Microbial Activity in Granular Activated Carbon Filters in Drinking Water Treatment

Aleksandra Knezev

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THESIS

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Aleksandra Knezev
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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Water treatment is needed to produce drinking water from sources contaminated with pathogenic micro-organisms, anthropogenic chemicals and/or containing an elevated concentration of natural organic matter (NOM) and/or inorganic compounds impacting drinking water quality, both from a public health point of view and/or its aesthetic appreciation, e.g. colour, taste, odour and turbidity. In the Netherlands, water treatment aiming at the removal of such constituents includes multiple barriers, e.g. coagulation/sedimentation, filtration, oxidation and disinfection. Granular activated carbon (GAC) filtration has become a main barrier in surface-water treatment for the removal of organic contaminants, but also removes NOM, including humic and fulvic acids and biodegradable compounds. This thesis describes investigations on microbiological processes in these GAC filters (GACFs). The general introduction provides information about (i) the application of GAC filtration in water treatment, (ii), GAC characteristics, (iii) bacterial attachment and growth, (iv) microbial activity in GACFs, and (v) the objectives and aims of the studies described in this thesis.

1.2 BIOLOGICAL FILTRATION IN WATER TREATMENT

1.2.1 Function and operational conditions

In the Netherlands, two thirds of drinking water is produced from groundwater and one third from surface water (rivers and lakes). Biological filtration processes, including soil passage (e.g. river-bank filtration, dune infiltration), rapid sand filtration (RSF), dual-media filtration (DMF), slow sand filtration (SSF) and GAC filtration, are applied in water treatment for the removal of biodegradable compounds. One or more of these processes are applied at different stages in the water treatment train, depending on the origin and composition of the source water and national regulations and traditions related to water treatment and water quality. Biological filtration processes differ in the operational conditions (empty bed contact time, flow rate, frequency of backwashing, and frequency of regeneration in the case of GACFs). All these conditions and the nature of the filter material have an impact on the intensity and effects of the microbiological activity in the filter bed.

RSFs are usually positioned at the beginning of the treatment train. In groundwater treatment RSFs are positioned after the aeration where they serve the removal of iron and manganese oxides, suspended solids and ammonia (nitrification). In surface-water treatment RSF or DMF are applied after flocculation or coagulation and sedimentation for the removal of suspended solids and are also often operated as biological filters for the removal of ammonium and biodegradable organic matter, including precursors for disinfection by-products (LeChevallier et al. 1992; Carlson and Amy 1998; Huck et al. 1994). Frequent backwashing (within several days) is necessary to remove particulate material and biomass that is accumulated in the filter bed, causing pressure drop.

SSFs are usually positioned at the end of treatment as a polishing step for the removal of turbidity and pathogenic microorganisms and biodegradable compounds. SSFs operate at much lower hydraulic and organic loading rates, and instead of backwashing the surface layer of

a SSF, also known as 'Schmutzdecke', is removed at a certain pressure drop or a certain time interval that is usually very long (months or years).

1.2.2 Biological stability of drinking water

Biological filtration processes can serve as a barrier against faecal contamination, in particular during soil passage, but play a major role in the removal of biodegradable organic compounds. These compounds are present in the raw water, but are also produced by oxidation processes applied in water treatment for the removal of anthropogenic organic compounds, e.g. ozonation (Servais et al. 2005). Removal from water is needed to prevent microbial growth in the distribution system (regrowth). Distribution of biologically instable water can cause various problems, such as noncompliance to water quality regulations (heterotrophic plate counts, coliforms), growth of opportunistic pathogenic microorganisms (*Legionella pneumophila, Pseudomonas aeruginosa* or certain nontuberculous mycobacteria), growth of free-living protozoa, fungi, invertebrates, discoloration and taste and odour problems (van der Kooij and van der Wielen 2014). In the Netherlands, drinking water is distributed without a disinfectant residual, and therefore the water supply companies aim at achieving treated water with a high level of biological stability.

Biological stability is related to the concentration of compounds that can support bacterial growth in drinking water, i.e. the microbial growth potential (van der Kooij 1992; van der Kooij and Veenendaal 2014; LeChevallier et al. 1991, LeChevallier 2014; Servais et al. 1991; Laurent et a. 2005). Such compounds are present in water leaving the treatment plants, either as dissolved or particulate organic carbon and biomass, but may also originate from the accumulated sediments or from the pipe materials (Schoenen 1986; Colbourne 1985; van der Kooij and Veenendaal 2001). A variety of methods are available for measurement of the microbial growth potential of water. The assimilable organic carbon (AOC) test measures the concentration of compounds promoting the growth of two bacterial cultures in samples of pasteurized water. The AOC concentration is calculated from the maximum colony counts of these bacteria and expressed as acetate-carbon equivalents L^{-1}, based on the yield factor of the test organisms for acetate (van der Kooij et al. 1982; van der Kooij 1992). Large databases of the AOC concentration in treated water in the Netherlands and in the USA are available (van der Kooij 1992; van der Kooij and Veenendaal 2014; Lechevallier et al. 1992). The concentration of biodegradable organic carbon (BDOC) can be measured as the difference between the initial and final DOC concentration after incubation with a mixed population in a static or dynamic test (Joret and Levi 1985; Servais et al. 1987; Kaplan et al. 1996).

Threshold levels for the concentration of AOC and BDOC in treated water have been proposed to define the treatment goals aiming at limiting regrowth in the distribution system. Van der Kooij (1992) showed that drinking water complied with the standard for the heterotrophic plate count (HPC) at an AOC concentration < 10 μg acetate-C eq L^{-1}. Below this concentration, no decrease of the AOC concentration was observed in the distribution system. However, growth of *Legionella* was observed in biofilms in an installation that was fed with drinking water containing c. 5 μg acetate-C eq L^{-1} (van der Kooij et al. 2005). LeChevallier et al. (1991) proposed AOC levels below 50 μg acetate C eq L^{-1} for controlling coliform regrowth. For the BDOC fraction a threshold for biological stability was set at 0.15-0.30 mg L^{-1} (Servais et al.

1991; Joret et al. 1994). The extend of microbial growth in distribution systems is also affected by e.g. water temperature, residence time, pipe materials and sediment formation.

1.3 GRANULAR ACTIVATED CARBON FILTRATION IN WATER TREATMENT

1.3.1 Position and role of GACF

Until 1960 mainly powdered activated carbon (PAC) was used in drinking water treatment for the removal of taste, colour and odour. Industrial and technological developments resulted in the large-scale production of a large variety of synthetic organic compounds that entered the aquatic environment and contaminated drinking water sources. Confronted with the presence of toxic substances in source waters, the water supply companies installed new technologies enabling the removal of these compounds. This development was also stimulated by the discovery of undesirable disinfection by-products (DBPs), such as trihalomethanes and other organohalogen compounds (Rook 1974). The application of GAC filtration was shown to be more effective in removing organic contaminants and precursors for DBP formation than PAC (Suffet 1980; Cairo et al. 1979; Anonymous 1980; Schalekamp 1979; Sontheimer et al. 1988; Snoeyink and Summers 1999). To date GAC is applied worldwide in drinking water treatment.

From studies conducted between 1960 and 1980 guidelines for the use of GAC filtration in drinking water treatment were set. It was shown that GAC filtration is most efficient when applied at the end of the treatment train to avoid frequent backwashing of the filter bed for the removal of accumulated particles. Consequently, conventional pre-treatment with coagulation, sedimentation and RSF or DMF before GACF was recommended (Sontheimer et al. 1988). The GACFs in the Netherlands are operated at empty bed contact times between 10 - 40 minutes at hydraulic loading rates between 2 – 30 $m^3m^{-2}h^{-1}$ and organic loading rates in the range of 5 – 55 g C $m^{-2}h^{-1}$ (Vries et al. 2012). GACFs are usually operated in a down-flow mode to minimize damage of filter material and release of particulate matter, and in a single or double series, or as pseudo-moving bed reactors. Backwashing is carried out either at fixed time intervals, or at the occurrence of a certain pressure drop. Regeneration of GAC to restore its adsorptive capacity can be carried out after a fixed period of operation or after a breakthrough of a target contaminant (Sontheimer et al. 1988).

In the Netherlands about 1200 x 10^6 m^3 of drinking water is produced annually. A total of 37.5% of this volume originates from fresh surface water and 62.5% comes from groundwater (Geudens 2012). Almost half (46 %) of this water is treated with GAC filtration, i.e. about 96% of the drinking water produced from surface water (430 x 10^6 m^3) and approximately one fifth of the groundwater (132 x 10^6 m^3), mainly at locations treating riverbank filtrate (25 locations, Vries et al. 2012).

GACFs are used for the removal of NOM, for colour and taste reduction, removal of micropollutants, often in combination with advanced oxidation, for the removal of oxidation by-products, for the removal of precursors for disinfection by- products in systems that apply chlo-

rination and for the improvement of the biological stability of treated water (Sontheimer et al. 1988; Billen et al. 1992; Huck et al. 1994; Orlandini 1999; Snoeyink and Summers 1999). The release of particulate matter (Camper et al. 1985) and invertebrates from GACFs (Schreiber et al. 1997) may cause problems in distribution systems. In the Netherlands, slow sand filtration (SSF), microsieves, or UV is sometimes applied after GAC filtration to control HPC levels.

Removal of organic compounds by GAC filtration is primarily determined by the adsorption capacity of GAC that depends on the nature of the raw material and the production process. The removal efficiency of GACFs depends also on the influent water quality, flow rate and contact time. These parameters are designed to achieve an optimal use of the adsorption capacity and attain long operational periods between the regenerations. Biological processes contribute to the overall removal of organic compounds in GACFs and are therefore considered as beneficial. The regeneration frequency of GAC usually depends on the threshold levels, usual for organic contaminants such as pesticides and pharmaceuticals, to maintain the desired effluent quality. When the removal of colour, taste, disinfection precursors, oxidation by-products or biological stability is the main focus, the concentration of NOM (DOC, TOC and UVA) is used to define the threshold levels (Graveland et al. 1994).

1.3.2 Characteristics of activated carbon

The ability of activated carbon to remove organic compounds from water is based on the properties of this material. These properties include: (i) a high specific surface area and (ii) specific chemical surface characteristics including hydrophobicity and presence of functional groups. The specific surface area varies between 400 and 1500 $m^2 g^{-1}$ and is a result of the high porosity of GAC. The shape, size, volume and surface area of the pores and their spatial distribution within the carbon particle characterize the porosity of GAC. These characteristics depend on the material from which the carbon is produced (bituminous coal, peat, lignite, petrol coke, wood and coconut shells). The diameter of the GAC particles applied in drinking water treatment generally ranges between 0.4 and 2.5 mm.

The internal structure of GAC is defined by crystallites similar to graphite (Fig. 1.1, left). The voids between the crystallites shape the micropores (<2 nm), which represent >99% of the internal surface area. The porous structure (Fig. 1.1, right) also comprises mesopores (2-50 nm) and macropores (>50 nm), representing a much smaller proportion of the internal surface. The surface of the micro- and mesopores and the volume distribution is determined from the amount of nitrogen required to form a complete monolayer on the carbon surface (Brunauer-Emmett-Teller (BET), Snoeyink and Summers 1999). Generally the BET surface is approximately 500 – 1000 $m^2 g^{-1}$ (Sontheimer et al. 1988; Bjelopavlic et al. 1999). The volume and surface distribution of the macropores and the mesopores is determined with mercury porosimetry. Typical curves of the cumulative pore volume show that mesopores occupy a small proportion of the internal particle surface c. 9% (81 $m^2 g^{-1}$ GAC), and that macropores occupy only c. 0.2 % (1.7 $m^2 g^{-1}$ GAC). The surface available for attachment of bacteria includes pores with radii > 500 nm, and occupies 0.008 % (0.070 $m^2 g^{-1}$ GAC) of the total adsorptive surface.

The surface area within the pores determines the adsorption capacity. The adsorption capacity for low molecular weight compounds (< 1000 g mol^{-1}) corresponds with the specific surface and volume of the micropores. The surface and volume of the mesopores and macropores determine the adsorption capacity for molecules ranging from 1000 to 70 000 g mol^{-1} (Kilduff et al. 1996; Newcombe et al. 1997; Thurman et al. 1982). Although the specific surface area of the macropores is small and contributes very little to the adsorption, the volume of macropores is important for the diffusion process (Sontheimer et al. 1988). Furthermore, macropores with a radius larger than 500 nm (> 0.5 μm) are available for bacterial adhesion.

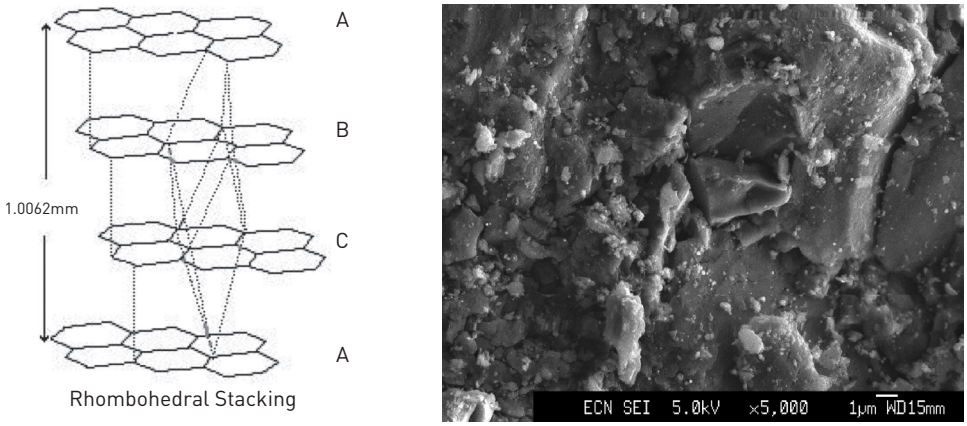

Figure 1.1 A schematic representation of the crystal structure of graphite (left, adapted from Amelinckx et al. 1965); B, a SEM image of the GAC surface (Norit GAC 830) provided by Cabot (2013).

The adsorption properties of activated carbon are also determined by the chemical characteristics of the GAC surface, which is hydrophobic by its nature. Sorption of dissolved oxygen on GAC leads to the formation of acidic surface oxides, thus increasing the polarity of the carbon surface and the adsorption capacity for carboxylic acid groups (Prober et al., 1975; Sontheimer et al., 1988; Snoeyink and Summers 1999). Generally 5 to 20 % of the activated carbon weight consists of other elements than carbon, from which metals and surface-bond oxygen are most important (Snoeyink and Weber 1967; Sontheimer et al., 1988). Oxygen-containing functional groups on GAC include carboxylic and anhydrides of carboxylic acids, phenolic, pyrone and quinon type of carbonyl, lactone, benzopyrylium derivatives etc. (Sontheimer et al., 1988). Results of some investigations indicate that the surface chemistry can be more important for the adsorption of NOM than the pore structure (Yapsakly et al. 2008). The occurrence of positive or negative charges on the surface of GAC is characterized by the point of zero charge (PZC) pH, which is the pH value at which the densities of positive and negative ions at the surface are equal. At a pH below PZC the surface is positively charged (acidophilic), and above PZC the surface is negatively charged (basophilic), the latter being the case for most GAC applications in water treatment. Furthermore, pH and ionic strength of the solution also affect the interactions between absorbate and the GAC surface (Bjelopavlic et al. 1999).

1.3.3 Adsorption

Adsorption is a process in which a molecule (adsorbate) from the liquid or gas phase is bound to the surface of a solid phase (adsorber) that is in contact with the liquid or gas. In case of *physical adsorption* van der Waal's and electrostatic interactions are responsible for the partitioning of the adsorbate between two phases. Non-polar compounds or hydrophobic parts of complex molecules are attracted to the lipophilic surface of GAC and regions of molecules with electrostatic charge are attracted to surface oxides with the opposite charge. Although physical adsorption is generally considered a reversible process (Moore 1972), desorption of complex molecules is unlikely to occur due to the multiple binding by different mechanisms (Bos et al. 1999). The adsorption may furthermore be a result of covalent interactions between adsorbate and adsorber. *Chemisorpion* is attributed to the presence of functional groups on activated carbon surface and is considered irreversible (Moore 1972). The desorption of adsorbed compounds requires an energy input equivalent to the energy of the bonds between adsorbate and surface. This energy input is achieved at high temperatures that are applied during thermal regeneration of GAC.

The adsorption process is described by the adsorption isotherm, the constant-temperature equilibrium relationship between the quantity of adsorbate per unit of adsorbent (q_e) and its equilibrium solution concentration (C_e). Several equations or models are available that describe this function, but the Freundlich and the Langmuir equations, are most frequently applied. The Freundlich equation (1) is an empirical equation, and the theory of adsorption that leads to the Freundlich equation was developed by Halsey and Taylor (1947).

$$q_e = KC_e^{1/n} \quad (1)$$

The parameters q_e and C_e are the equilibrium concentrations of a compound on surface and in solution, respectively. The parameter K is related to the capacity of the adsorbent for the adsorbate, and 1/n is a function of the strength of adsorption.

In the Langmuir equation (2),

$$q_e = \frac{q_{max}b}{1+bc_e} \quad (2)$$

the constant q_{max} corresponds to the surface concentration at a monolayer coverage and represents the maximum value of q_e that can be achieved as C_e is increased (Langmuir 1918). The constant b is related to the strength of the adsorption bond.

During GAC filtration of water containing a mixture of compounds with different values of the adsorption parameters, the equilibrium concentration of a compound is affected by competition. Sontheimet et al. (1985) developed a method for the analysis of the adsorption equilibria of multicomponent mixtures in GACFs. The large number of organic components are grouped into several pseudocomponents and adsorption competition within the mixture is assumed to obey the IAS-theory (Radke and Prausnitz 1972) or the SCA-model (Baldauf et al. 1977). Peel and Benedek (1980, 1983) developed a dual-rate kinetic model for the fixed-bed adsorber that explained two distinct stages during the approach to equilibrium in a GAC system by including the intraparticle kinetics to describe the behaviour of compounds in GACFs. In the first, fast

adsorption stage, the external film diffusion and transfer of the adsorbate to the solid-phase are the main driving forces. However, at an increasing solid phase concentration the specific rate of adsorption decreases as a consequence of diffusion along the walls of the macropores (surface diffusion), and diffusion from macropores into the micropores. As a result, breakthrough of different components present in the influent occurs at varying degrees, depending on contact time and operational period.

1.4 NATURAL ORGANIC MATTER IN WATER

The concentration of NOM in water used for the production of drinking water generally ranges from 0.5 to 5 mg L^{-1}. NOM includes a large diversity of substances with different biodegradation kinetics (Billen 1992; Amon and Benner 1996; Frimmel 1998). Generally two groups of compounds are recognized: a low molecular weight fraction (< 1 kDa) and a fraction comprised of large complex biopolymers (> 1 kDa) such as proteins, polysaccharides and humic substances (HS). HS in surface and groundwater comprise the majority (60-80%) of NOM (Thurman et al. 1982; Newcombe et al. 1997; Aiken and Costaris 1995; Kilduf et al. 1996). The apparent molecular weight of HS varies between a few hundred and several million Daltons (Choudhry 1984) containing c. 50% of carbon, 36-40% of oxygen, 3 – 4 % of hydrogen and 0.5-3 % of nitrogen (w/w) (Frimmel 1998). HS contains aromatic structures such as phenol or quinone bridged by ether (-O-), alkane (-CH_2-), amine (-NH-), imine (-N=) or other groups (Camper 2004). For a long period it was generally accepted that HS are built from covalently-bound repeating units. In the period 1986-1999 the theory was challenged by the micelle concept (Wershaw 1986; 1999; von Wandruska 1988) followed by the supramolecular concept (Piccolo 2001; 2002; Sutton and Sposito 2005). According to the supramolecular concept the low-molecular-weight components (building blocks) are held together by non-covalent van der Waals forces and hydrogen bonds. Hence, reassembling of the supramolecular aggregates to reach the most stable thermodynamic conformation will occur with changing conditions. The building blocks of HS in freshwaters include a diversity of low-molecular-weight molecules (500 Da), from which a portion is represented with biomolecules derived from lipids, lignin, nonlignin aromatic species, carbohydrates and proteins (Hoque et al. 2003; Reemtsma and Thesee 2003; Cooper et al., 2005; Hatcher et al. 2007; Seitzinger et al. 2005; Sutton and Sposito 2005).

The general perception is that low-molecular-weight organic compounds are degraded quickly while degradation of high-molecular-weight molecules requires more time (Saunders 1976; Hozalski and Bouwer 2001; Billen et al. 1992). Humic substances have long been considered as recalcitrant due to their ability to form complexes with extracellular enzymes that inhibit their activity (Wetzel, 1992). However, several studies have shown that bacteria in aquatic biofilms are capable of using humic substances (Amon and Benner 1996; Volk et al. 1997). More recently, it was demonstrated for drinking water that biofilms attached to corroding iron surfaces can utilize HS for bacterial growth (Camper 2004). Hence, under certain conditions, HS can serve as a carbon and energy source for growth of microorganisms.

