repulsion, impeding initial adherence. In addition, microorganisms generally attach more rapidly to rough surfaces (Denkhaus *et al.*, 2006). Indeed, we observed that the smooth surface of the HDPE plastic was difficult for the bacteria to colonize but once initial attachment took place, the irreversible attachment and further maturation was fast, resulting in an unevenly distributed biofilm. Thus, depending on the colonization level of the individual carriers, randomly taken out for denitrification rate measurement, the result varied. Assuming that with time all carriers would be fully colonized, high and stable denitrification rates would be obtained.

Wood chips turned out to provide a suitable surface for biofilm formation. The chips had irregular forms with cavities offering protection against chafing. The repeatedly high denitrification rates on wood chips indicated the presence of a strong biofilm. Biofilm formation is generally initiated by surface charge driven accumulation of inorganic solutes, glycoproteins, proteins and organic molecules at a surface. This attracts bacteria that move towards the surface with Brownian motion or by

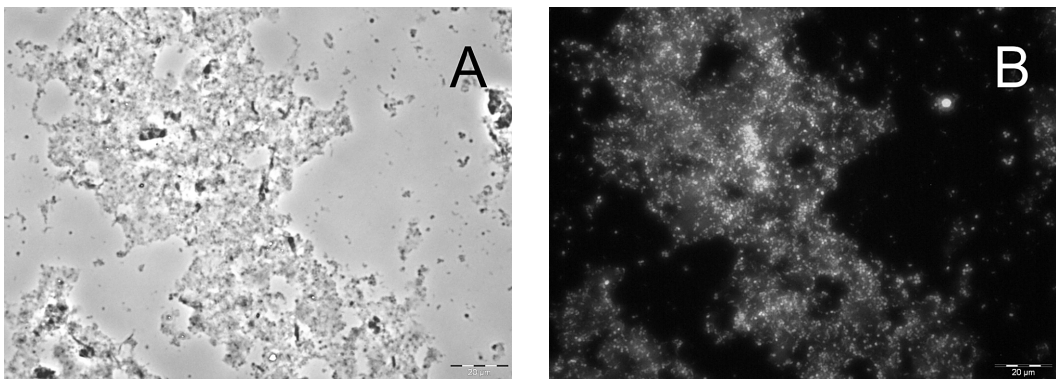

Figure 4. *Biofilm formed by B. denitrificans B79 on Kaldnes K1 after 5 weeks growth. The biofilms were detached by sonication and labeled with fluorescent dye using the FISH probe OTU6-178. A) is a phase contrast and B) is an epi-fluorescent micrograph. Scale bar 20 µm.*

chemotaxis (Qureshi *et al.,* 2005). The surface of wood is generally polar, allowing interaction with water molecules and water soluble compounds, facilitating the accumulation of molecules at the surface. In addition, wood is primarily composed of the organic molecules cellulose, hemicellulose and lignin, possibly providing organic carbon source for bacterial consumption from the base of the biofilm as a complement to the carbon sources in the liquid phase. Wood chips were in fact the carriers with fastest colonization of *C. denitrificans*. Despite the good qualities for biofilm formation and activity, it cannot be overlooked that wood is a biodegradable material that releases carbohydrates to the water phase and might thus not be suitable to use in a reactor. However, the carbohydrates in the wood have a potential of being used as reducing agent for NO_X in a post-denitrification system. This concept was examined by Mizoguchi et al. (2007) who obtained 100 % denitrification for more than 69 days without addition of external carbon source. Postdenitrification with addition of carbon source generally manages higher volumetric rates than predenitrification (Lazarova & Manem. 2000) stressing the potential of a wood based system. The actual amount of organic carbon release, the possible release of phenols and the stability over time in such system must be assessed before any conclusions regarding such systems can be made.

LECA materials are often used in horticulture or construction works. Like K1, expanded clay materials are also currently used in different wastewater treatment applications (http://www.filtralite.com/26631). The porosity and surface roughness makes it easily accessible for bacterial attachment. LECA materials are cheap and made of natural clay but the manufacturing process requires a great amount of energy, leading to a large environmental impact. Pumice is in many ways similar to LECA, the surface properties, chemical composition and performance in the denitrifying activity test (Fig. 3). But pumice is an unprocessed natural material formed during volcanic eruptions, leaving no environmental impact from the manufacturing process. The pumice used in this study came from Eritrea and is not a locally available material from a Swedish point of view. It is, however, found in many parts of the world where a low cost carrier material is needed. Reports of successful use of pumice in biofilters for removal of toxic compounds from industrial WW have been published (Di Lorenzo *et al.,* 2005; Kitis *et al.*, 2005). Based on the quite high and stable denitrification activity over time, the good mechanical properties, the low cost and the low energy requirements for production, we consider pumice to be the most potential material in this study. Further investigations on the performance and durability of pumice in laboratory scale reactors have been initiated.

Conclusions

In this study we show that the carrier materials LECA, Pumice, Wood chips and Kaldnes K1 supported formation of denitrifying biofilms of *C. denitrificans* and *B. denitrificans*. Pumice stands out as the most promising material considering costs, environmental impact and performance. Further studies of pumice as a carrier material with focus on process development and performance are in progress.

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