Pre-treatment processes affect the concentration and nature of NOM in the influent of GACFs. Oxidative pre-treatment increases charge, polarity and the hydrophilic nature of NOM, while

the biodegradation decreases the charge of NOM indicating that biodegradable NOM fraction has a more negative charge (Ypsalsky et al. 2009). Beside the humic compounds, NOM also contains low molecular weight (<500 Da) compounds including carboxylic acids, aldehydes, ketones and monomeric or oligomeric units of various biopolymers, such as amino acids, peptides or saccharides (Koudjonou et al. 2005). Most of the easily degradable low molecular weight compounds are utilised in biological filtration in the conventional pre-treatment, but some is also present in the GAC influent. However, oxidative pre-treatment increases the concentration of easily assimilable organic carbon (van der Kooij et al. 1989; Carlson and Amy 1997). Various anthropogenic micropollutants (pesticides, pharmaceuticals, additives etc.) and their metabolites can also be present in water as individual compounds or in association with HS (Houtman 2010). The hydrophilic low-molecular-weight components generally are more susceptible to the microbial degradation than the hydrophobic fraction that in turn adsorbs better on the GAC surface. However, many anthropogenic hydrophilic, low molecular weight molecules are recalcitrant and are not removed in GACFs (Schoonenberg et al. 2010).

1.5 MICROBIOLOGICAL ACTIVITY IN GAC FILTER BEDS

1.5.1 Attachment of bacteria to surfaces

In nature and in engineered systems such as water treatment processes and distribution systems, most microorganisms are present attached to water-exposed surfaces. The initial attachment of bacteria is a result of the physicochemical interactions controlled by van der Waals and electrostatic forces and is generally explained by the Derjaguin, Landau Verwey and Overbeek (DVLO) theory of colloidal stability (van Loosdrecht et al. 1990; Bos et al. 1999). The initially-reversible attachment is followed by the formation of extracellular polymers that irreversibly anchor the organism to the surface. This process is associated with the uptake of compounds from the water phase. The extracellular polymeric substances (EPS) are characterized by hydrophobic, and charged hydrophilic moieties, ensuring binding to surfaces differing in hydrophobicity and charge. The irreversible nature of the attachment is due to the irreversible nature of the adsorption of EPS. The extent of attachment is affected by both the nature of the organism and the nature of the surface. Thermodynamically, adsorption onto a hydrophobic surface is most favourable, because of the reduction of the surface-free energies of both the microorganism and the involved surface.

Bacteria and other microorganisms also interact actively or passively with the surface of GAC in contact with water. These interactions lead to attachment, retention, and consequently to growth and maintenance of bacteria in GACFs. The extent of growth and maintenance depends on the availability of compounds serving as a source of energy and/or carbon. Only the surfaces of the pores on the outer part of a GAC particle with a radius greater than 500 nm are potentially available for bacterial attachment. The fraction of the GAC surface comprised by these pores represents only a small faction (0.008%)of the total GAC surface, but due to the roughness and porosity of GAC this surface is much larger when compared with other materials of the same particle diameter used in drinking water treatment, such as sand or anthracite. The specific surface area of sand (diameter 1 to 2 mm), with a filter bed porosity of 39% is approximately 35 -70 cm^2 cm^{-3}, respectively. The surface of GAC that includes pores with radii

> 500 nm that are available for bacterial attachment, calculated from mercury intrusion, is approximately 350 cm^2 cm^{-3}. Hence, GAC provides a larger surface area available for bacterial attachment than sand. Furthermore, the hydrophobic nature and the presence of functional groups additionally strengthen the attachment of bacteria to GAC surface.

1.5.2 Microbial substrate utilization and growth

Microbial utilization of biodegradable compounds is characterized by the rate of uptake and the formation of biomass. The relationship between the rate of growth and the concentration of the growth-limiting compound (energy source) is given by the Monod equation, i.e.

$$\mu = \mu_{max} \times S/(K_s+S) \quad (1)$$

where: μ (h^{-1}) is the specific growth rate of the microorganisms, i.e. the increase of microbial biomass (M) per unit of biomass (M) and time [(dM/(M×dt)]; μ_{max} (h^{-1}) is the maximum specific growth rate of the microorganisms; S is the concentration of the limiting substrate for growth; Ks is the "saturation constant" or the value of S at $\mu / \mu_{max} = 0.5\ \mu_{max}$ and Ks are empirical coefficients and are determined by the ability of a species to utilize a specific substrate. The rate of growth corresponds with the rate of uptake of the involved substrate. The relationship between substrate uptake and growth is defined by the yield coefficient (Y) with:

$$Y = dS/dM \quad (2)$$

Y depends on the nature of the substrate and the nature of the microorganism. For easily biodegradable organic compounds, Y is about 0.5 mg biomass C mg^{-1} substrate C (Heijnen and van Dijken 1992).

The efficiency of a species to utilize a specific substrate is related to the composition of the substrate and the kinetics of the critical enzymatic reaction involved in the transformation of the involved substrate. Passive non-specific transport through the bacterial cell wall and the porins in the outer membrane into the periplasmic space is restricted to compounds with a molecular weight < 700 Da (Decad and Nikaido 1976; Nikaido 2003). Specific porins may transport specific compounds. The transportation from the periplasmic space to the cytoplasm via the cytoplasmic membrane is carrier mediated and highly specific, requiring membrane transport proteins. This energy requiring transportation results in high concentrations of the transported molecule in the cytoplasm. Easily biodegradable low molecular weight compounds such as monosaccharides, amino acids and (hydroxy)carboxylic acids, are rapidly transported through the porins into the periplasmic space and their transport is most likely diffusion limited.

Large complex molecules including biopolymers must first be transformed by exo-enzymatic hydrolysis before they can be transported into the cell and utilized. The utilization rate of large molecules will therefore be affected by the rate and efficiency of binding to the cell and the subsequent effect of the exo-enzymatic reactions (Hoppe 1991). Extracellular enzymes can be classified as ectoenzymes and truly extracellular enzymes. Ectoenzymes are associated either with the cytoplasmic membrane or the cell wall of viable cells. Strict-sense extracellular

enzymes occur in free form and catalyse reactions detached from their producers. Bacterial extracellular enzymes may be actively secreted by intact viable cells, released into the environment by viral lysis or predation (Wetzel 1991). Overall, the utilization of compounds that cannot pass through the porins (in gram-negative bacteria) requires adsorption to specific binding proteins, followed by hydrolysis. In contrast to the low-molecular-weight compounds, utilization of the high-molecular-weight compounds may be limited by the kinetics of adsorption and hydrolysis. Sack et al. (2014) demonstrated that polysaccharides at concentrations of 10 μ g L^{-1} can cause biofilm formation on a glass surface. The substrate concentration (S) at the surface (in the biofilm) depends on the transportation from the bulk water to the microorganisms and the utilization by the micro-organisms. Transportation from the bulk water to the attached microorganisms is the result of convection and diffusion. In granular media filters (GACF and RSF), the flow pattern is turbulent, resulting in an effective transportation of the substrate to the laminar layer at the solid phase. Diffusion subsequently determines the transportation of the substrate to the microorganisms. Studies of biofiltration in drinking water indicate that diffusion and external mass-transport resistance in the biofilm have a negligible effect on the performance of a biofilter (Wang and Summers 1995; Gagnon and Huck 2001; Urfer 1998; Uhl 2000; Huck et al. 1991; Zhang and Huck 1996).

1.5.3 Microorganisms in GACFs

Microbiological activity in GACFs was recognized already in early studies on GAC filtration as elevated colony counts in the effluent, oxygen consumption by the filterbeds and release of GAC fines with attached bacteria in the filtered water (Melbourne and Miller 1974; Weber et al. 1978; Cairo et al. 1979; van der Kooij 1983; Bancroft et al. 1983; Chudyk and Snoeyink 1984). Initially, the presence of bacteria raised doubts about health risks associated with the introduction of GAC filtration in water treatment. Elevated heterotrophic plate counts (HPC) in effluents of GACFs were repeatedly reported by operators and researchers, especially during the startup period. In the process of filter maturation, HPCs in the effluent decrease and stabilize usually at levels one order of magnitude lower than in the startup period (Klotz et al. 1975; van der Kooij 1975; Werner et al. 1978; Cairo et al. 1979; den Blanken 1982; Wilcox et al. 1983; Chudyk and Snoeyink 1984; McElhaney and McKeon 1985; Servais et al. 1991). HPCs in the GAC filtrate can be controlled by treatment with chlorine, chlorine dioxide, UV light, or slow sand filtration (Camper et al. 1986; Graveland et al. 1994; IJpelaar et al. 2007). Multiplication of pathogenic bacteria of faecal origin or faecal indicator bacteria does not occur in GACFs under the usual operating conditions (Camper et al. 1986; Stewart et al. 1990). Furthermore, GACFs with a long running time are able to remove these microorganisms from the influent (van der Kooij 1983; Hijnen et al. 2010). Nevertheless, certain undesired bacteria, e.g. coliforms may survive in GAC filterbeds and enter a distribution system when attached on fines for which disinfection is not efficient (Camper et al. 1985; Camper et al. 1986; Stewart et al. 1990).

Bacteria have been isolated from GACFs by using classical culture techniques with plate count agar or R2A agar (Reasoner and Geldreich 1985). Subsequently, these organisms were identified by determining a number of metabolic characteristics for classification. The isolated bacteria have been identified as members of *Achromobacter, Arthrobacter, Alcaligenes, Acinetobacter, Aeromonas, Bacillus, Caulobacter, Chromobacterium, Corynebacterium, Flavobacterium, Morax-*

ella, Micrococcus, Microcyclus, Nitrosomonas, Nitrobacter, Paracoccus, Pseudomonas, and actinomycetes (van der Kooij 1975; Werner et al. 1978; Cairo et al. 1979; Parsons 1980; den Blanken 1982; 1983; Wilcox et al. 1983; McElhaney and McKeon 1985; Camper et al. 1986; Burlingame et al. 1986; Rollinger and Dott 1987). None of the isolates was identified as an organism associated with a potential health risk. However, many isolates remained unidentified due to the low discriminatory capacity of the classical identification methods. By using fatty acid analysis for bacterial identification, Norton and LeChevallier (2000) found that the largest proportion of isolates from GAC belonged to the genera *Acidovorax, Hydrogenophaga, Pseudomonas, Xanthomonas,* and *Sphingomonas*. Also rotifers, nematodes, copepods, oligochaeta and tardigrada were detected in high numbers in GACFs and could not easily be removed or controlled by backwashing (van der Kooij 1983; Schreiber et al. 1997; Scholz and Martin 1997; Wang et al. 2014).

1.5.4 Biological activity and biodegradation in GACFs

Much research has been conducted to evaluate the contribution of microbial activity to the removal of organic contaminants and NOM from the influent. Investigations showed that oxidative pre-treatment with ozone extends the running time between regenerations and improved the removal of toxic substances and dissolved organic carbon (Sontheimer et al. 1978). It became evident that beside adsorption, biodegradation also plays a role in the removal of organic compounds in GACFs, and that optimal utilization of GAC filtration in water treatment also requires knowledge about biological processes and biodegradation in GACFs. A variety of techniques has been used to assess the activity or the concentrations of bacteria in GACFs (Table 1.1).

Table 1.1 Methods for the assessment of the quantity and microbial activity in biological filters.

Reference	Parameter
Klotz et al. 1975; van der Kooij 1983; Urfer and Huck 2000	Oxygen consumption
Weber 1978; Cairo et al. 1979	Scanning electron microscopy
Werner et al. 1979; Camper et al. 1985	Heterotrophic plate counts
DiGiano et al. 1992	Total cell counts
Wang et al. 1995; Carlson et al. 1996	Phospholipids
White et al. 1977; Servais et al. 1994	Uptake of labelled substrates
Fonseca et al. 2001	Reduction of INT*
Velten et al. 2007	ATP

* 2-(p-iodo-phenyl)-3(p-nitrophenyl)-3 phenyl tetrazolium chloride

These methods have been applied to study the development of biomass and to relate biomass activity with the removal of organic carbon from the influent. In order to distinguish between

adsorption and biodegradation, methods for characterization and biodegradability of the natural organic matter have also been developed (van der Kooij et al. 1982; 1992; Vartiainen et al. 1987; Servais et al. 1989; Peuravuori and Pihlaja 1997). Measurement of the concentration of microorganisms or microbial activity repeatedly revealed initial growth of bacteria in GACFs up to a certain level, which then remained stable (Bancroft et al. 1983; Billen et al. 1992; Wang et al. 1995; Carlson and Amy 1998; Velten et al. 2007). The distribution of biomass through the filterbed was reported to include a maximum biomass at the top and a decreasing concentration or activity with the filter depth. Filters that received pre-ozonated water developed more biomass than filters that received non-ozonated water (Billen et al. 1992; Wang et al. 1995; Carlson and Amy 1998; Urfer and Huck 2000; Huck et al. 2000; Fonseca et al. 2001; Velten et al. 2007). This stimulation of microbial growth was attributed to the formation of easily biodegradable compounds from humic and fulvic acids by partial oxidation (van der Kooij et al. 1982; 1989; Gagnon et al. 1997; Carlson and Amy 1997).

The number of bacteria present on the GAC particles is 2-3 orders of magnitude higher than those in the filtrates. Hence, the biological processes in GACFs proceed almost entirely on the GAC surface (van der Kooij 1983; Servais et al. 1994). The elevated specific rate of removal of natural organic matter in mature GACFs as compared to SSF and the higher oxygen consumption in GACFs than in filters with sand, anthracite or non-activated granular carbon (NAGC) was observed in different investigations (van der Kooij 1983; Nijishima et al. 1992; Wang et al. 1995; Carlson and Amy 1998). These findings suggested that GAC enhances microbial activity when compared to the inert filter materials.

In many studies that compared GACF to filters with inert media, an elevated specific rate of NOM removal and higher microbial activity was repeatedly reported (Table 1.2).

Table 1.2 Enhancement of the removal of NOM in GACF relative to the removal in filters with inert media.

Filter material	Removal enhancement by GAC (%)	Reference
GAC/Sand	50	Sontheimer and Hubele 1986
GAC/Anthracite/Sand/Gravel	50-62	LeChevallier et al. 1992
GAC/Sand	60-75	Servais et al. 1994
GAC/Anthracite/Sand	23-26	Wang et al. 1995
GAC/Pumice/Glass/Sand	52-82	Uhl 2000
GAC/Sand	n.o.	Huck and Sozansky 2008
GAC/Anthracite	<5	Chowdhurry et al. 2013

n.o. not observed

The enhanced removal was often attributed to enhanced microbial activity (Shimp and Pfaender 1982; Chudyk and Snoeyink 1984; Goeddertz et al. 1988; Nishijima et al. 1992; Servais et al. 1994; Wang et al. 1995), and microbial utilization of compounds adsorbed onto GAC (Eberhardt 1975; Sontheimer et al. 1978; Weber et al. 1978; Chudyk and Snoeyink 1984;

Kim et al. 1997; Summers et al. 2010). However, utilisation of adsorbed compounds has been denied by other investigators (Peel and Benedek 1983; van der Kooij 1983; Sontheimer and Hubele 1986). To date, the mechanisms of the enhanced biological activity in GAC filterbeds remain unresolved.

A number of authors have suggested that the enhancement of microbial activity is related to the accumulation of organic matter on the GAC surface by adsorption (Eberhardt 1975; Sontheimer et al. 1978). Another explanation is related to the nature and size of the specific surface area of GAC that is available for bacterial attachment (van der Kooij, 1983; Carlson and Amy, 1998; Wang et al. 1995). The enhancement of microbial activity by the adsorption process was questioned when GAC types with considerably different adsorptive properties showed similar biological activity (Jekel 1979; Wang et al. 1995). Furthermore, in batch experiments with river water and preloaded GAC or NAGC, bacteria died equally soon indicating that adsorbed compounds are not available for growth (van der Kooij 1983). However, enhanced microbial activity and increase of the adsorption capacity of GAC observed as the extension of filter life time and postponed breakthrough of NOM and/or organic micropollutants are commonly related phenomena (Peel and Benedek 1983; Chudyk and Snoeyink 1984; Goeddertz et al. 1988; Speitel et al. 1989; Hutchinson and Robinson 1990; Nishijima et al. 1992; Servais et al. 1994; Wang et al. 1995; Kim et al. 1997; Jones et al. 1998; Nagayev and Sirotkin 1998; Orlandini 1999; Klimenko et al. 2004; Aktas and Cecen 2007).

The improved performance of GACFs has also been explained by the biodegradation of assimilable organic compounds from the water phase. Compounds that would normally adsorb onto GAC are biodegraded, and saturation of GAC adsorption capacity is postponed. Improved performance of GACFs receiving water pre-treated with ozone demonstrates that increase in biodegradability of NOM in the influent is leading to better removal of organic micropollutants and NOM when compared to filters receiving water without oxidative pre-treatment (Sontheimer et al. 1978; Orlandini 1999; van der Aa et al. 2012).

Peel and Benedek (1980) gave another explanation for the continuing removal of organic compounds in GAC filters and demonstrated that kinetic limitation of the diffusion in the micropores can result in a continuing adsorption long after apparent breakthrough has been achieved. This is attributed to very slow adsorption and described by a dual-rate kinetic model that produce breakthrough curves very similar to adsorption curves observed in practice. This second slow removal phase is usually seen as biodegradation, but the authors anticipated that some of the removal that was attributed to biodegradation might be purely adsorptive (Peel and Benedek 1980; 1983).

The improvement of GAC performance due to enhanced microbial activity is often explained by *bioregeneration,* the idea based on the disproportion between oxygen consumption and the removal of organic compounds (Sontheimer 1979; Jekel 1979). However, van der Kooij (1983) suggested that an elevated exceeding oxygen consumption is due to biomass accumulation. The research on bioregeneration focused mainly on restoration of adsorption capacity for a single solute as phenol, naphthalene (Chudyk and Snoeyink 1984; Goeddertz et al. 1988; Speitel et al. 1989; Hutchinson and Robinson 1990) or atrazine (Jones et al. 1998). Restoration

of 50-100% of adsorptive capacity for single solutes (phenol, naphthalene, dichloromethane, 3-chlorobenzoic acid etc.), and 20-40 % for humic substances has been reported for different systems of continuous or offline bioregeneration (Nagayev and Syrotkin 1998).

Two mechanisms are usually proposed to explain bioregeneration: exoenzymatic activity (Pierrotti and Rodman 1974) and reverse-concentration gradient (Andrews and Chi Tien 1981), the latter being more widely accepted and supported by experimental observations (Chudyk and Snoeyink 1984; Goeddertz et al. 1988; Speitel et al. 1989; Hutchinson and Robinson 1990). In both proposed mechanisms bioregeneration involves desorption of sorbed molecules, either following the action of exoenzymes or by the effect of microbial activity in the biofilm on the concentration and diffusion of a biodegradable solute on the surface and inside the GAC particle.

Beside the removal of organic contaminants, GAC filtration also affects the biological stability of water (van der Kooij 1984, 1987; Zang and Huck 1996; Servais et al. 1991; LeChevallier et al. 1992). For this purpose, GACFs are often operated long after the saturation of adsorption capacity as biological filters and the effect of biomass on the removal was addressed in different investigations (Huck et al. 1994; Wang et al. 1995; Carlson and Amy 1998; Laurent et al. 1999). Empirical and conceptual models have been developed to explain and predict the removal of biodegradable organic matter (as BDOC, AOC) in GACFs that were operating as biological filters (Billen et al. 1992; Huck et al. 1994; Hozalsky et al. 1995; Wang and Summers 1995; Huck et al. 2000). In empirical models, a linear relationship that was found between the influent quality (BDOC, AOC) and the removal rate was used to predict the removal at different operating conditions (van der Kooij 1987; Huck et al. 1994; Hozalsky et al. 1995; Wang and Summers 1995; Huck et al. 2000). Wang et al. (1995) and Carlson and Amy (1998) observed that the removal is controlled by the biomass concentration only up to a certain level, as markedly different biomass concentration in different filters was able to remove equivalent BDOC quantity.

Based on studies on GACFs in Parisian suburbs, a deterministic model for prediction of BDOC removal in biological filters, the Chabrol model, was developed (Billen et al. 1992). The model predicted the performance of a biological filter in the steady state mode and involves a total of three groups of parameters that characterize: (i) the interactions with solid support ii) the physiology of the microorganisms and iii) the bacterial mortality. Parameters describing the solid support are determined experimentally, but most of the parameters describing physiology, including non-grazing mortality, were adopted from the literature with the assumption that these are constant characteristics of naturally occurring bacteria in aquatic environments.

1.6 STUDY OBJECTIVES

GAC filtration is applied in water treatment in the Netherlands for the removal of organic micropollutants and for the reduction of colour, and in combination with advanced oxidation for the improvement of the biological stability of finished water. The efficiency of GAC filtration is limited, and GAC regeneration is needed when breakthrough of a target compound is reached

(Wiesner et al. 1973; Suffet 1980; Kruithof and van der Leer 1983; Sontheimer and Hubele 1987). Thermal regeneration of GAC is expensive, and the costs of the annual regeneration of GAC at location Weesperkarspel of Amsterdam Water Supply comprise 46% of the total operational costs for GAC filtration, and 15% of the drinking water production costs (Barrios et al. 2008).

After several decades of research, the relationship between microbial activity in GACFs and the surface characteristics of GAC is still not elucidated. Optimisation of the biological activity in GAC filtration may lead to further optimization of this treatment process. Such optimisation requires a fundamental understanding of the biological processes, the adsorption processes and their interactions, which may be influenced by water composition (raw water, pre-treatment), temperature (seasonal effects) and process conditions, respectively.

A research project was defined aiming at collecting such knowledge in a series of investigations including observations in full-scale treatment plants, laboratory experiments, and pilot plant studies. For this purpose, both adsorption processes (R. van der Aa, TU Delft) and microbiological processes were studied. The investigations described in this thesis aim at analysing the microbiological processes occurring in GACFs used in water treatment. In particular the contribution of bacteria to the removal of organic compounds is studied. To elucidate the contribution of the bacterial activity to the effects of GAC filtration on the removal of NOM from the water the study focused on the quantification and the characterisation of the bacterial community in GACFs.

The following goals of this investigation have been defined:

1. Development and evaluation of a method for measuring the concentration of active microorganisms in GAC filter beds;
2. Characterisation of the bacterial activity in GACFs, which includes:
   a. Isolation and identification of predominating bacterial species;
   b. Elucidation of physiological properties of the predominant species
3. Analysis of the interactions between process conditions and the microbiological processes during GAC filtration.

To this end, **Chapter 2** describes a method for measuring the concentration of active bacterial biomass in GACFs, focusing on the removal of the attached bacteria with high-energy sonication and their subsequent quantification of adenosine triphosphate (ATP) and total cell counts. Furthermore, the application of this method in full scale and pilot plant GACFs , RSFs and SSFs is described to obtain quantitative information about the level of microbial activity in GACFs as compared to RSFs and SSFs in relation to biofilms in drinking water treatment.

In **Chapter 3**, the use of genomic fingerprinting and 16S rRNA gene sequence analysis is described to identify the predominant cultivable bacteria from full-scale GACFs in water treatment plants in the Netherlands. **Chapter 4** continues with the cultivation-independent assessment of bacteria in full-scale GACFs based on the sequence analysis and t-RFLP fingerprinting of PCR-amplified bacterial 16S rRNA genes aiming to identify the predominant types.

The nutritional versatility and growth kinetics of two *Polaromonas* strains, representing the predominating bacteria in the studied GACFs, were analyzed in batch culture growth experiments with mixed and individual substrates at concentrations typical for drinking water (**Chapter 5**). The information about the nature of favourite substrates and kinetic parameters of growth for these two strains, together with the information about the concentration of biomass in a GAC filter is then applied in **Chapter 6** to improve our understanding of the removal of organic matter by the biodegradation processes in a GAC filter.

Obtained results and insights are integrated with available relevant data from the literature in a final synthesis in **Chapter 7** with focus on the effect of the GAC surface characteristics on the microbial activity and biodegradation in GACFs, the mechanisms behind the enhanced performance of GACFs and the significance of insights obtained in this study for drinking water treatment practice.

Several organisations have contributed to this joint research project, including Amsterdam Water Supply (Waternet), Water Supply Midden Nederland (Vitens), Norit (Cabot), KWR Watercycle Research Institute (previously Kiwa Water Research), Delft University (TUD) and Wageningen University (WU). This study on the microbiological processes in GAC filters was financially supported by the Ministry of Economic Affairs, Project number BTS99160.

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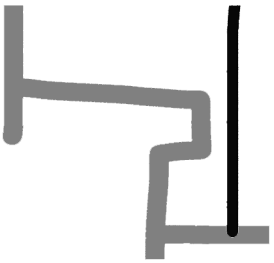

CHAPTER

2

OPTIMISATION AND SIGNIFICANCE OF ATP ANALYSIS FOR MEASURING ACTIVE BIOMASS IN GRANULAR ACTIVATED CARBON FILTERS USED IN WATER TREATMENT

This chapter is based on publication:

Magic-Knezev A. and Van der Kooij D. (2004) Optimisation and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment. *Water Research,* 38, 3971–3979.

ABSTRACT

A method for determining the concentration of active microbial biomass in granular activated carbon (GAC) filters used in water treatment was developed to facilitate studies on the interactions between adsorption processes and biological activity in such filters. High-energy sonication at a power input of 40 W was applied to GAC samples for the detachment of biomass which was measured as adenosine triphosphate (ATP). Modelling of biomass removal indicated that a series of six to eight sonication treatments of two minutes each yielded more than 90% of the attached active biomass. The ATP concentrations in 30 different GAC filters at nine treatment plants in The Netherlands ranged from 25 to 5000 ng ATP cm^{-3} GAC, with the highest concentrations at long filter run times and pretreatment with ozone. A similar concentration range was observed in nine rapid sand (RS) filters. ATP concentrations correlated significantly ($p<0.05$) with total direct bacterial cell counts in each of these filter types, but the median value of the ATP content per cell in GAC filters (2.1×10^{-8} ng ATP/cell) was much lower than in the RS filters (3.6×10^{-7} ng ATP/cell). Average biofilm concentrations ranging from 500 to 10^5 pg ATP cm^{-2} were calculated assuming spherical shapes for the GAC particles but values were about 20 times lower when the surface of pores > 1 µm diameter is included in these calculations. The quantitative biomass analysis with ATP enables direct comparisons with biofilm concentrations reported for spiral wound membranes used in water treatment, for distribution system pipes and in other aquatic environments.

***Key words* – granular activated carbon, adenosine triphosphate, biological filtration, biofilms, ultrasound.**

Nomenclature

ATP = adenosine triphosphate
CFU = colony-forming units
DOC = dissolved organic carbon
GAC = Granular Activated Carbon
HES = high-energy sonication
HPC = heterotrophic plate count
LES = low-energy sonication
NOM = natural dissolved organic matter
NOX = *Spirillum sp.* strain NOX
P17 = *Pseudomonas fluorescens* strain P17
TDC = total direct cell count
UST = ultrasonic treatment

2.1 INTRODUCTION

Granular activated carbon (GAC) filtration is used in water treatment for the removal of organic compounds that cause undesirable colour, odour or taste, and for the removal of pesticides and other xenobiotics, respectively. Adsorption and biodegradation occur simultaneously during GAC filtration.

A significant part of the costs of GAC filtration in water treatment is caused by the required thermal regeneration. Ozonation increases the biodegradability and reduces the adsorbability of natural organic matter (NOM) dissolved in water (Sontheimer et al. 1978; Van Leeuwen et al. 1985; Carlson et al. 1996). Consequently, preozonation enhances the microbial activity in GAC filters and delays the occupation of adsorption sites by NOM, resulting in postponed regeneration of GAC for the removal of persistent xenobiotics (Orlandini 1999). More detailed knowledge about the interactions between treatment conditions and biodegradation in GAC filters may lead to a more efficient use of GAC filters in water treatment. A method for determining the concentration of active biomass on the surface of GAC was developed as a first step toward elucidation of these interactions.

A variety of techniques has been used to assess the activity or the concentrations of bacteria present in GAC filters, e.g. oxygen consumption (Klotz et al. 1975; Van der Kooij 1983; Urfer and Huck 2000), heterotrophic plate counts (Klotz 1979; Camper 1985), total direct cell counts (DiGiano et al. 1992), scanning electron microscopy (Weber 1978; Cairo et al. 1979), phospholipids (Wang et al. 1995 and Carlson et al. 1996), uptake of labelled substrates (White et al. 1977; Servais et al. 1994) and reduction of 2-(p-iodo-phenyl)-3(p-nitrophenyl)-3 phenyl tetrazolium chloride (INT) (Fonseca et al. 2001), respectively. Factors hampering the general application of one of these methods include the laborious nature of the analytical procedure and difficulties with the interpretation of the data: e.g. oxygen uptake by activated carbon itself, the large percentage of viable but uncultivable microorganisms and no differentiation between viable and non active bacteria, respectively. This paper describes an ATP-based method for determining the concentration of the active biomass in GAC filters. ATP was selected because of the speed and accuracy of the analysis and its general use in aquatic microbiology (Karl 1980; Webster et al. 1985; Van der Kooij et al. 1995). The micro-organisms were removed from GAC surface with ultrasound, a technique that is often used for the detachment of particle-associated microorganisms and biofilms (Mathieu at al. 1993; Van der Kooij et al. 1995). However, sonication can inactivate microorganisms (Hua and Thompson 2000) and therefore the effects of ultrasonic treatment on the ATP concentration and cultivability of pure cultures and detached biofilm bacteria were investigated in this study.

The main objectives of this investigation were:

1. To optimise ultrasonic treatment for the removal of bacteria from GAC in combination with the use of ATP analysis for biomass quantification;
2. To determine the relationship between ATP concentrations and selected biomass parameters (HPC, TDC) in GAC filter beds and obtain information about effects of process conditions on the biomass concentration in these filters. For this purpose a limited survey on biomass con-

centrations in GAC filters, rapid sand (RS) filters and slow sand (SS) filters as applied in water treatment was conducted.

2.2 MATERIALS AND METHODS

2.2.1 Method development

Method development was conducted in four phases: (i), determination of the effects of ultrasound on the ATP concentration and cultivability with selected pure cultures of aquatic bacteria (*Spirillum sp*., strain NOX and *Pseudomonas fluorescens*, strain P17) and suspensions of mixed bacterial communities detached from GAC samples. The suspensions were treated (10 times) with ultrasound and after each treatment ATP and cultivability were determined. Untreated suspensions served as a control; (ii), determination of the effect of the adsorptive properties of GAC and the release of fines on the concentration of free ATP. Free ATP was added to a suspension of virgin GAC or GAC with developed biomass and incubated for two hours. A solution of free ATP in autoclaved tap water was used as control;. (iii), testing of the efficiency of the sonication treatment to remove attached bacteria with bacterial biofilms on plasticized polyvinylchloride (PVCp), GAC and sand. The materials were subsequently treated up to 20 times with ultrasound at different power inputs and after each treatment biomass removal was measured as the ATP concentration. The ATP yield was plotted against the number of treatments; (iv), from the results of these experiments an optimal method for the removal and measurement of active biomass was defined and applied to a series of samples from different filter beds.

2.2.2 Samples from filter beds

Samples of GAC, sand and anthracite were obtained from nine water treatment plants in The Netherlands. Filter material (10-100 g) was collected from below the filter bed surface (2-10 cm) with a properly cleaned multisampler (Eijkelkamp) and stored in 100 cm^3 screw-capped borosilicate flasks in the filtrate of the sampled unit at 4°C for a maximum period of 36 hours before the analysis. The specific density of the filter material was calculated from the weight of 50 cm^3 of GAC, sand or anthracite that had been dried for four hours at 105° C. The volume is determined with a measuring cylinder (100 cm^3). Prior to its use in experiments, virgin carbon (Norit 0.8 ROW Supra) was kept in demineralised water for 48 hours to allow saturation with oxygen. Subsequently, acid (1M HCl) was added to obtain pH 8.

2.2.3 Calculation of external surface of GAC

Data for the external surface of the GAC particles assuming spherical shapes were derived from the literature (Sontheimer et al. 1985). The estimation of the surface of the pores with a diameter $> 1\ \mu$m is based on data provided by the manufacturer about GAC porosity as based on penetration of butan at increasing pressure (Wheeler et al. 1983) and the assumption that pores had a cylindrical shape.

2.2.4 Test strains, mixed GAC community and stationary biofilm

Spirillum sp. strain NOX and *Pseudomonas fluorescens* strain P17 were obtained from the stationary growth phase of AOC tests in treated water as described earlier (Van der Kooij 1992). A mixed microbial community as present on GAC was obtained with a two minutes low energy

sonication (LES) treatment of a GAC sample (2 g wet weight) in 50 cm^3 autoclaved tap water. Subsequently, the suspended bacteria were separated from the GAC particles and kept in autoclaved tap water for 4 hours at 20°C to allow adaptation to the new environment. A biofilm was grown on pieces (20 cm^2) of plasticized PVC (PVC-P) incubated for 10 days at 15 °C in 600 cm^3 of tap water inoculated with 1 cm^3 of filtered river water (1.2 μm membrane filter) and enriched with nitrogen and phosphorus to prevent growth limitation (Van der Kooij and Veenendaal 2001).

2.2.5 Ultrasonic treatment

A total of two to five gram of wet filter material was added to 50 cm^3 of autoclaved tap water (pH 8.4±0.2) in a 100 cm^3 screw-capped borosilicate flask. Sonication was applied for two minutes. A volume of five cm^3 of the obtained suspension was collected and kept on ice for examination. The surplus liquid was decanted, 50 cm^3 autoclaved tap water was added and sonication was repeated. This procedure was repeated several times depending on the objective of the experiment. Low-energy sonication (LES) was applied with a Branson sonication unit 5050 at a constant frequency of 43 kHz and 180 W power output. Samples contained in 50 cm^3 of autoclaved tap water were placed in the sonication chamber with 10 L of demineralised water. High-energy sonication (HES) was applied with a Sonifier II W-250 at a constant frequency of 20 kHz and an adjustable power output. Ultrasound was applied to the sample via a titanium microtip (D = 5 mm), with power inputs ranging from 10 to 40 W.

2.2.6 ATP measurement

The measurement of ATP is based on the production of light in the luciferine-luciferase assay. ATP was released from suspended cells with nucleotide-releasing buffer (NRB, Celsis). The intensity of the emitted light was measured in a luminometer (Celsis AdvanceTM) calibrated with solutions of free ATP (Celsis) in autoclaved tap water following the procedure as given by the manufacturer. The detection limit was 1 ng ATP L^{-1} of sample. Standard additions of free ATP dissolved in autoclaved tap water were used for recovery experiments.

The influence of the adsorptive properties of GAC on the ATP measurement was tested by addition of free ATP to suspensions of virgin and preloaded GAC in autoclaved tap water (0.04 - 0.1 g GAC cm^{-3}, dry weight). The concentrations of added ATP were of 2, 100, 600 and 3000 ng L^{-1} respectively. Autoclaved tap water with similar concentrations of free ATP was used as control. Recovery of added ATP was calculated from the measured ATP concentrations in the controls and in samples, which were corrected for the concentration of autochthonous ATP. The influence of carbon fines on the efficiency of light measurement was determined with GAC sampled from a filter bed and suspended in autoclaved tap water at concentrations of 0.01 to 0.3 g GAC cm^{-3} (dry weight) and subsequently sonicated. The liquid phase with GAC fines was separated from the GAC particles by decantation. After the measurement of autochthonous ATP, free ATP was added and the recovery was calculated from the measured values in the samples and the control solutions, respectively.

2.2.7 Total direct cell count (TDC)

A defined volume of the microbial suspension as obtained by sonication of GAC samples was filtered through a 0.22 μm polycarbonate filter, stained with acridine orange as described by

Hobbie et al. (1977) and observed with epifluorescence microscopy (1000 x, Leica DM RXA). A total of ten random fields, each containing 20 to 200 cells, were analysed.

2.2.8 Heterotrophic plate counts (HPC)

Volumes of 0.05 cm^3 of appropriate decimal dilutions of microbial suspensions obtained by sonication of GAC, sand or anthracite in autoclaved tap water were spread in triplicate over the surface of R2A agar (Oxoid Ltd.) plates, which were incubated during 10 days at 25° C.

2.2.9 Statistic analysis

The statistical significance of the effects of ultrasonic treatment on microbial ATP and cultivability was determined by a t-test after correction of the data for the values measured in the controls.

2.3 RESULTS

2.3.1 Effects of sonication on microbial ATP and cultivability

The concentration of ATP in a suspension of NOX and P17 grown in treated water and in suspensions of the microbial community obtained from GAC samples (n=3) remained unaffected (102±2%) after ten LES treatments of two minutes each. This treatment also had no effect on the HPC values in these samples (105±15%). Ten HES treatments of two minutes each at power inputs of 20 W and 40 W also did not significantly affect the ATP concentration in microbial suspensions of NOX and P17 and mixed microbial communities obtained from GAC (recovery > 90%; n=4). HPC values increased after the first few treatments at both power inputs but further sonication obviously damaged the bacteria. This damage occurred after four to six treatments in the samples treated with 20 W and after two to four treatments in the samples that were treated with 40 W. Typical examples of this effect are shown in Figure 2.1. The decrease of the HPC values following HES treatment fitted to an exponential function:

$$B_T = B_0 \cdot e^{-k.t} \quad (1)$$

where B_0 is the HPC value in the untreated sample, and k (min^{-1}) is the coefficient of exponential decrease. Values of k for strain NOX, strain P17 and a mixed microbial community obtained from a GAC filter were calculated from the effects of ten subsequent HES treatments at power inputs of 20 and 40 W (Table 2.1). From these values it can be derived that the HPC values of strain NOX and the mixed microbial community decreased with 1 to 6 % for each minute of HES treatment in most cases but with strain P17 also decreases of 15 to 20% were observed (Fig. 2.1b). HES treatment affected strain P17 significantly ($p < 0.05$) more than strain NOX and the mixed microbial community from GAC. Furthermore, treatment at 40 W gave significantly larger k values than treatment at 20 W ($p < 0.05$).

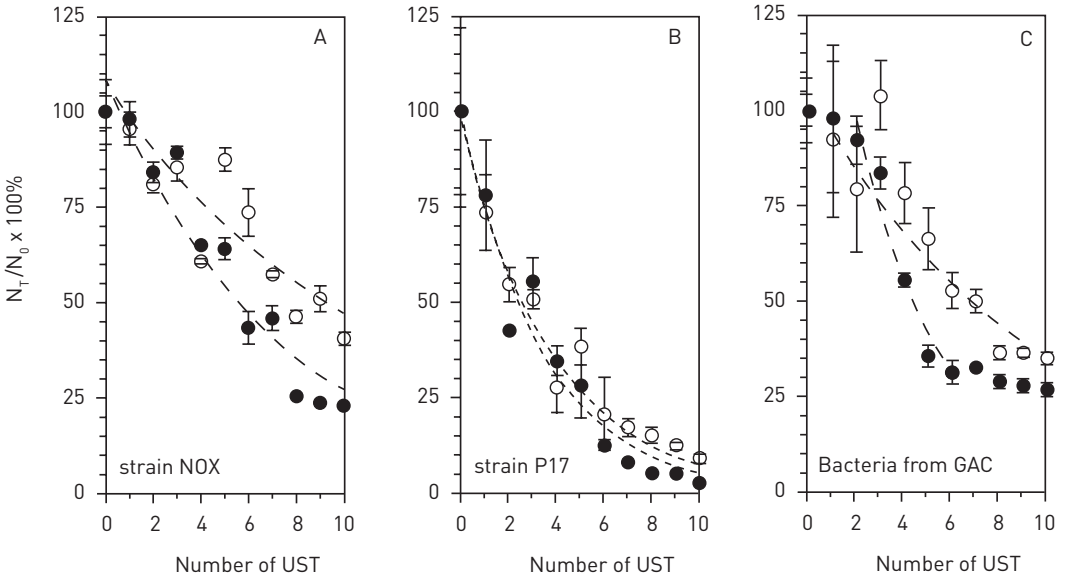

Figure. 2.1 Effect of HES treatment on the HPC values of strain NOX and strain P17 grown in tap water and a mixed microbial community obtained from a GAC filter. Abbreviations: N_0, concentration at start (time zero, untreated sample); N_T, concentration at time T. Symbols: ○, 20 W; ●, 40 W.

Table 2.1 Coefficients of exponential decrease (k, min^{-1}) of the cultivability of strain NOX, strain P17 and a mixed microbial community obtained from a GAC filter, after ten subsequent HES treatments at two power inputs.

Organism	High energy sonication (HES)	
	20 W	40 W
Spirillum sp. strain NOX	0.018 ± 0.006 (r^2= 0.59) 0.029 ± 0.002 (r^2= 0.96) 0.041 ± 0.01 (r^2 = 0,82)	0.064 ± 0.006 (r^2= 0.95) 0.041 ±0.004 (r^2= 0.94) 0.078 ± 0.01 (r^2 = 0.93)
Pseudomonas fluorescens strain P17	0.040 ± 0.007 (r^2= 0.82) 0.163 ± 0.006 (r^2 = 0.99) 0.122 ± 0.01 (r^2 = 0.97)	0.071 ± 0.012 (r^2= 0.85) 0.226 ± 0.03 (r^2 = 0.94) 0.147 ± 0.02 (r^2 = 0.95)
Mixed GAC community	0.007 ± 0.004 (r^2= 0.59) 0.054 ±0.01 (r^2 = 0.94)	0.035 ± 0.002 (r^2= 0.99) 0.101 ± 0.02 (r^2 = 0.82)

2.3.2 Stability of ATP in the presence of GAC

The effect of the adsorptive properties of GAC on the concentration of free ATP was tested with the internal standard addition method. The average recovery of free ATP was 104±7% (n=8) five minutes after the addition to tap water suspensions of virgin GAC and GAC from a filter bed. Two hours after the addition, the recovery of free ATP was 74±13% (n=4) for virgin

GAC and 91±12% (n=4) for GAC from a filter bed. The average recovery of ATP observed after standard addition (97 ±3%; n=48) further demonstrated that carbon fines produced during sonication of GAC had no significant effect on the ATP measurement.

2.3.3 Removal of microorganisms from GAC

Three samples from GAC filters and two sand samples from RSF filters were added to autoclaved tap water (0.1-0.3 g filter material cm^{-3}) and subjected to LES or HES at 40 W for two minutes in a series of 6 to 20 treatments of two minutes each. The cumulative ATP yield of the biomass obtained with LES was 50±8 % (n = 5) of the biomass obtained with HES. GAC samples from two different filter beds and samples of PVC-P with attached microorganisms were treated with HES at power inputs of 10 to 40 W. Maximum removals were achieved at power inputs above 20 W (Fig. 2.2a). Subsequently, the effects of sequential HES treatments at 40 W on biomass removal was determined for GAC sampled from seven different filter beds. Four samples had ATP yields below 1000 ng ATP cm^{-3} of GAC and in 3 samples the ATP yield varied between 1000 and 3200 ng ATP cm^{-3} GAC. Typical examples of cumulative ATP yields are shown in Figure 2.2b. The data fitted well with the saturation kinetics as described by the Michaelis-Menten equation ($0.91 \leq r^2 \leq 0.99$; n=7). In 5 of 7 samples the cumulative ATP yield as measured after six treatments was 90±3% of the maximum values calculated with this equation. GAC particles that had been sonicated ten times at 40 W cm^{-3} were stained with acridine orange and observed using fluorescence microscopy. The untreated particles emitted a high level of fluorescence but no fluorescent objects were observed on the treated particles (data not shown). Obviously, ten treatments had removed almost all attached biomass.

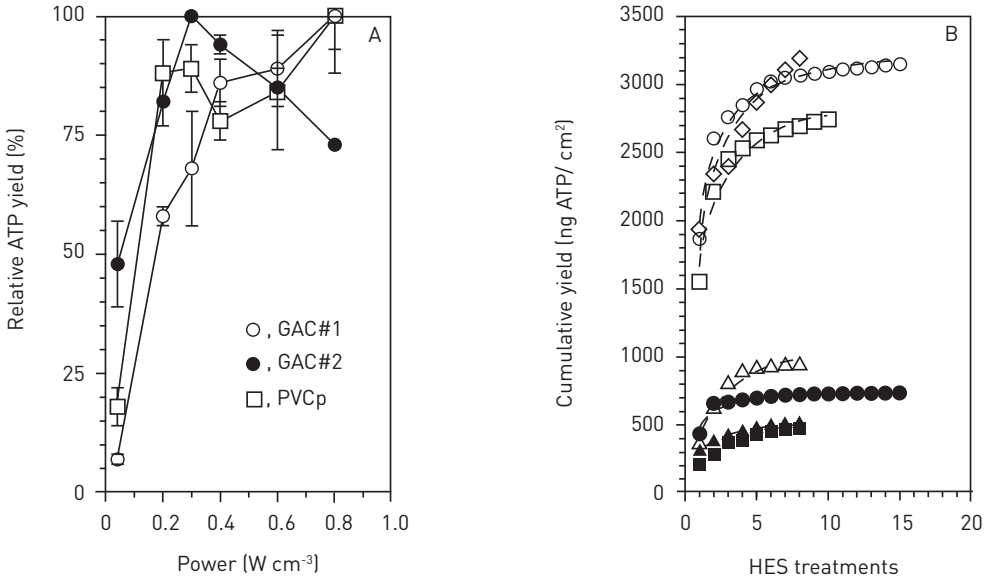

Figure 2.2 A. Removal of attached bacteria from GAC and plasticized (PVC) PVCp at different power inputs of HES; B, Cumulative ATP-yield as a function of the number of sonications in seven different GAC samples. The relative ATP yield (A) is Maximum observed value at applied power range.

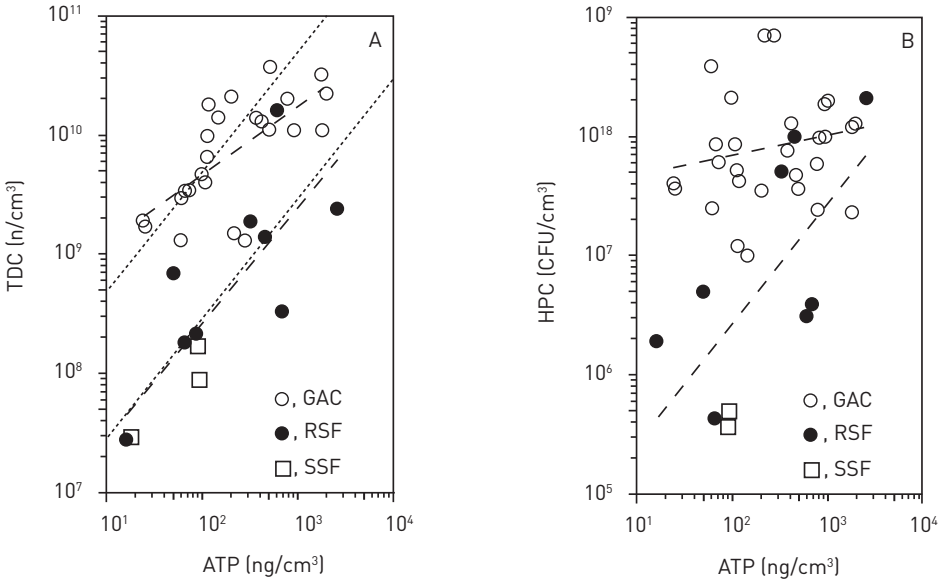

Figure. 2.3 Relationships between the ATP concentrations and TDC values (A) and HPC values (B), respectively in samples of filter material. GAC, granular activated carbon; RFS, rapid sand filters; SSF, slow sand filters. Broken lines represent calculated relationships; dotted lines represent proportional relationships based on the median values of the ATP content per cell (A).

2.3.4 Active biomass in GAC filters, rapid sand filters and slow sand filters

From a total of nine water treatment plants in The Netherlands samples were collected from 30 different GAC filters, nine rapid sand filters (RSF) and three slow sand filters (SSF) and analysed for ATP, HPC and TDC, respectively. These samples were selected to include different conditions of filtration, viz. raw water source, DOC concentration, pretreatment, filter run time, filtration rate and carbon type (Table 2.2). Measurements on GAC samples were all conducted in duplicate. The median value of the variation coefficient for the ATP analysis (6%) was slightly lower than the variation coefficients for TDC (14%) and HPC (15%), respectively. Significant correlations were observed between values of ATP and TDC ($p < 0.05$) in GAC filters and in RS filters ($p < 0.05$), respectively, but not between values of ATP and HPC (Fig. 2.3). The ATP concentrations in the GAC samples ranged from 25 to 5000 ng cm^{-3}, thus covering more than two orders of magnitude. The ATP concentrations of the RSF samples also covered a wide range but values were below 100 ng ATP cm^{-3} in the SSF samples (Fig. 2.4). ATP concentrations below 100 ng cm^{-3} were observed in GAC filters that had been in operation for a few weeks (samples 3 and 4). After 330 days the ATP concentration in these filters had increased tenfold (samples 21 and 22). The ATP concentration in GAC filters supplied with ozonated water (samples 15 and 40) was about 2 to 3 times higher than in filters operating under similar conditions but supplied with non-ozonated water (samples 7 and 32). At two of the nine investigated locations, two-stage GAC filtration was applied. The ATP concentration in the first stage filters at both locations (samples 20 and 37) was higher than in the second stage filters (samples 19 and 18).

Table 2.2 Operational parameters of filtration units and ranges of biomass concentrations on filter material. CT contact time, V filtration rate.

Filter type (N.samples)	Run time (days)	CT (min)	V ($m\ h^{-1}$)	DOC ($mg\ l^{-1}$)	ATP ($ng\ cm^{-3}$)	HPC ($CFU\ cm^{-3}$)	TDC ($Cells\ cm^{-3}$)
SSF (n=3)	675-2000	30-240	0.25-0.5	1.4-3.2	18-93	$4\ 10^5$- $5\ 10^5$	$3\ 10^7$ – $2\ 10^8$
RSF (n=9)	820-7300	5-20	3-11	2.0-3.2	16-2592	$4\ 10^5$ - $1\ 10^8$	$3\ 10^7$ – $2\ 10^{10}$
GAC (n=30)*	7-1580	10-45	3-10	1.8-5.4	24-5067	$1\ 10^7$ - $1\ 10^9$	$1\ 10^9$ – $4\ 10^{10}$

*number of samples tested for ATP concentration

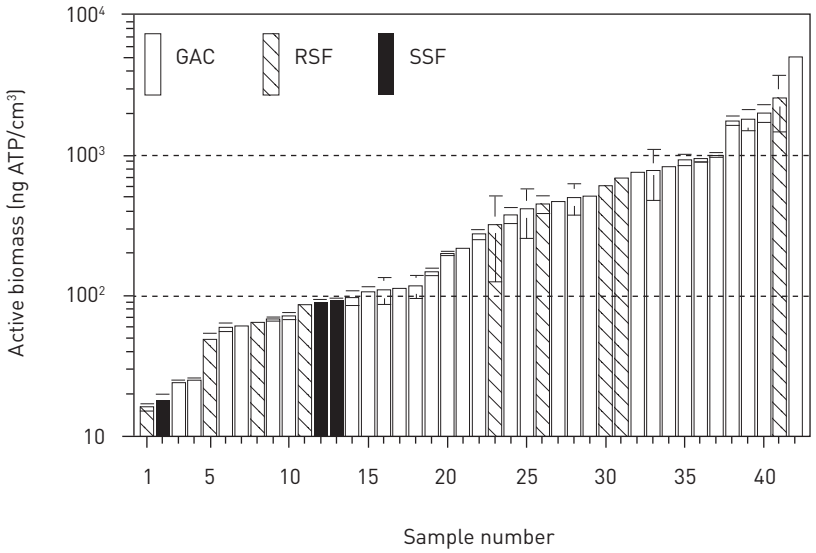

Figure. 2.4 Active-biomass concentrations in GAC, RSF and SSF samples from 9 water treatment plants in the Netherlands.

2.4 DISCUSSION

2.4.1 Method development

Removal of attached biomass from the GAC surface and its subsequent quantification by ATP are essential steps in the determination of the concentration of active biomass on GAC with the method developed in this study. LES treatment did not affect the ATP concentration and cultivability of the sonicated bacteria but was clearly less effective in biomass detachment than HES treatment. The first HES treatment incidentally caused some increase of the ATP concen-

tration but subsequent sonications slightly decreased the ATP concentration, with the total decrease remaining less than 10% after ten HES treatments. The slight initial increase of the ATP concentration and the HPC values after HES treatment may be attributed to a disintegration of clusters of bacteria during sonication and a subsequent better performance of nucleotide releasing reagent on individual bacteria. However, further HES treatment clearly damaged bacteria, as is demonstrated by the decreasing HPC values with increasing number of sonications (Fig. 2.1). The effect depends on the nature of the bacteria and the intensity of sonication, with HES treatments at 40 W damaging significantly more bacteria than treatment at 20 W (Table 2.1). The observed inactivation of strain NOX and the mixed microbial community of 3 to 9 % for each minute of HES treatment at 40 W agrees well with the inactivation (7 to 10%) reported for Escherichia coli for each minute of HES treatment (Hua and Thompson 2000).

Both virgin and colonised GAC did not effect the concentration of added ATP when the measurement was performed five minutes after addition. This observation demonstrates that free ATP (i) is relatively stable in the presence of GAC, (ii) does not adsorb rapidly onto GAC and (ii) is not rapidly utilised by the attached microorganisms. Also carbon fines eroding from GAC during sonication did not have a significant effect on the light yield at the applied sample volumes. The removal of attached bacteria depends on the power input of ultrasound. Maximum removal of attached bacteria from GAC is obtained with HES treatment at power inputs above 20 W (Fig. 2.2a). Six to eight HES treatments removed about 90% of the maximum biomass yield as calculated with the Michelis-Menten equation (Fig 2.2b), but these treatments may significantly affect the cultivability of the bacteria (Fig. 2.1). Based on these observations the following procedure for removal of bacteria from GAC was established. A total of 2 to 5 gram of wet filter material is added to 50 cm^3 of autoclaved tap water and treated with a series of six HES treatments at 40 W of two minutes each. After each HES treatment, the detached bacteria are separated from the material to avoid the damaging effect of subsequent treatments and stored on ice (< 30 min) prior to biomass analysis (ATP, HPC).

2.4.2 Biomass concentration in GAC filters

The significant correlation with TDC values and the low value of the variation coefficient of the ATP measurement confirmed that ATP is a suited parameter for the quantification of active biomass in GAC filters. Attractive properties of the ATP analysis include the short time required for analysis, the low detection level and the knowledge about the ecological and physiological significance of ATP Limitations of the parameter are the variations of the ATP content of cells depending on the growth phase and temperature and the sensitivity of the enzymatic reaction for certain inorganic compounds, e.g. copper and calcium (Karl 1980). Hence, the analytical procedures must be conducted under strictly defined conditions.

The concentrations of ATP in most GAC samples in this study were clearly higher than the values (60-200 ng ATP cm^{-3} GAC) reported by Van Leeuwen et al. (1985). The ATP concentration of 80 ng ATP g^{-1} dry weight sand as reported for an SSF sample (Seger and Rothman 1996) was similar to the values found in this study. The low biomass concentrations in SSF can be attributed to the low load with biodegradable compounds, viz. a low filtration rate (0.25 to 0.5 m h^{-1}) in combination with a low concentration of biodegradable compounds in the influents of these filters which are at the end of the treatment chain. The similarities between concentra-

tions of active biomass in GAC and RS filters may be explained by similarities in operational process parameters viz. filtration rate (3 - 10 m h^{-1}) and water quality. This study showed that GAC filters supplied with ozonated water contained about 2 to 3 times more active biomass than filters fed with non-ozonated water operating under the same hydraulic conditions. This observation is consistent with the observation that biomass levels as determined with INT in biofilter systems operated with ozonated water were about 50% higher than in identical systems operating with non-ozonated water. (Fonseca et al. 2001). Furthermore, a 50% reduction of biomass (measured as phospholipids) on GAC was observed after switching from ozonated to non-ozonated the influent water (Wang et al. 1995). Hence, preoxidation, running time and also the position of the filter beds in water treatment are dominant parameters affecting the biomass concentration in GAC filters. Quantification of these effects, and also the impact of carbon type, requires further investigation

The surface area, which is important for the transport of substrate from the water phase to the GAC surface, can be estimated at approximately 50 to 100 $cm^2\ g^{-1}$ when spherical shapes are assumed for the GAC particles (Sontheimer 1985). The observed values of 25 to 5000 ng ATP cm^{-3} of GAC, with an average dry weight of about 0.5 g cm^{-3}, thus correspond with 500 to $2x10^5$ (median: 10^4) pg ATP cm^{-2} of this surface. These values are in the same range as biomass concentrations in fouling spiral wound membranes used in water treatment and on plastic materials in contact with biologically stable water but much higher than most biofilm concentrations observed in distribution systems in the Netherlands (van der Kooij 1999; Vrouwenvelder et al. 1998). The concentrations of biomass carbon on GAC, as calculated with the conversion factor C = 250 x ATP (Karl 1980), ranged from 6.3 to 1250 (median: 80) μg C cm^{-3} of GAC. These values clearly exceed the biomass concentrations previously reported for GAC (< 10 μg C cm^{-3}) used in water treatment (Servais et al. 1991) and are also higher than the value for the maximum capacity of GAC for bacterial fixation (20 μg C cm^{-3}) and the maximum adsorption capacity for bacteria (40 μg C cm^{-3}) as defined in the Chabrol model (Billen et al. 1992). Most biomass carbon concentrations estimated for the surface of spherical GAC particles (0.13 to 50 μg C cm^{-2}) are also higher than those (0.1 to 1.2 μg C cm^{-2}) reported for drinking water distribution systems (Niquette et al. 2000). The surface of GAC potentially available to microbial attachment, which includes the surfaces of all pores larger than 1 μm, is about 20 times larger than the surface of the spheres (Wheeler et al. 1983). The average biomass concentrations calculated for this surface (25 – 10,000 pg ATP cm^{-2}; 0.01 – 2.5 μg C cm^{-2}) are similar to concentrations observed on surfaces exposed to treated water.

2.4.3 Relationships between HPC, TDC and ATP analysis

The HPC values in GAC samples measured in this study correspond well with values reported earlier (Klotz 1975; Van der Kooij 1983; Cairo et al. 1979) and also with the numbers of active bacteria calculated from the respiration of radiolabeled substrate (Servais et al. 1994). Only small fractions of all bacteria (0.02 to 9%) produced colonies on R2A medium. The impact of sonication on cultivability is limited in comparison to these low percentages. The observed TDC values (up to 4 10^{10} cells cm^{-3} GAC) are clearly higher than the values obtained with scanning electron microscopy (10^8 cells g^{-1} wet GAC; Klotz 1979) and fluorescence microscopy (10^9 cells $gram^{-1}$ wet GAC; DiGiano et al. 1992). The relatively high TDC values may be explained by the release of microbial cells from the pore surfaces, which are not visible with microscopy.

From the TDC values it can be derived that the present cells do not form a real biofilm on the GAC surface. The average coverage of the total accessible surface (up to 4000 cm^2 cm^{-3} GAC) is less than 5 % at TDC values below 10^{10} cells cm^{-3}. This low percentage indicates that the growth of bacteria on the GAC surface of the pores larger than 1 μm is limited by the availability of biodegradable compounds.

The average ATP content per cell, which is a measure for metabolic activity, ranged from 7 10^{-9} to 2 10^{-7} ng ATP $cell^{-1}$ for the filter bed materials (Table 2.2). The median ATP content of cells on GAC (2.1 10^{-8} ng ATP $cell^{-1}$) is at the low range of the ATP content reported for groundwater bacteria (2 10^{-8} to 4 10^{-7} ng ATP $cell^{-1}$; Jensen 1989, Metge et al. 1993) and bacteria on membranes used in water treatment (2 10^{-8} to 7 10^{-7} ng $cell^{-1}$; Vrouwenvelder et al. 1998). This median value is also lower than 10^{-7} ng ATP $cell^{-1}$ reported for starving cells (Webster et al. 1985), indicating rather low metabolic activities of the cells on GAC. The median value of the ATP content of the cells in RS filters (3.6 10^{-7} ng/cell) was about 10 times higher than the value obtained for the GAC filters. These observations indicate that (i), the cells in GAC filters were smaller and/or less active than those in RS filters and (ii), TDC values are not suited for quantitative assessment of the biomass concentration but give valuable information when used in combination with measurements of ATP or cell size. The high ATP content of the cells in RS filters suggests a relatively high growth rate, which may be explained by the frequent and intensive backwashing of these filters. The effects of the difference in cell activities (and growth rates) between GAC and RS filters on biodegradation processes are not yet clear. The biomass activity in GAC filters will be further investigated by determining the identity and physiological properties of the predominating bacteria.

CONCLUSIONS

1. HES treatment is more effective than LES treatment for the removal of biomass from GAC; it does not affect the concentration of free ATP, but reduces cultivability. Modeling indicates that more than 90% removal of attached biomass (ATP) is achieved with a series of six to eight HES treatments at 40 W power input.
2. The uptake of free ATP by cells or GAC is negligible in the applied procedure when the analysis is conducted five minutes after sonication and ATP analysis is not affected by carbon fines released during sonication. ATP concentrations in GAC filters and in RS filters correlate significantly with the TDC values in each of these filter types, but the median value of the ATP content of the cells in GAC filters is about 10 times lower than in RS filters;
3. ATP concentrations in GAC filters in water treatment cover a wide range of concentrations. Biomass concentration on GAC was affected by running time and pretreatment with ozone. The concentrations of active biomass on GAC calculated for the total accessible surface are at the same range as concentrations of biofilms in distribution systems, indicating a limited availability of growth substrates.

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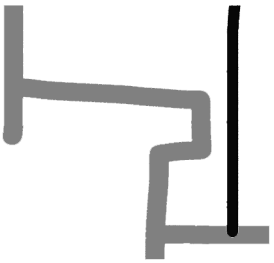

CHAPTER

3

POLAROMONAS AND *HYDROGENOPHAGA* SPECIES ARE THE PREDOMINANT BACTERIA CULTURED FROM GRANULAR ACTIVATED CARBON FILTERS IN WATER TREATMENT

This chapter is based on publication:

Magic-Knezev A, Wullings B., Van der Kooij D. 2009. *Polaromonas* and *Hydrogenophaga* species are the predominant bacteria cultured from granular activated carbon filters in water treatment. *Journal of Applied Microbiology* 107(5), 1457-1467

ABSTRACT

Aim: Identification of the predominating cultivable bacteria in granular activated carbon (GAC) filters used in a variety of water treatment plants for selecting representative strains to study the role of bacteria in the removal of dissolved organic matter.

Methods and Results: Bacterial isolates were collected from 21 GAC filters in nine water treatment plants treating either ground water or surface water with or without oxidative pretreatment. Enrichment of samples in dilute liquid medium improved culturability of the bacteria by approximately log unit, to 9% up to 70% of the total cell counts. Genomic fingerprinting and 16S rRNA sequence analysis revealed that most (68%) of the isolates belonged to the *Betaproteobacteria* and 25% were identified as *Alphaproteobacteria*. The number of different genera within the *Betaproteobacteria* was higher in the GAC filters treating ozonated water than in the filters treating nonozonated water. *Polaromona* was observed in nearly all of the GAC filters (86%), and the genera *Hydrogenophaga*, Sphingomonas and Afipia were observed in 43%, 33% and 29% of the filter beds, respectively. AFLP analysis revealed that the predominating genus *Polaromonas* included a total of 23 different genotypes.

Conclusions: This study is the first to demonstrate that *Polaromonas*, which has mainly been observed in ultraoligotrophic freshwater environments, is a common component of the microbial community in GAC filters used in water treatment.

Significance and Impact of the Study: The predominance of ultraoligotrophic bacteria in the GAC filters indicates that very low concentrations of substrates are available for microbial growth. *Polaromonas* species are suited for further studies on the nutritional versatility and growth kinetics enabling the modelling of biodegradation processes in GAC filters.

ECN SEI 5.0kV X500 10µm WD13mm

3.1 INTRODUCTION

Granular activated carbon (GAC) filters are used in water treatment for the removal of natural organic matter (NOM) and organic micropollutants. The main removal mechanism is adsorption, but part of the dissolved organic compounds is also removed by biological activity in these filters. Biodegradation processes improve the biological stability of the water by removing biodegradable nonadsorbable organic compounds that may cause bacterial regrowth and biofilm formation in drinking water distribution systems (LeChevallier et al. 1987; van der Kooij et al. 1989; Servais et al. 2004). Furthermore, biodegradation of adsorbable organic compounds delays the saturation of adsorption sites, thereby increasing the quantity of organic matter that can be removed by a GAC filter in a certain period of time (Wilcox et al. 1983). The concentration of micropollutants in filtered water most often determines the operational period of a GAC filter and postponed breakthrough of these compounds therefore reduces the costs of water treatment (Orlandini et al. 1997).

The efficiency of the biodegradation processes depends on the concentration and activity of the microbial biomass and the nutritional properties of the microbial community. The properties of the microbial community are in turn determined by the composition and concentration of NOM and by the operational parameters (filtration rate, contact time and GAC properties) and temperature. Several methods are available for determining the concentration (or activity) of biomass in GAC filters, e.g. oxygen consumption, tetrazolium reduction, phospholipids and adenosine triphosphate (ATP) (Camper et al. 1985; Urfer and Huck 2000; Fonseca et al. 2001; Magic-Knezev and van der Kooij 2004; Velten et al. 2007). Based on the data about ATP concentrations observed in the GAC filters, it can be estimated that at least 6–125 kg of dry weight of microbial biomass carbon is present in a filter bed containing 100 m^3 of GAC (Magic-Knezev and van der Kooij 2004).

Information about the nutritional properties of the predominating bacteria is needed to elucidate the biodegradation capacity of GAC filters. Bacteria isolated from GAC filters have been identified in most cases as members of the genera Pseudomonas, Acinetobacter, Caulobacter, Alcaligens, Flavobacterium and Bacillus (McElhaney and McKeon 1978; Wilcox et al. 1983; Stewart et al. 1990). However, these bacteria may not represent the microbial community because substrate-rich solid media was used with an incubation time of a few days, yielding only small fractions of the bacteria present in oligotrophic environments (Reasoner and Geldreich 1985). Furthermore, many aquatic isolates remained unidentified because of restricted reactions with substrates used at high concentrations in the classic tests for the identification of bacteria. To date, molecular analytical methods enable effective identification of isolated bacteria. Furthermore, culture- independent techniques revealed the existence of many unknown and uncultured bacteria and stimulated researchers to adapt the cultivation techniques for bacteria from environmental samples (Button et al. 1993; Bussmann and Schink 2001; Page et al. 2004). An advantage of using cultivation methods is that organisms are obtained for further studies.

The objectives of our study were: (i) identification of cultivable bacteria predominating in GAC filters used in water treatment and (ii) selection of representatives of these bacteria for

further study. In this study, the number of cultivable bacteria was determined in GAC filters at a variety of full-scale water treatment plants in relation to the total microscopic count using enrichment media with low nutrient concentration to favour growth of aquatic bacteria. Subsequently, isolates were collected and identified based on 16S rRNA sequences, and fingerprinting techniques were applied to determine the diversity of pre- dominant isolates.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of predominant bacteria

Bacteria were isolated from 21 different GAC filters, the sand of six dual media (anthracite /sand) filters and one slow sand filter (SSF), respectively, at nine full-scale water treatment plants in The Netherlands. The GAC filters were operated at different conditions. The empty-bed contact time varied between 10 and 45 min and the filtration rate was in the range of 3–10 m h^{-1}. The concentration of dissolved organic carbon varied between 1.5 and 5.5 mg L^{-1} and the water temperature ranged from 5 to 21°C. Filter material was collected, stored and treated with ultrasound to obtain suspended biomass as described previously (Magic-Knezev and van der Kooij 2004). The bacteria in the obtained suspensions were cultivated on solid medium. Appropriate decimal dilutions of the obtained bacterial suspensions were spread in triplicate over the surface of R2A agar (Oxoid) plates and incubated during 10 days at 25°C (Reasoner and Geldreich 1985). The colonies were counted and from these counts, the number of colony-forming units (CFU) cm^{-3} was calculated. From each sample, three colonies of the three most abundant colony types and colonies with a distinctive morphological feature were collected for further analysis.

The most probable number (MPN) method was used for enumeration of the concentration of the bacteria in samples of nine GAC filters. The liquid medium used for this purpose was prepared from the autoclaved influent of the GAC filter enriched with 5 mg L^{-1} of yeast extract (YE). The obtained biomass suspensions were decimally diluted in autoclaved tap water and inoculated in fivefold in 9-ml volume of the YE medium. Growth was monitored with ATP analysis during 14 days of incubation at 22°C. Subsequently, the concentration of bacteria was determined according to MPN method (Clesceri et al. 1996). Volumes of 0.1 ml from the highest dilution with growth were plated on R2A medium to obtain pure cultures. A total of 186 isolates were collected from the R2A plates and 25 isolates were collected from the liquid medium.

3.2.2 Identification of isolated bacteria

Collected isolates were screened with the repetitive extragenic palindrome rep-PCR to identify colonies with similar appearance and select the representatives for the sequencing of 16S rRNA. Full sequences were used to determine the phylogenetic position of an isolate and partial sequences were used to confirm the identity of isolates with rep-PCR pattern similarity >90%. AFLP fingerprints were used to evaluate the diversity within predominant genus.

3.2.3 Rep-PCR analysis

Freshly grown colonies on R2A medium were suspended in 500μl of DNA-free water and DNA was extracted using the Bio-Rad DNA Extraction Kit, following the procedure prescribed by the manufacturer. Subsequently, a genomic fingerprint was generated using specific primers for the REP regions of DNA as described previously (Versalovic et al. 1991). The PCR products were separated using agarose gel electrophoresis (2.5% fine NuSieve 3:1; BMA, Rockland, ME, USA) at 60 V for 16 h. Gels were stained for 30 min with Cyber gold and digitized with Bio-Rad Gel Documentation System (Bio-Rad). REP- patterns were compared by the bionumerics software ver. 3.2 (Applied Maths NV) after the normalization to the 100-bp standard reference lanes (Invitrogen 100-bp DNA ladder, Cat no. 15628019), applying the unweighed pair group method. Rep-PCR fingerprints were generated from DNA of *Aeromonas hydrophila* (strain M800), *Ps. fluorescens* (strain P17) and *Spirillum sp*. (strain NOX) in six to nine series to determine the variability of the procedure. These strains were grown and maintained on Lab-Lemco Agar (Oxoid).

3.2.4 AFLP analysis

The AFLP technique for DNA fingerprinting adapted for analysis of *Campylobacter* strains was used (Duim et al. 1999). The final products were diluted 1 : 1 together with an internal lane standard (PE Applied Biosystems) and analysed on a short capillary/POP four polymer by using a model ABI 310 automated DNA sequencer. The obtained chromatograms were normalized with internal lane stan- dard and compared using bionumerics software ver. 3.2.

3.2.5 Sequencing and phylogenetic analysis

The 16S rRNA was amplified after DNA isolation with PCR using Taq DNA polymerase with primers for conserved domains (Devereux and Willis 1995). Full 16S rDNA sequences were analysed using two internal primers (530R16 and 907F16) and two terminal primers (8R16 and 1510R16) and partial 16S rRNA sequences were analysed using one internal primer (907F16). The 16S rRNA sequences were assembled with the Croma Tool and the dnastar software package (DNAStar Inc., WI). Automatically aligned and subsequently manually corrected sequences were added to the ARB SSU rDNA database tree (Technische Universität München, Freising, Germany) using the quick parsimony tool without filters. The identity of isolates on the genus level was derived from their position in the ARB SSU rDNA database tree (Ludwig et al. 2004). Sequences were also compared with the sequences from the GenBank data-base using the Blast tool (http://www.ncbi.nlm.nih.gov/ blast) to identify the closest relatives.

3.2.6 Statistical analysis

The differences between the proportions of different bacterial species or groups originating from the filters treating different water types were tested using the Z-test (http://onlinestatbook.com/rvls/) on difference between proportions at a confidence level of 95%.

3.2.7 Nucleotide sequence accession numbers

All partial or full-length sequences from 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers EU130949–EU131006 and EU180508–EU180567.

3.3 RESULTS

3.3.1 Isolation of predominant cultivable bacteria

The colony counts of bacteria on R2A medium ranged from $1 \cdot 10^7$ to $5 \cdot 10^9$ CFU cm^{-3} and represented 0.2– 9% of the total direct cell count (TDC) values that ranged from $1 \cdot 10^9$ to $4 \cdot 10^{10}$ cell per cm^3 GAC (Magic- Knezev and van der Kooij 2004). The predominating col- ony types represented 20–50% of the colony counts and the second and third most abundant colony types represented 10–20% of these counts. A total of 186 isolates originating from different filter materials were collected from R2A plates. The number of isolates obtained from the GAC filters treating surface water (SW) exceeded the number of isolates from the GAC filters treating ground water (GW) as a result of the larger variety of colony types on R2A plates inoculated with biomass obtained from filters treating SW. The first, second and third dominant colony types yielded a total of 86, 50 and 33 isolates, respectively. The remaining 17 isolates represented less abundant colony types with a distinctive morphologic feature, not found in other isolates, e.g. colour, shape or roughness of the colony.

The concentration of cultivable bacteria, estimated as MPN in liquid medium, ranged from $1.1 \cdot 10^8$ to $4.3 \cdot 10^{10}$ cells per cm^3 GAC. These concentrations were 0.4–2.2 log units (median 0 · 98) higher than the corresponding HPC values (Table 1). A total of 25 isolates were obtained as pure culture from the highest dilution with growth in liquid medium.

3.3.2 Selection and identification of cultured bacteria

The pattern similarity of the rep-PCR genomic fingerprint determined for three control strains in multiple experiments varied between 84% and 98% for *Aer. hydrophila* strain M800 (n = 6) and *Ps. fluorescens* strain P17 (n = 8) and from 89% to 99% for *Spirillum sp*. strain NOX (n = 9). The rep-patterns within a species show more variation than between strains and a rep-PCR pattern similarity >90% was used for clustering of the isolates. Rep-PCR fingerprints were generated for all isolates collected from the solid medium. A total of 130 (70%) of the isolates were divided into 39 clusters, each with 2–12 isolates, with >90% similarity. Furthermore, 56 isolates (30%) occurred individually in the rep-PCR dendrogram. For representatives of each cluster and all individually occurring isolates, the sequence of 16S rDNA gene was determined. Full sequences were used to determine the phylogenetic position of an isolate and partial sequences were used to confirm the identity of isolates with rep-PCR pattern similarity >90%. Isolates within clusters with rep-PCR pattern similarity >90% showed >97% 16S rDNA sequence similarity confirming the validity of the applied selection criterion for rep-PCR pattern similarity (>90%). A total of 112 selected isolates obtained from R2A plates and 25 isolates grown in the MPN tests were identified based on the complete or partial 16S rDNA sequence.

The phylum of the Proteobacteria represented about 94% of all isolates (Table 2). The proportions of *Alpha-* and *Betaproteobacteria* differed for the two culturing methods. *Alphaproteobacteria* represented 22% of the isolates obtained from the solid medium and 44% of the isolates obtained from the liquid medium.

A total of 25% of the identified isolates were assigned to the class *Alphaproteobacteria* of which *Sphingomonas* (7.1%) and *Afipia* (4.3%) were most frequently represented. The iso-

lates identified as members of the genus *Sphingomonas* were found in 33% of the filter beds (7/21) and had >97% sequence similarities with five different *Sphingomonas sp*. strains (AB033945, AF395031, AF428806, AY038702 and AY509378). The isolates identified as members of the genus *Afipia*, which were found in 29% of the filter beds (6/21), had >97% sequence similarity with *Afipia genosp. 11* (AGU87782 and AGU87779) or *Afipia massiliensis* (AY568510).

Table 3.1 Concentration of bacteria from 9 GAC samples cultivated on solid (HPC) and in liquid medium (MPN).

Sample	HPC CFU/ml (stdev)	MPN N/ml (Lower – Upper*)	Log (MPN/HPC)
A	$1.2\ 10^7(1.3\ 10^6)$	$1.2\ 10^8$ ($3.7\ 10^7$ – $4.2\ 10^8$)	1.0
B	$4.0\ 10^7$ ($5.4\ 10^6$)	$1.1\ 10^8$ ($3.6\ 10^7$ – $3.8\ 10^8$)	0.4
C	$5.9\ 10^7$	$1.4\ 10^8$ ($3.6\ 10^7$ – $4.2\ 10^8$)	0.4
D	$1.9\ 10^8(1.3\ 10^7)$	$9.3\ 10^8$ ($1.8\ 10^8$ – $4.2\ 10^8$)	0.7
E	$2.0\ 10^8$ ($2.3\ 10^7$)	$1.1\ 10^9$ ($4.0\ 10^8$– $2.9\ 10^9$)	0.7
F	$2.4\ 10^8$ ($6.1\ 10^7$)	$2.3\ 10^9$ ($4.6\ 10^8$ – $9.4\ 10^9$)	1.0
G	$3.6\ 10^8$ ($6.9\ 10^7$)	$4.3\ 10^{10}$ ($9.0\ 10^9$ – $1.8\ 10^{10}$)	2.1
E	$4.9\ 10^8$ ($8.5\ 10^7$)	$2.0\ 10^{10}$ ($4.5\ 10^9$ – $4.2\ 10^{10}$)	1.6
H	$7.1\ 10^8$ ($1.8\ 10^8$)	$7.5\ 10^9$ ($1.7\ 10^9$ – $2.0\ 10^9$)	1.0

*] 95% confidence limits.

The Betaproteobacteria represented 68% (143/211) of all isolates, of which the *Comamonadaceae* represented 76% (108/143) and included 51% (108/211) of all collected isolates. The most frequently represented genus of this group was *Polaromonas* (26.5%), which represented 50% of the predominating colony types and 43% of the second predominating colony types. *Polaromonas*-related bacteria were isolated from 86% (18/21) of the filter beds (Table 2). Their concentration estimated from the colony counts ranged from $5 \cdot 10^6$ to $3 \cdot 10^9$ CFU cm^{-3} GAC and represented 1–8% of the TDC values. The concen- tration of *Polaromonas*-related bacteria in GAC filters estimated from the MPN values ranged from $5 \cdot 10^7$ to $3 \cdot 10^{10}$ cells per cm^3 GAC, i.e. 5–75% of the TDC values.

The genus Hydrogenophaga comprised 13.7% of all isolates and 20% of the *Betaproteobacteria*. *Hydrogenophaga*-related bacteria were isolated from 43% (9/21) of the filter beds investigated (Table 2) and were dominant in two filter beds. These bacteria had >97% sequence similarity with five sequences in the NCBI database and represented mainly *Hydrogenophaga palleronii* (AF445679, AF523030, AF523047 and AF523069) or *Hydrogenophaga taenospiralis* (AF078768). Isolates identified as *Hydrogenophaga* were 21% of the second predominant colony types and 26% of the third predominant colony types. The concentration of *Hydrogenophaga* in GAC filters was estimated at $2 \cdot 10^6$–$1 \cdot 10^9$ CFU cm^3 GAC, i.e. 0.2–3% of the TDC values. The concentration of *Hydrogenophaga*- related bacteria in GAC filters obtained from the MPN

Table 3.2 Identity and number (proportion) of bacteria, number (proportion) of filter beds in which a certain group is detected and number of filter beds in which this group was predominant.

Identity	Number of isolates (%)	Present in filter beds (%)	Predominant in filterbeds (%)
Betaproteobacteria			
Polaromonas	56 (26.5)	18 (86)	11 (52)
Hydrogenophaga	29 (13.7)	9 (43)	2 (10)
Methylibium	13 (6.2)	4 (19)	3 (14)
Ultramicrobacterium	9 (4.3)	2 (10)	1 (5)
Aquaspirillum	8 (3.8)	4 (19)	0
Ideonella	6 (2.8)	2 (10)	0
Acidovorax	4 (1.9)	1 (5)	0
Comamonadaceae. unclassified	3 (1.4)	1 (5)	0
Variovorax	3 (1.4)	1 (5)	0
Alcaligenes	2 (0.90	1 (5)	0
Burkholderia	2 (0.9)	1 (5)	0
Pseudomonas	2 (0.9)	1 (5)	0
Rubrivivax	2 (0.9)	1 (5)	0
Xylophilus	2 (0.9)	1 (5)	0
Aquamonas	1 (0.5)	1 (5)	0
unclassified	1 (0.5)	1 (5)	0
Alphaproteobacteria			
Sphingomonas	15 (7.1)	7 (33)	2 (10)
Afipia	9 (4.3)	6 (29)	1 (5)
unclassified	6 (2.8)	4 (19)	1 (5)
Bradyrhizobium	5 (2.4)	2 (10)	0
Brevundimonas	4 (1.9)	2 (10)	0
Rhodobacter	4 (1.9)	2 (10)	0
Methylomonas	3 (1.4)	1 (5)	0
Roseomonas	2 (0.9)	1 (5)	0
Acetobacter	1 (0.5)	1 (5)	0
Caulobacter	1 (0.5)	1 (5)	0
Deviosa	1 (0.5)	1 (5)	0
Rhizobium	1 (0.5)	1 (5)	0
Actinobacteria	9 (4.3)	3 (14)	0
Bacteriodetes	4 (1.9)	3 (14)	0
Gammaproteobacteria	1 (0.5)	1 (5)	0
Firmicutes	2 (0.90)	2 (10)	0

tests was estimated at $2 \cdot 10^7 – 1 \cdot 10^{10}$ cells per cm^3 GAC, i.e. 2–25% of the TDC values. A total of 7.6% of all isolates were related to a methyl tert-butyl ether (MTBE), degrading bacterium (AF176594) of the unclassified *Burkholderiales*. These isolates had >97% sequence similarity with four sequences (AY622242, AY662010, AY212718 and AF176594).
Cultivation in the liquid medium did not yield different genera when compared with the isolates from the solid medium.

3.3.3 Diversity of *Polaromonas*

Most (66%) of the *Polaromonas* isolates had 99% similarity with an uncultured bacterium clone C-15 (AF523046) of the Comamonadaceae, obtained from bottled mineral water (Loy et al. 2005). The remaining isolates of the *Polaromonas* group had >97% sequence similarity with five other sequences (AY250094, AY752100, AF523013, AF407400 and AF523046). The complete or partial 16S rDNA sequences of these isolates showed >97% similarity with the sequences of *Polaromonas vacuolata* (Irgens et al. 1996), *Polaromonas naphthalenivorans* (Jeon et al. 2004) and *Polaromonas aquatica* (Kampfer et al. 2006; Fig. 1). AFLP analysis revealed that the isolates could be divided into 23 genotypes with >90% internal similarity (Fig. 2)

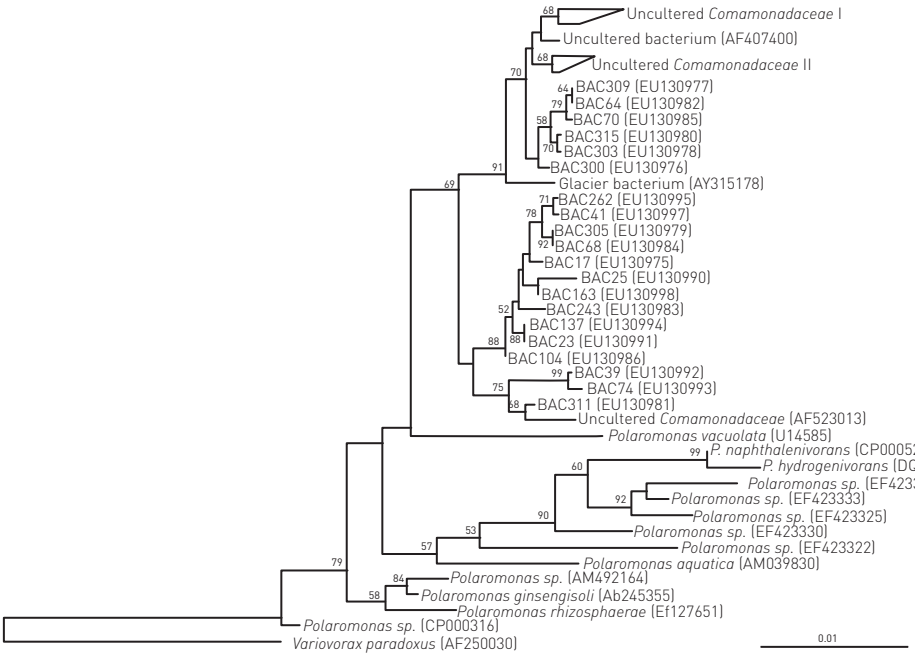

Figure 3.1 Phylogenetic tree showing the diversity and the relatedness of isolates from GAC filters classified as members of genus *Polaromonas*. The tree is constructed from full-length 16S rRNA sequences using the neighbour-joining method with Felstenstein correction and termini filter using 1000 bootstrap replicates. Bootstraps values above 50% are shown. The scale bar represents 1% evolutionary distance. *Variovorax paradoxus* is used as the out-group.

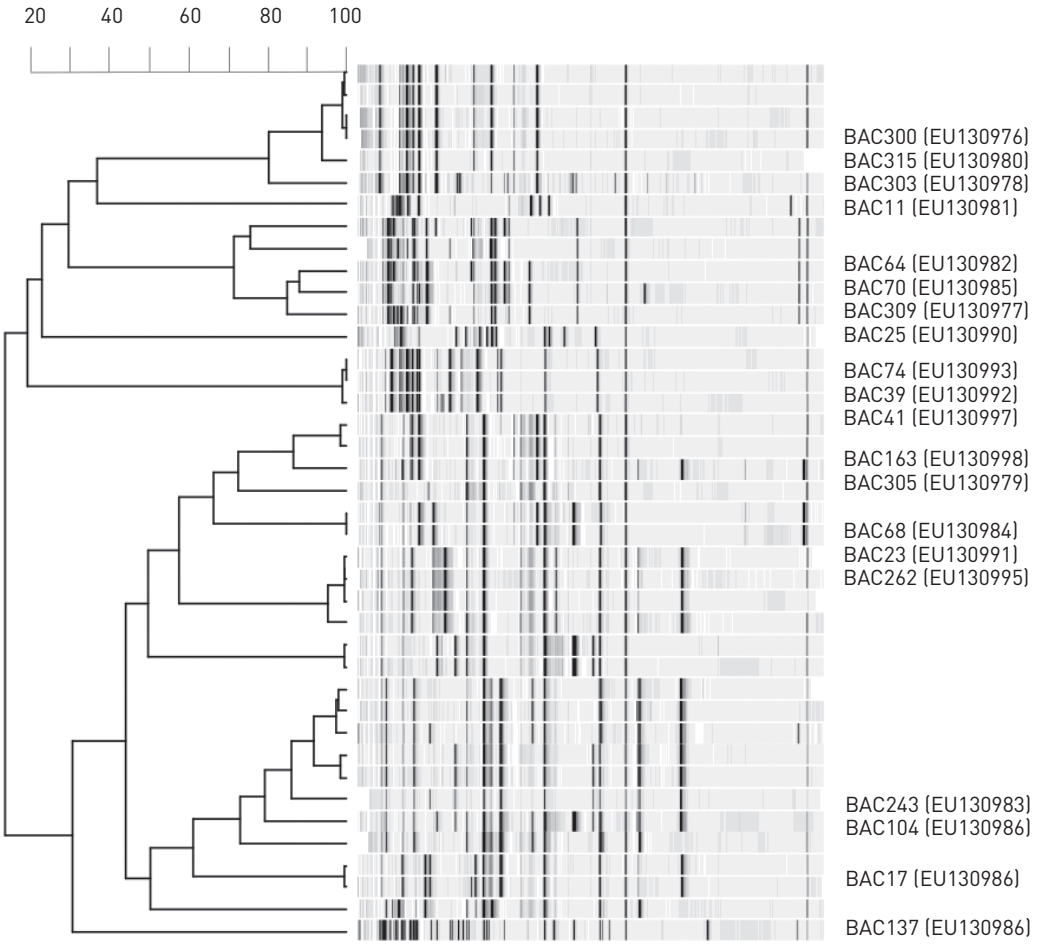

Figure 3.2 Pearson correlation dendogram from AFLP patterns of *Polaromonas*-related isolates from GAC filters. The accession numbers were shown only for the isolates that were used to build the phylogenetic tree based on 16S rRNA sequences in Fig. 1.

3.3.4 Identity of the predominating cultured bacteria in relation to water origin and pretreatment

The GAC filters studied were supplied with different water types. The GAC filters treating either GW or SW showed similar relative abundances of the Betaproteo- bacteria (data not shown). The genus *Polaromonas* represented a significantly ($P < 0.01$) larger proportion (33%) of the isolates cultured from GAC filters supplied with ozonated water (OW) than from filters without oxidative pre-treatment (16%). Isolates of the genus *Variovorax* were only observed in filters treating nonoxidized groundwater. The genus *Sphingomonas* represented the predominating *Alphaproteobacteria* in GAC filters treating SW and the genus *Afipia* represented the predominant

Alphaproteobacteria in the filters treating GW. The genera *Caulobacter*, *Devosia*, *Methylobacterium* and *Rhodobacter* were only obtained from filters treating nonoxidized ground water, whereas members of the family *Acetobacteraceae* and the genera *Brevundimonas, Rhizobium* and *Roseomonas* were obtained only from filters treating OW. The latter genera and Rhodobacter were specific for filters treating SW and members of the genera *Caulobacter, Devosia* and *Methylobacterium* were specific for filters treating GW.
The isolates collected from sand and anthracite layers of the rapid sand filtration (RSF) or slow sand filtration (SSF) were identified as members of the same genera that were observed in GAC filters. Also in these filters, members of the *Comamonadaceae* family were predominant (53%).

3.4 DISCUSSION

3.4.1 Abundance and diversity

The range of HPC values in GAC samples observed in this investigation correspond well with values reported earlier for GAC filters in water treatment (Camper et al. 1985; Stewart et al. 1990). These values represent a minor proportion (0.2–9%) of the TDC values and similar frac- tions of cultivable bacteria have been reported for oligotrophic aquatic environments and soil (Reasoner and Geldreich 1985; Bruns et al. 2001; Page et al. 2004; Janssen 2006). Adjustment of the culturing technique improved the cultivability of bacteria from GAC filters by approximately 1 log unit (median value; Table 2). This observation is consistent with results of other studies, viz. an increase of the average cultivability of lake bacteria with 1.25 log units when the substrate and oxygen concentration were adjusted and for marine bacteria, a cultivability of 2–60% was reported with the dilution-culture technique (Button et al. 1993; Bussmann and Schink 2001). However, we did not observe differences in the identity of isolates harvested with the two culturing techniques. Hence, the dilution-culture technique yields isolates representing a significant proportion of the total bacterial community, which therefore can be used for further studies.

In previous studies, a limited number of rapidly growing bacteria, which were able to metabolize substances at high concentrations, and bacteria with specific features, e.g. formation of spores, were isolated from GAC filters, viz. members of the genera *Pseudomonas, Alcaligenes, Acinetobacter, Flavobacterium, Caulobacter* and *Bacillus* (Parsons et al. 1980; Stewart et al. 1990; White et al. 1996). The identity of the isolates collected in this study differed clearly from the identity of bacteria isolated in earlier studies on GAC filters. The differences may be attributed to: (i) optimized removal of bacteria from GAC surface, (ii) adjustment of culturing techniques and (iii) use of molecular methods for identification of isolated bacteria. Furthermore, the nomenclature of many previously isolated bacteria has been adapted since the introduction of phylogenetic analysis based on 16S rDNA sequences. The species *Alcaligenes, Ps. acidovorans* and *Ps. testosteroni* have been reclassified to the *Comamonadaceae* as *Variovorax paradoxus, Comamonas acidovorans* and *Comamonas testosteroni*, respectively (Willems et al. 1991). Bacteria previously identified as *Pseudomonas* are presently classified as *Sphingomonas* (White et al. 1996). In this study, *Polaromonas* was identified as predominant genus in GAC filters and these bacteria represent a high proportion of the TDC values (up to 70%). To our

knowledge, representatives of the genus *Polaromonas* have not been reported in earlier studies on GAC. Only a few species of this genus have yet been described (Irgens et al. 1996; Jeon et al. 2004; Kampfer et al. 2006). The genomic diversity of the isolates identified in this study as *Polaromonas spp*. (Figs 3.1 and 3.2) suggests that further classification into additional species may be expected in the future.

Comparison with the bacterial communities of freshwater habitats, including SW, treated water and bottled water and of soil, yield a number of similarities and differences, but this comparison is biased by the application of cultivation in this study. The predomination of the *Betaproteobacteria* (61–80%) in GAC filters in water treatment is consistent with the position of this bacterial class in communities of biofilms and planktonic bacteria in natural freshwater environments (Zwart et al. 2002; Brummer et al. 2003; Bruns et al. 2003; Eiler and Bertilsson 2004; Page et al. 2004; Gich et al. 2005) and in drinking water distribution systems (Kalmbach et al. 1997). The smaller proportion of *Alphaproteobacteria* is consistent with studies on natural fresh water, but these bacteria represented more than 90% of isolates from a distribution system simulator with a monochloramine residual and from a chlorinated drinking water distribution system (Williams et al. 2003). Alphaproteobacteria also are major constituents of the microbial communities in soil (Bodour et al. 2003; Janssen 2006), and in particle- associated and free-living bacterial communities in water treatment systems and river water (Crump et al. 1999; Macdonald and Brozel 1999). The small fractions of representatives of the *Gammaproteobacteria* (<1%), the *Actinobacteria* (4Æ3%) and the *Firmicutes* (<1%) are consistent with studies on natural freshwater communities, bottled natural mineral water and soil (Eiler and Bertilsson 2004; Loy et al. 2005). However, the *Bacteriodetes* (1.9%) were a significant fraction (>25%) of the bacterioplankton community in Swedish lakes (Eiler et al. 2004) and the Actinobacteria equalled the presence of the *Alpha-* and *Betaproteobacteria* in a number of lakes and rivers (Zwart et al. 2002). These variations may be due to both differences in water composition and comparing clones with isolates. The relatively high proportion of *Alphaproteobacteria* among isolates cultivated in liquid medium (44%) suggests that the abundance of *Alphaproteobacteria* in GAC filters may be underestimated by cultivation on R2A medium.

3.4.2 Relationship between identity of predominating bacteria and environmental conditions in GAC filters

The presence of bacteria in GAC filters depends on the interactions between environmental conditions and their physiological properties. The significant contribution of a few bacterial types found in the GAC filters treating different water types indicates that these bacteria are typical constituents of the bacterial community in GAC filters used in water treatment (Table 2). On the contrary, bacterial types represented by only a single isolate (singletons) also constituted a relatively large proportion (30%) of the isolated bacteria. The bacteria isolated from the GAC filters may have different origins, viz. (i) the feed water, (ii) the biomass accumulating between the GAC particles and (iii) the bacteria attached to GAC surface. Previous observations showed that the main proportion of bacteria in GAC filters is present on the surface of GAC particles (Magic-Knezev and van der Kooij 2004). Therefore, the predominating bacterial types represent mainly attached bacteria and/or bacteria present as accumulated biomass. The predominating types of bacteria cultured from the GAC filters belonged to a few

genera, of which *Polaromonas* was most frequently observed. Some genera were only incidentally observed but such observations could not be attributed to differences in environmental conditions.

The concentration of biodegradable compounds in the feed water of the GAC filters usually is relatively low. Oxidative pretreatment of the water increases the concentra- tion and diversity of biodegradable compounds leading to the increased bacterial abundance in these filters (Gagnon et al. 1997). Concentrations of easily assimilable organic carbon (AOC), mainly including carboxylic acids and aldehydes, may attain a level of 40–150 µg of C/l after ozonation. Without such oxidation, the AOC concentration remains <10 µg CL^{-1} in GW and <35 µg CL^{-1} in SW (van der Kooij et al. 1989; van der Kooij 1992). Descriptions of the nutritional versatility of the predominating genera confirm that *Polaromonas, Hydrogenophaga* and *Afipia* prefer carboxylic acids and/or amino acids as growth substrates but are limited in the utilization of carbohydrates and generally do not hydrolyse proteins, carbohydrates or fats, (Garrity et al. 2005; Magic-Knezev and van der Kooij 2006). *Polaromonas spp*. generally are not observed in natural freshwater environments (Zwart et al. 2002; Eiler and Bertilsson 2004), but the organism predominated in an ultraoligotrophic Crater lake, in contaminated GW and in bottled natural mineral water (Jeon et al. 2004; Page et al. 2004; Loy et al. 2005). Representatives of the genera *Pseudomonas, Aeromonas, Aquaspirillum* and *Flavobacterium* can multiply at concentrations of a few µg L^{-1} of low molecular weight compounds (van der Kooij et al. 1982; van der Kooij and Hijnen 1984, 1985), but were not detected in GAC filters or are present at a very low concentration. Obviously, *Polaromonas spp*. multiply even more efficiently at low concentrations of biodegradable compounds and/or attach more efficiently onto the GAC surface than these organisms. Indeed, the batch tests with *Polaromonas* isolates collected from the GAC filters revealed that these bacteria are able to multiply at very low concentrations of carboxylic acids and aromatic acids (Magic-Knezev and van der Kooij 2006). Elucidation of the predominant position of *Polaromonas* requires further investigations, e.g. survival at a low flux of nutrients, attachment properties and utilization of NOM present in SW and GW. Furthermore, the contribution of these bacteria to the microbial community in GAC filters should be determined with culture-independent methods

3.4.3 Microbiological contaminant removal

Adsorption processes play a major role in the removal of organic contaminants with GAC filtration in water treatment (Orlandini 1999). Hydrogenophaga spp. can degrade MTBE (Hatzinger et al. 2001; Kane et al. 2007). *Polaromonas*-related bacteria can utilize *cis*-dichloroethane or naphthalene as sole carbon source at a concentration of a few mg L^{-1} (Coleman et al. 2002; Jeon et al. 2004). Furthermore, *Sphingomonas spp*. are capable degrading a wide variety of xenobiotic compounds, including pesticides (White et al. 1996). The concentrations of MTBE and pesticides in SW used for water treatment usually is low (<1 µg L^{-1}; Achten et al. 2002) and it is uncertain if biodegradation of these compounds occurs in GAC filter beds in the presence of clearly higher concentrations of biodegradable NOM. In addition, a contribution of these bacteria to the removal of xenobiotic organic compounds at suddenly elevated concentrations is uncertain, because adaptation seems to be slow (Coleman et al. 2002).

3.5 ACKNOWLEDGEMENTS

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CHAPTER

4

IDENTITY OF BACTERIA PREDOMINATING IN GRANULAR ACTIVATED CARBON FILTERS FOR DRINKING WATER TREATMENT

Knezev A., Wullings B., Smidt H. and van der Kooij D.

This chapter has been submitted for publication

Abstract

The sequence and T-RFLP fingerprint analysis of 16S rRNA genes of bacteria from full-scale and pilot-plant granular activated carbon filters (GACFs) operating under different conditions at five drinking water treatment plants were studied. A total of 135 operational taxonomic units (OTUs), retrieved from a clone library comprising 288 partial or full 16S rRNA gene sequences, represented 76% of the average bacterial richness in the filters as estimated by Chao1. The most abundant OTUs retrieved from GACFs belonged to *Betaproteobacteria* (43% of all sequences), *Alphaproteobacteria* (38%) or *Acidobacteria* (7%). Most of the *Betaproteobacteria* belonged to the TRA3-20 group of yet-uncultured *Betaproteobacteria* (34% of betaproteobacterial sequences) and to the *Comamonadaceae* family (43% of betaproteobacterial sequences) with the genus *Polaromonas* being found in 80% of the examined GACFs and representing 25% of the *Betaproteobacteria*. The majority of *Alphaproteobacteria* belonged to the families *Bradyrhizobiaceae* (23%) and *Hyphomicrobiaceae* (17%) from the order *Rhizobiales* (55% of alphaproteobacterial sequences). Members from the order *Rhodospirillales* represented 19% of the *Alphaproteobacteria*. The T-RFLP analysis revealed a relatively high similarity (>60%) between the communities from GACFs at different locations and a temporal and spatial stability over a one-year period. A high Shannon evenness index (0.82 ± 0.06) of T-RFLP profiles suggests an even distribution of predominant species. An increase in richness of bacterial OTUs in a pilot GACF during a one-year maturation period was accompanied by an increase in relative abundance of predominant fragments. Oxidative pre-treatment appeared to affect the bacterial community composition. The metabolic properties of cultured relatives of the predominant bacteria from GACFs suggest that bacteria on GAC are chemo-organothrophic aerobes that can grow at low concentrations of carboxylic acids and aromatic compounds or chemolitotrophs that utilize ammonium or nitrite as source of energy. Representatives of *Polaromonas*, *Bosea*, *Afpia*, *Sphingomonas*, *Variovorax* and unclassified *Commamonadaceae* were ususally isolated from the same GAC filters indicating their importance in the biodegradation processes in GACFs.

ECN SEI 5.0kV X500 10μm WD13mm

4.1 INTRODUCTION

Bacteria in aquatic environments attach to and grow on water-exposed solid surfaces and form biofilms. Biofilm formation is conditioned by the continuous supply of nutrients provided by the water flow (Marshall 1997; Flemming et al. 2014). The removal of dissolved organic matter by biofilms is widely used in treatment of water and wastewater. A commonly applied process for the removal of micro-pollutants and natural organic matter (NOM) in water treatment is granular activated carbon (GAC) filtration. The removal is primarily achieved through the adsorption of organic compounds on the GAC surface (Sontheimer et al. 1988). In addition bacteria attach to the GAC surface and utilize biodegradable organic compounds from the influent (LeChevallier et al. 1987; van der Kooij et al. 1989; Servais et al. 2004). By reducing the concentration of growth-promoting compounds the bacteria in GAC filters contribute to the control of biofilm formation, accumulation of sediments, multiplication of pathogens and invertebrates and corrosion in distribution systems (LeChevallier et al. 1987; Sontheimer et al. 1988; Servais et al. 1994). Furthermore, biodegradation extends the lifetime of a GACF and decreases the frequency of GAC regeneration (Orlandini, 1999; van der Aa et al. 2011). In order to further improve the performance of GAC filters, additional understanding is needed for the optimal employment of biodegradation processes in these filters.

The quantitative aspects of microbial activity in GAC filters have been investigated in various studies (Urfer and Huck 2000; Fonseca et al. 2001; MagicKnezev and van der Kooij 2004; Velten et al. 2007). Characterisation of bacteria from GACFs by classical techniques yielded a broad range of genera including *Achromobacter, Arthrobacter, Alcaligenes, Acinetobacter, Aeromonas, Bacillus, Caulobacter, Chromobacterium, Corynebacterium, Flavobacterium, Moraxella, Micrococcus, Microcyclus, Nitrosomonas, Nitrobacter, Paracoccus, Pseudomonas,* and *actinomycetes* (van der Kooij McElhaney and McKeon 1985; Wilcox et al. 1983; Rollinger and Dott 1987; Parsons 1980; Camper et al. 1985). However, many isolates remained unidentified due to low discriminatory capacity of classical identification methods. Norton and LeChevallier (2000) used fatty acid composition to identify bacteria from GAC and species from genera *Acidovorax, Hydrogenophaga, Pseudomonas, Xanthomonas*, and *Sphingomonas* were detected as predominant. Recently, predominating cultivable bacteria from GAC filters were isolated using nutrient-poor media and identified with molecular tools as members of the genera *Polaromonas* and *Hydrogenophaga* within the *Comamonadaceae* family (Magic-Knezev et al. 2009; Niemi et al. 2009). The metabolic properties of two *Polaromonas* strains isolated from GAC filters indicated that these slowly growing bacteria are specialized in the utilization of carboxylic and aromatic acids at concentrations of $<10\ \mu g/l$ (Magic-Knezev and van der Kooij 2006).

Cultivation independent studies on bacteria in GAC filters have been conducted mainly in pilot plant filters, to study regeneration or focusing on a certain process, such as nitrification (Li et al. 2010; Liao et al. 2012, 2013). In this study molecular methods were applied to identify the predominant bacteria in full-scale GACFs and to assess the effects of operational conditions on the diversity of bacterial communities. For this purpose bacterial communities from full-scale and pilot-plant GACFs at drinking water treatment plants in the Netherlands were investigated

by constructing clone libraries of PCR-amplified 16S ribosomal RNA genes and by using 16S rRNA gene targeted T-RFLP fingerprinting.

4.2 MATERIALS AND METHODS

4.2.1 GAC filters

A total of 18 GACFs at five full-scale drinking water treatment plants and five filters at three pilot plants in the Netherlands were included in the study. A total of two rapid sand filters (RSF) from location A2 and A6, were also included in the study. Surface water from four different sources and one groundwater source was used (Table 4.1). After conventional pre-treatment and softening the water was treated with ozone at the locations A1, A6, A7 and by advanced oxidation (peroxide-UV) at location A4. At locations A2, A3 and A5 no oxidative process was applied before GAC filtration. The numbers of bacteria and the bacterial biomass concentration in the examined GAC filters have been reported previously (Magic-Knezev and van der Kooij 2004).

The GAC samples for clone library construction and the T-RFLP analysis were collected once from full-scale filters of different operational periods in a cold and in a warm season. The GAC samples from the surface water pilot-plant (location $A7^{PI}$) were collected monthly during a period of one year, and samples from the groundwater pilot-plant (location A1, A2) were collected once in two months during one year. The GAC samples from the pilot-plant filters treating SW with peroxide and UV (location A4) were sampled only once in the warm period for the T-RFLP analysis. Methods used to collect GAC from the filter beds and harvesting of bacteria from GAC have been described previously (Magic-Knezev and van der Kooij 2004). Briefly,

Table 4.1 Influent water quality of GAC filters at eight different locations.

Location	Water* source	Oxidation	DOC** ($mg\ l^{-1}$)	UVA# (m^{-1})	AOC# ($\mu g\ l^{-1}$)	Temp. (°C)	pH
A1	GW (PP[a])	O_3/H_2O_2	1.5-1.8	2.6-2.8	10-50	12	8.3-8.5
A2	GW (PP)	N.O.[c]	1.5-1.8	3.4-4.0	2-5	12	8.4-8.6
A3	SW (FS[b])	N.O.	1.2-2.6	6.2-6.9	170	2-25	7.9
A4	SW (FS)	UV/H_2O_2	1.4-2.2	6.6	40-80	2-25	8.3-8.5
A5	SW (FS)	N.O.	3.4	3.0-3.9	n.a.	2-25	7.9
A6	SW (FS)	O_3	2.1	3.0-3.9	n.a.	2-25	8.0
A7	SW(FS)	O_3	5.5-6.5	10-12	40-150	2-25	7.6-7.8
$A7^{PI}$	SW(PP)	O_3	5.5-6.5	8-11	8-12	2-25	7.6-7.8

* GW – groundwater; SW – surface water;** DOC dissolved organic carbon; #ultraviolet light absorbance (254 nm); ##AOC-assimilable organic carbon; [a]PP-pilot-plant; [b]FS-full-scale; [c]N.O-pre-oxidation not applied.

GAC samples (10 – 20 g) were collected aseptically from the filter beds and kept submerged in the effluent at 4°C. Bacteria attached to GAC were harvested within 24 hours by applying a series of six two-minute treatments of a GAC sample (2 – 5 g wet GAC) in 50 ml of autoclaved tap water with high-energy ultrasound of 20kHz via a titanium microtip (D = 5 mm), at a power input of 40 W.

4.2.2 DNA extraction.

Bacterial suspensions obtained by sonication of GAC samples were concentrated using a polycarbonate filter (diameter 25 mm, 0.22 μm pore size, type GTTP; Millipore). Subsequently, DNA from bacteria retained on the filter was extracted and purified by the FastDNA Kit for soil (BIO 101, Vista, CA, USA) as described by the producer. Extracted DNA was stored at -20°C.

4.2.3 T-RFLP analysis.

T-RFLP analysis was done as previously described (Dunbar et al. 2001). Shortly, the 16S rRNA gene of bacteria harvested from GAC was amplified with Platinum Taq polymerase (Hot-Start Taq polymerase, Invitrogen, Life Technology Corporation, CA, USA) using 8F-FAM (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGTACA-3') primers and the iCycler IQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). The following thermal cycling was applied: 30 cycles 95°C for 45 s, 57°C for 60 s and 72°C for 2 min; and a final extension at 72°C for 7 minutes. PCR products were purified (DNA Clean & Concentrator-5 Kit van Zymo Research Corporation, Orange, CA, USA) and digested with restriction enzymes HhaI (Promega Corporation Madison, WI, USA) at 37°C for 4-6 hours. Digestion products were purified, and after addition of the internal standard (Genescan –500 ABI 401734, Life Technology Corporation, Carlsbad, CA, USA) and loading buffer (Hi-Di formamide) DNA was denatured during 3 min at 95°C and transferred directly to ice for a minimum of 13 min. Electrophoresis was performed in the short capillary/POP polymer in the Run module "GS STR pop 41 ml D 28 min" on an ABI 310 automated DNA Sequencer (AME Bioscience A/S, Toroed, Norway) with extended running time of 35 minutes. T-RFLP data was generated for 70 samples (Supplemental Material Table 4.S1A).

The T-RFLP fingerprints were compared using Pearson correlation. Dendrograms were constructed by the unweighted pair-group method using arithmetic averages (Bionumerics software version 3.0, Applied Maths NV, Sint-Martens-Latem, Belgium). The peak height was used as an estimate of the relative abundance of an individual fragment as the ratio to the total peak height in a sample. The Shannon and Simson's indices (and the reciprocals) were used to evaluate diversity (Simpson 1949; Shannon and Weaver 1963).

4.2.4 Cloning and sequencing of PCR products

The 16S rRNA gene was amplified with Taq DNA polymerase (Hot-Start Taq polymerase, Invitrogen, Life Technology Corporation, CA, USA) and primers targeting conserved domains (8F and 1392R). The PCR products were extracted and purified from the 1 % agarose gel (DNA Clean & Concentrator-5 Kit van Zymo Research Corporation, Orange, CA, USA) and cloned into pGEM-T Easy Vector System II (Promega, Leiden, The Netherlands). A total of 288 white colonies were obtained from 10 samples. The number of clones obtained per sample varied

between nine and 47 (Table 4.1B, Supplemental Material). White colonies were screened by colony PCR using standard cloning primers sp6 and T7agarose gel electrophoresis for the correct size of the insert. The DNA insert was sequenced either with one internal primer (F907) to obtain partial sequences of approximately 200-400 base pairs (bp) or by using primers T7 and SP6 and internal primers (F907, R530) to obtain full length sequences.

4.2.5 Nucleotide sequence data analysis.

Sequences were assembled using the software package DNA Star Seqman II (Lasergene Inc., Madison, WIS., USA). ARB software was used to determine the phylogenetic position of sequenced fragments (Ludwig et al. 2004). Sequences were aligned with the SINA online aligner service available within the ARB-Silva platform (Prüsse et al. 2007; Prüsse et al. 2012), and imported to the non-redundant SSURef_NR99_115_SILVA_20_07_13 database. A distance matrix of sequences was calculated without a filter and corrections. Aligned sequences were added to the main tree by the parsimony tool with local optimization. The phylogenetic identity of the sequences at the genus, family or class level was determined using the classification tool of the SINA aligner, as well as based on their position in the main tree. The number of unique sequence types, operational taxonomical units (OTU's) and richness (CHAO1 index, Chao 1984) were calculated from the distance matrix generated by ARB using DOTUR software (Schloss and Handelsman 2005).

Nucleotide sequence accession numbers. All partial and full length 16S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers KJ615071-KJ615358.

4.2.6 Enzymatic properties of Polaromonas strains

A total of eight strains of *Polaromonas* isolated from GAC filters (EU130976, EU130977, EU130978, EU130979, EU130980, EU130981, EU130988 and EU130989; Magic-Knezev and van der Kooij 2009) were tested for enzymatic activity with the apiZYM test (Biomerieux, Durham, NC/USA) as described by the producer.

4.3 RESULTS

4.3.1 Clone Library

A library of 288 clones was generated from the bacterial communities associated with ten GAC filters (Table 4.S1B). A total of 68% of the sequences showed >97% similarity with any sequence in the reference database used for the alignment. A total of 135 operational taxonomic units (OTUs, defined by a threshold of 97% sequence similarity), were detected, representing 76 % of the average species richness estimated with Chao1 (Table 4.2).

Table 4.2 Number of sequences and OTUs, defined using a threshold of 97% 16S rRNA gene sequence similarity, and the total species richness estimation for the collective clone library derived from ten GAC filters and an individual clone library (GAC- GW) from a GAC filter treating ozonated ground water. Low and High represent 95% confidence interval.

Origin of samples	Number of sequences	Number of OTUs*	Total species richness (Chao1 estimation)		
			Avg.	Low	High
GAC filters (n=10)	288	135	194	178	287
GAC-GW (n=1)	48	27	56	33	146

*sequence similarity > 97%

About half (52%) of the identified families were represented by ≤1% of the sequences (n=1-4); one third (27%) was represented by 1-5% of the sequences (n=4 -14) and one fifth (21%) was represented by 5 – 16 % of the sequences (n=14 – 47). The majority of the sequences and OTUs were classified as *Betaproteobacteria* (43% of sequences, 31% of OTUs), *Alphaproteobacteria* (38%, 36%) or *Acidobacteria* (7%, 9%). A detailed overview of the classification of the retrieved sequences is provided in Tables 4.3, 4.4 and 4.5, respectively.

A total of 42 (37%), 17 (15%) and 17 (15 %) sequences from the *Betaproteobacteria* were identified only to the class, order or family level, respectively. A total of 42 OTUs (124 sequences) from the *Betaproteobacteria* represented 7 families from 5 orders with the *Burkholderiales* being most abundant (43% of the sequences, 40% of the OTUs). The majority (91%) of the sequences from the *Burkholderiales* belong to the family *Comamonadaceae*, with genus *Polaromonas* representing 65% of the sequences from this family. The *Comamonadaceae* were found in 8 of the 10 studied filters and the genus *Polaromonas* in 6 of the filters.

A total of 34% of the betaproteobacterial sequences, representing 19% of the OTUs could be classified to the candidate order "TRA3-20" using the SINA classifier, and all showing 90% or higher sequence similarity, with the majority of sequences comprising an approximate genus-level group at ≥ 95% sequence similarity. These sequences were most closely related to an uncultured bacterium TRA3-20 (AF047644, Edwards et al. 1999) and an uncultured bacterium clone OC35 (AY491578, Phung et al. 2004). The order *Nitrosomonadales* represented 5 % of the betaproteobacterial sequences, and was detected in GACFs at 3 from 6 locations treating oxidized surface water (locations A4, A6 and A7).

The majority of sequences of the *Alphaproteobacteria* retrieved from GACFs grouped within the order of *Rhizobiales* (63%), representing the families *Bradyrhizobiaceae (39%)*, *Hyphomicobiaceae* (28%) and *Rhodobacteraceae* (9%). The most frequently observed genera were *Bosea, Afipia and Bradhyrizobium*. The families *Acetobacteraceae* and *Rhodospirillaceae* of the order of *Rhodospirillales, represented* 17% of the sequences (18% of OTUs) of the *Alphaproteobacteria* and the order *Sphingomonadales* represented 11% of the sequences (6% of OTUs).

Table 4.3 Identity and abundance of 124 sequences and 42 OTUs from the *Betaproteobacteria*, retrieved from 10 GAC filters at different influent water quality

Order	Family	Genus	No. seq (OTUs)	No. FB (locaties)	Locations
TRA3-20	N.I.	N.I.	42 (8)	11 (7)	A1, A2, A3, A4, A5, A6, A7
Burkholderiales	*Comamonadaceae*	*Polaromonas*	31 (7)	6 (5)	A1, A2, A3, A5, A7
		N.I.	7 (3)	5 (5)	A1, A2, A5,A6,A7
		Acidovorax	5 (2)	3 (3)	(A1, A6, A7)
		Comamonas	3 (1)	1 (1)	A6
		Hydrogenophaga	2 (1)	2 (2)	A5, A6
	Alcaligenaceae	*Achromobacter*	4 (2)	2 (2)	A6, A3
	N.I.	N.I.	1 (1)	1 (1)	A5
Nitrosomonadales	*Nitrosomonadaceae*	N.I.	5 (3)	3 (3)	A4, A6,A7
	Oxalobacteraceae	N.I.	1 (1)	1 (1)	A7
Methylophilales	*Methylophilaceae*	*Massilia*	3 (1)	1 (1)	A5
		N.I.	3 (2)	2 (2)	A3, A4, A5
Rhodocyclales	*Rhodocyclaceae*	N.I.	6 (2)	4 (3)	A4, A6, A7
N.I.	N.I.	N.I.	1 (1)	1 (1)	A6
N.I.	N.I.	N.I. (*Variovorax*)	7 (5)	2 (2)	A1, A2
N.I.	N.I.	N.I.	3 (2)	2 (2)	A2, A4

N.I. not identified;

The remaining sequences (19%) were classified either as *Gammaprotebacteria* (3%) or *Deltaproteobacteria* (1%) or as other phyla, from which the *Acidobacteria* (7%) and *Nitrospirae* (2.4%) were most abundant (Table 4.5). *Acidobacteria* were detected in most of the filters (80%) treating surface water. The sequences of *Acidobacteria* were distributed among three phylogenetically distinct groups.

Table 4.4 Identity and abundance of sequences and OTUs from *Alphaproteobacteria*, retrieved form GAC filters at different locations

Order	Family	Genus	No. Seq. (OTUs)	No. FB (Loc)	Locations
Rhizobiales	*Hyphomicrobiaceae*	N.I.	13 (8)	5 (3)	A1, A3, A6,
		Pedomicrobium	2 (1)	2 (2)	A1, A3
		Sideroxydans	2 (1)	1 (1)	A7
		uncultured	2 (1)	1 (1)	A7
	Bradyrhizobiaceae	*Bosea*	14 (5)	2 (2)	A1, A2
		Afipia	7 (3)	2 (1)	A1
		Bradyrhizobium uncultured	6 (2)	4 (3)	A1, A3, A4,
	Rhodobacteraceae	N.I.	12 (4)	5 (5)	A, A3, A4, A6, A7
Rhizobiales	*Methylobacteriaceae*	uncultured	5 (2)	3 (2)	A3, A5
	Xanthobacteraceae	uncultured	3 (1)	3 (3)	A3, A4, A7
	MNG7	N.I.	1 (1)	1 (1)	A7
	uncultured	N.I.	2 (1)	1 (1)	A4
Rhodospirillales	*Acetobacteraceae*	N.I.	5 (3)	3 (3)	A2, A6, A7
	N.I.	N.I.	5 (2)	2 (2)	A3, A6
	Incertae Sedis	*Reyranella*	3 (1)	2 (1)	A3
	Rhodospirillaceae	uncultured	3 (1)	1 (1)	A6
		N.I.	1 (1)	1 (1)	A3
	N.I.	N.I.	1 (1)	1 (1)	A7
Sphingomonadales	*Sphingomonadaceae*	GOBB3-C201	7 (1)	1 (1)	A5
		Novosphingobium	3 (1)	2 (1)	A1, A2
	uncultured (13)	N.I.	2 (1)	1 (1)	A6
Rickettsiales	*Holosporaceae*	N.I.	1 (1)	1 (1)	A7
	SM2D12	N.I.	3 (2)	2 (2)	A2, A6
Caulobacterales	*Hyphomonadaceae*	*Woodsholea*	3 (2)	2 (2)	A6, A7
N.I.	N.I.	N.I.	1 (1)	1 (1)	A5
N.I.	N.I.	TK34	2 (1)	1 (1)	A6

Table 4.5 Identity and abundance of sequences and OTUs from proteobacterial classes other than *Alpha*- and *Betaproteobacteria* and from other phyla retrieved from GAC filters

Phylum (Class)	Order	Family/Genus	No Seq. (OTUs)	No. FB (Loc)	Locations
Acidobacteria		s.g.[a] 4	7(5)	4 (3)	A4, A6, A7
		s.g. 6	4(4)	5 (4)	A2, A4, A6, A7
		f.i.s. *Bryobacter*/s.g. 3	2 (2)	4 (2)	A3, A4
(*Holophagae*)	*Holophagales*	f.i.s. *Blastocatella*/ s.g. 10	1 (1)	1 (1)	A4
Nitrospirae (*Nitrospira*)	*Nitrospirales*	*Nitrospiraceae*/ *Leptospirillum*	2 (2)	1 (1)	A2
		Nitrospiraceae /N.I.	1 (1)	1 (1)	A6
		Nitrospiraceae / N.I.	2 (1)	2 (2)	A6, A7
Proteobacteria (*Gammaproteobacteria*)	*Legionellales*	*Coxiellaceae/ Aquicella*	2 (2)	2 (2)	A6
	Legionellales	*Coxiellaceae*	2 (1)	1 (1)	A6
	N.I.	N.I.	5 (4)	3 (3)	A1, A6, A7 -
	Chromatiales	*Ectothiorhodospiraceae*	1 (1)	1 (1)	A7
	Xanthomonadales	*Xanthomonadaceae*	1 (1)	1 (1)	A4
	Bdellovibrionales	*Bdellovibrionaceae*	1 (1)	1 (1)	A6
(Deltaproteobacteria)	*Myxococcales*	N.I.	1 (1)	1 (1)	A3
	N.I.	N.I.	1 (1)	1 (1)	A6
Bacteroidetes (*Cytophagia*) (*Sphingobacteriia*)	*Cytophagales*	*Cytophagaceae/ Flexibacter*	1 (1)	1 (1)	A7
	Sphingobacte-riales	*Chitinophagaceae* /N.I.	3 (3)	3 (3)	A1, A7
		Saprospiraceae N.I.	1 (1)	1 (1)	A7
Actinobacteria	*Propionibacteri-neae*	*Nocardioidaceae/ Nocardioides*	1 (1)	1 (1)	A6
Chloroflexi (*Caldilineae*)	*Caldilineales*	*Caldilineaceae*	1 (1)	1 (1)	A6
Cyanobacteria		*Chloroplast*	1 (1)	1 (1)	A6
Planctomycetes	*Planctomycetales*	N.I.	1 (1)	1 (1)	A4
		uncultured	1 (1)	1	A4
Unclassified		uncultured	1 (1)	1	A7
Candidate div.		TM7	1 (1)	1	A1
N.I.		TA06	1 (1)	1	A7

N.I. not indentified; f.i.s. : Family Incertae Sedis; s.g. subgroup; [a] ,s.g. subgroep.

4.3.2 T-RFLP analysis

T-RFLP patterns were obtained from a total of 48 samples collected from 21 GACFs. Six samples were analysed in duplicate to determine the variability of the T-RFLP method. The similarity between duplicate patterns from the same DNA extract ranged between 86% and 94%. The position of a T-RFLP fragment obtained from a single sequence varied with ± two apparent base pairs (Table 4.S3).

The T-RFLP patterns of 67 GAC-associated bacterial communities, with a pattern similarity for all samples > 60%, were arranged in three major groups. The first group (similarity > 68%) comprises patterns derived from filters treating oxidized surface water; the second group (similarity >73%) is from filters treating groundwater and the third group (similarity >63%) includes patterns from the filters treating non-oxidized surface water (Fig. 4.1). These three groups were further divided into subgroups according to oxidative pre-treatment (communities from filters treating groundwater) and location. Grouping revealed that the patterns from different filters at the same location were highly similar; effect of filter age and season were not observed. The communities from the rapid sand filters (A2-SF and A6-SF) clustered separately from the GACF communities. The similarity with communities from GACFs was <65%. The bacterial diversity in the examined GACFs (n=67) was highly similar (Table 4.S5). The average value of the Shannon evenness index of 0.82±0.06 (0.66 – 0.88) indicated a relatively even distribution of the predominant species in GACFs. The number of T-RFLP fragments with a relative abundance above 0.5% was between 10 and 62 (37±12, P50=12), and the number of fragments with a relative abundance above 5% was between 3 and 8 per sample. The fragments with a relative abundance above 5% represented on average 48±19% of the total pattern (p75 < 62%) and were grouped around 5 positions (Table 4.S2) from which three positions, representing 200, 340 and 360 bp apparent fragment size, were detected in the majority of studied GAC communities.

T-RFLP patterns of selected isolates and clones from GACFs were used to identify the dominant fragments in the community patterns (Table 4.S3). Members from the family *Comamonadaceae* produced fragments of approximately 200 bp (196-204bp). The members of *Alphaproteobacteria* (genera *Bradyrhizobium, Pedomicrobium, Hyphomicrobium*) produced fragments of 340 bp, and clones (KJ615152, KJ615160, KJ615167) related with TRA3-20 produced a fragment of 360 bp.

The bacterial community from the pilot-plant GACFs treating ozonated surface water (location A8) was analysed monthly during one year (Fig. S1). The most abundant fragments were present already after two weeks of operation and remained predominant during the whole period (Chapter 6). The patterns from the communities obtained in the first 2 months of operation (short running times) were clustered separately from the patterns of the communities obtained in the period between 2 and 12 months. Similarity values <75 % were found for short running times (<60 days). The similarity of the communities obtained in de period between 2 and 12 months of operation was above 80%. The patterns showed minor differences in the beginning of spring and in the fall. Furthermore, the predominant fragments were at the same positions as those retrieved from full-scale filters (Fig. S1). No differences in composition of the community in the top and in the bottom part of the filter were observed.

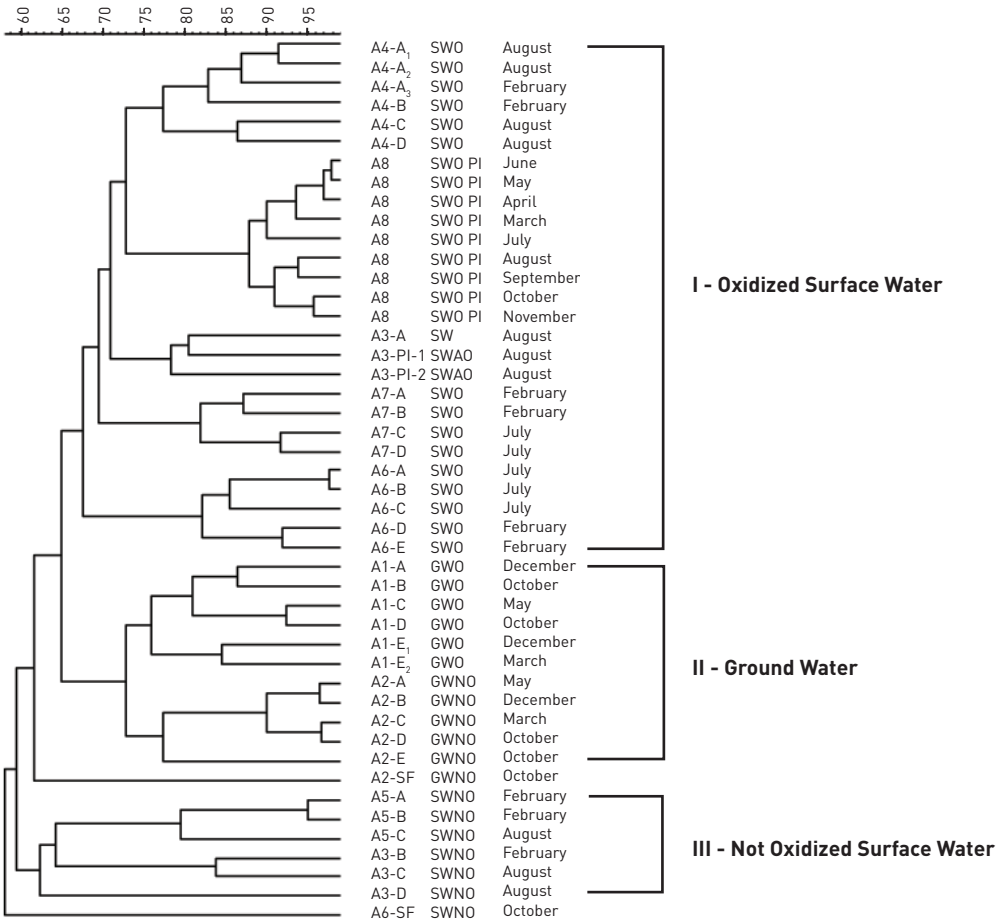

Figure 4.1 Clustering of T-RFLP patterns of 48 bacterial communities obtained from 18 full-scale GAC filters and 3 pilot-plant filters. The T-RFLP fingerprints were compared with the unweighted pair-group method using arithmetic averages (Bionumeric software version 3.0. Applied Maths NV., Sint-Martens-Latem, Belgium). A2- SF = Rapid sand filter at location A2, A6-SF = rapid sand filter at location A6.

4.3.3 Comparison of clone library and T-RFLP fingerprints

The communities from two GACFs at locations A1 and A6 were studied in more detail. T-RFLP patterns were generated for a number of abundant sequences and isolates from these GACFs (Table 4.S3). The predominant fragments in the T-RFLP patterns were identified based on similarity to the T-RFLP patterns of representative clones and isolates. The identity of the predominant species derived from the T-RFLP pattern was compared with the identity derived from the sequence analysis of the clones (Table 4.S4). In the GAC pilot filter treating ozonated groundwater (location A1), both methods indicated the predominance of *Polaromonas* and *Bradhyrizobium* in the community. The sequences of *Hyphomicrobium* spp. and *Methylobacterium* spp. detected in the clone library could not be confirmed by T-RFLP because the position of fragments for these sequences was not determined experimentally. However, fragments of 340

and 342 bp were assigned to *Pedomicrobium* sequences (Table 4.S3), which are closely related to *Hyphomicrobium* (Garrity et al. 2005). Furthermore, the in silico predicted fragment size for *Hyphomicrobium* spp. (AF279791) (339 bp) is very similar with fragments of *Pedomicrobium*. In the full-scale GAC filter treating oxidized surface water (location A7) both analyses indicated the predominance of unidentified *Betaproteobacteria* related with clone TRA3-20 (AY491578), *Bradhyrizobium, Polaromonas*, *Pedomicrobium* and uncultured *Commamonadaceae* (Table 4.S4).

4.3.4 Enzymatic activity of Polaromonas strains isolated from GAC filters

Polaromonas was identified as dominant genus in a previous cultivation dependent study (Magic-Knezev at al. 2009) and therefore cultured representatives were available for physiological characterization (Magic-Knezev and van der Kooij 2006). Additional tests on the enzymatic activity were conducted to analyse the role of these bacteria in GAC filters. All tested isolates exhibited activity of 4 out of 20 tested enzymes: esterase (C4), esterase-lipase (C8), leucine and naphthol-AS-BI-phosphohydrolase. Alkaline and acid phosphatase, lipase, valine- and cystein arylamidase, trypsin and alpha-chymotrypsin and enzymes for carbohydrate degradation were not active in tested strains.

4.4 DISCUSSION

4.4.1 Identity of bacteria predominating in GAC filters

This study focused on the identification of the predominant species (>1 %), and therefore an approach was chosen comprising the analysis of a relatively limited number of clones from an individual GAC filter (Table 4.S1A-B) in combination with T-RFLP profiling, that has previously been shown to be well suited for that purpose, in line with its inherent limitations in estimation of the true richness (Bent et al. 2007). From a total of 24 groups of the *Betaproteobacteria* detected in a previous culture-based study (Magic-Knezev and van der Kooij 2009) and this study, four groups were found in both studies, viz. *Polaromonas*, *Hydrogenophaga*, *Variovorax* and unclassified *Comamonadaceae*. From a total of 28 groups of *Alphaproteobacteria* detected in the culture-based and the culture-independent study, five were found in both studies (*Sphingomonas*, *Bradyrhizobiaceae*, *Afipia*, *Bosea* and unclassified *Rhodobacteriaceae*). Hence, the predominance of genera from the family *Comamonadaceae*, *Hyphomicrobiaceae*, *Rhodobacteriaceae* and *Bradyrhizobiaceae* reported here (Table 4.3, Table 4.4) is in concordance with the culture-based study of the communities from the same GACFs (Magic-Knezev et al. 2009). The culture-independent study additionally revealed the importance of microorganisms related to the uncultured betaproteobacterium TRA3-20, *Acidobacteria* and *Nitrosomonadaceae*.

The number of OTUs in samples from GACFs estimated by methods applied in this research varied between 33 – 146 OTUs for the clone library (Table 4.5, GAC-GW) and 25 – 49 species for the T-RFLP (>0.5% relative abundance). The estimated richness found here was similar to some of the previously reported data for freshwater and drinking water systems (Table 4.4). Recent studies that used pyrosequencing of PCR-amplified 16S rRNA gene fragments revealed a higher richness in GAC filters (Lautenschlager et al. 2014; Liao et al. 2013), with an increasing richness being observed with increasing numbers of sequences analysed per sample (Table 4.6).

The even distribution of the predominant species (E_H=0.82±0.06; Table 4.S5) and the high number of single sequences is typical for oligotrophic aquatic environments, and indicates unfavourable conditions for predominance of a single species (Hughes et al. 2001). Several studies demonstrated that rare species (<1%) in biofilm communities represent the majority of richness in these communities (>75%, Kwon et al. 2011, >92% Lautenschlager et al. 2014). Also Liao et al. (2013) reported a relative abundance of less than 6% for all genera in the studied GACFs.

However, the relatively high similarity between the communities from different filters observed in our study (>60%, Fig. 4.1) and temporal and spatial stability of the bacterial composition (Figure 4.S1) suggest that the predominant species are similar for all investigated GAC filters.

Table 4.6 Species richness in different aquatic environments and different methods targeting the 16S rRNA gene

Environment	Method	Species richness	Sequence depth (# sequences)	Reference
Surface water (SW)	S.S.[a]	48	239	Crump et al. 1999
SW, combined	S.S.	34	689	Zwart et al. 2002
SW [3] ; humic lake	S.S.	62	179	Burkert et al. 2003
Dental unit water sys.	S.S.	40	170	Singh et al. 2003
Chlorinated DW [2]	S.S.	23-55		Williams et al. 2004
DW distribution	S.S.	83	288	Martiny at al. 2003
DW distribution	S.S.	24-55	216	Eichler et al. 2006
DW	SARST[4]	62-332		Poitelon et al. 2009
Groundwater	T-RFLP	95		Danovaro et al. 2006
DW Biofilm	DGGE	11-21		Emtiazi et al. 2004
SW – river	DGGE	16		Winter et al. 2007
GAC filters	S.S.	36-70	768	Li et al. 2010
RSF	S.S.	65	431	White et al. 2012
GACF	S.S.	6-27	52-55	Liao et al. 2012
GACF	Pyroseq. [b]	2441-4139	10890-13659	Liao et al. 2013
GACF	Pyroseq. [b]	300	2140	Lautenschlager et al. 2014
GACF	DGGE	24		

[a] Sanger sequencing, [b] Pyrosequncing [2] DW = drinking water; [3] SW= surface water; [4] Serial analysis of ribosomal sequences

4.4.2 Comparison with bacterial communities in aquatic environments.

Most bacteria identified in the GACFs are common constituents of freshwater environments and biofilms (Zwart et al. 2002; Eichler at al. 2006; Williams et al. 2004; Newton at al. 2011; Zeng et al. 2013; Liao et al. 2013; Vaz-Moreira et al. 2013; Lautenschlager et al. 2014). The predominance of the phylum *Proteobacteria* in drinking water systems has been previously reported (Lautenschlager et al. 2014; Zeng et al. 2013; Liao et al. 2013; Vaz-Moreira et al. 2013; Liao et al. 2012; Pinto et al. 2012). However, we found a higher proportion of proteobacteria in GACF's than reported by Liao et al. (2013) (45-65%) and Lautenschlager et al. (2014) (38 – 74%). The proportion of classes within the *Proteobacteria* varies for different aquatic environments. For GACFs three independent, culture-based studies identified *Betaproteobacteria* and in particular the family *Comamonadaceae*, with the genera *Polaromonas* and *Hydrogenophaga*, as predominant in GAC filters (Magic-Knezev and van der Kooij 2009; Niemi et al. 2009; Norton and LeChevallier 2000). Liao et al. (2013) observed a decrease in the abundance of *Betaproteobacteria* in GACFs amended with ammonium. Lautenschlager et al. (2014) found a total of three predominant groups of sequences (15-20%) in filters from drinking water treatment (SSF, RSF, GACF), including *Bradyrhizobium*, unclassified *Betaproteobacterium* and unclassified *Rhodocyclales*, from which the unclassified *Betaproteobacterium* showed higher abundance in GACFs than in SSF and RSF. A high relative abundance of the *Comamonadaceae* has frequently been reported for aquatic environments (Emtiazi et al. 2004; Burkert et al. 2003; Crump et al. 1999; Niemi et al. 2009; Kampfer et al. 2006; Kalmbach et al. 1997; Poitelon et al. 2009). Remarkably, Liao et al. (2013) found only members of the genus *Hydrogenophaga*, although also in their study the class of *Betaproteobacteria* was abundant, especially in a filter with a low concentration of ammonia in the influent. The abundance of members from the alphaproteobacterial order *Rhizobiales* in GAC filters has also been reported by Liao et al. (2013) and Lautenschlager et al. (2014) using pyrosequencing of PCR-amplified 16S rRNA gene fragments.

A total of 9 from the 22 genera frequently identified in the pyrosequencing study (Liao et al. 2013) were also found in our study (*Bradyrhizobium, Hyphomicrobium, Pedomicrobium, Bryobacter, Flexibacter, Nitrospira, Novosphingobium and Rhodobacter*), but authors reported a much lower abundance of these sequences (0.2 – 5.3%). Ammonia-oxidizing bacteria from the betaproteobacterial order of *Nitrosomonadales* are common constituents of aquatic environments and soil (Eichler et al. 2006; Garity et al. 2005). In contrast, alphaproteobacterial nitrifiers predominate in drinking water with chlorine (Williams et al. 2004), and in some distribution systems *Nitrospirae* are more abundant than nitrifying *Betaproteobacteria* (Martiny at al. 2003).

Only a few sequences of the *Gammaproteobacteria* were retrieved from the investigated GACFs, although the members of this class (as *Pseudomonas, Aeromonas, Legionella*) are common constituents of aquatic environments and used as parameters for microbiological quality of drinking water (Danovaro et al. 2006; Williams et al. 2004; Zwart et al. 2002; Crump et al. 1999). However, these organisms are usually present in very low numbers (1 in 100 ml) and identification of many more clones from a single community (e.g. $>10^7$ at 10^5 bacteria ml^{-1}) would be needed for detection of these organisms. In line with this notion, Liao et al. (2013) was able to reveal the presence of *Gammaproteobacteria* in the GAC filters at much higher sequencing effort (2441-4139 sequences per sample) especially in the filters with higher ammonium con-

centration, but reported only sequences from genus *Lysobacter* as frequently observed (0,5%). Consequently, the use of qPCR methods is more efficient for detection of specific bacterial genera and species.

The presence of *Acidobacteria* in GACFs has also been reported in other studies at a similar frequency (Liao et al. 2014 (5,3 – 5,9%); Lautenschlager et al. 2014; Shiary et al. 2012).

Although similarities with communities from other aquatic and terrestrial environments are present, the specific predominance of *Polaromonas* in GAC filters (Magic-Knezev et al. 2009; Niemi et al. 2009; Jeon et al. 2004; Coleman et al. 2002) and the group TRA3-20 may indicate specific conditions in GAC filters that enhance growth of these bacteria. The findings further suggest that members form *Rhisobiales* (*Hypomicrobiaceae*, *Bradyrhizobiaceae* and *Rhodobacteraceae*) also play important role in GACFs.

4.4.3 Metabolic properties of bacterial types predominating in GAC filters

Culture based and culture independent studies revealed the importance of several bacterial groups in GACFs, vz. TRA3-20, *Polaromonas* and members from the order *Rhizobiales* and the phylum *Acidobacteria*.

The candidate order TRA3-20 represents 15 % of the retrieved sequences, and to date has no cultured representatives. Hence, the functional role of this important group in GACFs cannot be revealed beyond mere speculation due to the absence of cultured relatives from this group.

Polaromonas spp. accounted for 11 % of the identified clones (Table 4.3) and 26.5% of the identified bacterial colonies (Magic-Knezev et al. 2009). Representatives of this genus have also been found in groundwater contaminated with *cis*-dichloroethene and GACFs from drinking water treatment (Jeon et al. 2004; Coleman et al. 2002), and in bottled water after storage (Loy et al. 2005), indicating their preference for ultra-oligotrophic environments. This preference is consistent with physiological characteristics of *Polaromonas* spp. strains previously isolated from GAC filters that were shown to grow at microgram-per-liter levels of carboxylic and aromatic compounds. These organisms also assimilate amino acids but do not utilize carbohydrates (Magic-Knezev and van der Kooij 2006). Furthermore, the genome analysis of a *Polaromonas sp*. strain isolated from a GACF indicated a potential for utilization of halogenated alkanes, cyclic alkanes and (poly)aromatic compounds (Mattes et al. 2008). The enzymatic activity measurements of eight of the strains isolated from GACFs indicated the ability to hydrolyze N-terminal amino acids from peptides, amides or arylamides (arylamidase), water-soluble short acyl chain esters (esterase) and also less soluble long-chain fatty acids (esterase-lipase) (Chahinian and Sarda 2009). The *Polaromonas sp*. strains tested here also could utilize the hydrophobic amino acid leucine (leucine arylamidase) and phosphate (phosphohydrolase). The absence of enzymes for the hydrolysis of proteins and carbohydrates is consistent with the isolation of *Polaromonas* spp. from oligotrophic aquatic environments where proteins and carbohydrates are not the main substrate.

The importance of *Rhizobiales* in biofilters has been reported previously (Shiray et al. 2012; White et al. 2012 and Liao et al. 2013). The high relative abundance of *Rhizobiales* (20% of the

clones) found in our study indicates some similarity in the conditions in soil and in GAC filters, that may be related to limited availability of nitrogen and carbon sources (Batjes 1996). The growth of *Hyphomicrobium* may be stimulated by soil extract, and these organisms can utilize one-carbon compounds such as methanol or methylamine (Garrity et al. 2005). This suggests that *Hyphomicrobium* can utilize low molecular weight compounds from humic origin that are also important substrates in GACFs. The presence of bacteria known to be involved in nitrogen cycling, such as *Bradyrhizobium* (nitrogen fixation), *Nitrospirae* (nitrite oxidation) and *Nitrosomonadales* (ammonium oxidation) indicate that in GACFs nitrification processes occur despite the low ammonium concentration in the influent (usually below 0.02 mg N L^{-1}), as ammonium is removed in conventional treatment preceding the GAC filtration (van der Wielen et al. 2009). Presence of nitrogen metabolizing bacteria in GACFs can therefore indicate i) incomplete removal of ammonia in the pretreatment or/and ii) secondary production of ammonia and amino acids by the protozoan activity in GAC filters (Sherr and Sherr 2002). Furthermore, presence of species able to fix nitrogen indicates that nitrogen fixation may also occur in GAC filters. As an energetically expensive process nitrogen fixation is more probable to occur when the fixed nitrogen is depleted and phosphorus and carbon are still available. Concentration of nitrogen and phosphorous in facilities treating surface water vary with season and nitrogen concentration in form ammonium may reach values of 0.2 mgL^{-1} but is occasionally also below the detection limit (0.001 mg L^{-1}). However, nitrate is available in the influent (0.8-7 mg L^{-1}) indicating that nitrogen fixation is less likely to occur in GAC filters.

Analysis of complete genomes of three strains from subdivisions 1 (*Acidobacterium capsulatum*, strain Ellin345) and 3 (strain Ellin 6076) within the phylum *Acidobacteria* suggested that these organisms are heterotrophs that utilize simple or complex sugars and are capable of nitrate and nitrite reduction (Ward et al. 2009). The examined *Acidobacteria* strains exhibit slow metabolic rates under low-nutrient conditions. It should be noted, however, that most of the sequences retrieved from GAC filters in this study were classified as members of subdivisions 3, 4 and 6, and hence possibilities for extrapolation with respect to the physiology of the corresponding bacteria remain limited.

4.4.4 Factors affecting the composition of the bacterial community in GACFs

The high similarity between the GAC communities from different locations and the low similarity to bacterial communities from the rapid filters (A2-SF, A6-SF, Fig. 4.1) indicate specific conditions in GACFs. Shirey et al. (2012) also observed a low temporal variability in GACFs after a period of maturation and difference in the communtiy structure between anthracite and GACFs.

The variation in the bacterial community structure of GACFs from different locations is mainly related to the water source and the (oxidative) pre-treatment, but the effect of the seasonal changes in water quality and the maturation of a filter is not very clear from the data (Fig. 4.1), possibly due to (i) the low sensitivity of applied methodology, i.e. the small number of clones, (ii) the low resolution of the T-RFLP analysis, or (iii) a limited effect of seasonal changes in water quality on predominant bacteria in GACFs. Nevertheless, in all examined filters, predominant bacterial groups TRA3-20, *Polaromonas* and *Rhizobiales* represented the most abundant bacteria.

This suggests that the nature of the GAC surface and the nature of substrates available for growth (natural organic matter NOM) play an important role in the selection of the dominant populations. Most of the investigated GACFs are positioned at the end of the treatment train where the concentration of organic matter is low (1-6 mg DOC L^{-1}), and readily degradable carbon and energy sources are already utilized in the previous steps (Table 4.1). Moreover, strong adsorptive properties of GAC furthermore reduce the concentration of available organic carbon in the water phase (Chapter 6). However, the species that are among the first to colonize GAC remain predominant (Fig. 4.S1) suggesting that these organisms are best adapted to the conditions specific for GACFs where they carry the main role in the biodegradation processes.

The most important factor that distinguishes GAC filters from the aquatic and soil environments is the specific nature of the GAC surface (Sontheimer et al. 1988). The enhancement of bacterial growth when compared to filters with inert material is frequently reported for GAC (Nishijima et al. 1992; Servais et al. 1994; Wang et al. 1995). Factors affecting selection of bacteria in GAC may be related to: i) the effect of GAC surface properties on strength of bacterial attachment that was demonstrated by Magic-Knezev and van der Kooij (2004) and Billen et al. (1992) ii) the effect of GAC geometry on the protection of bacteria from share forces and predation iii) the effect of GAC on the availability of organic compounds for growth. Furthermore, low density of GAC hampers rigorous backwashing and provides conditions for the accumulation of particulate organic matter and protists between the GAC particles (Scheriber et al. 1997; Wang 2014) resulting in biomass turnover and production of ammonia (Sherr and Sherr 2002; Pernthaler et al. 2005) that may explain the relatively important fraction of *Rhizobiales* in GAC filters (Table 4.4). Such conditions in GACFs lead to preferential survival of small bacteria that are strongly attached and better protected inside the macropores on the GAC surface.

To be able to survive, attached bacteria must also be capable in utilization of organic carbon available in GACFs. The metabolic properties of cultured relatives of the predominant bacteria in GACFs suggest that low molecular weight compounds are the major source of carbon for growth and maintenance. The majority of organic carbon in GAC is comprised of high molecular weight humic substances (HS), while the low molecular weight compounds represent a smaller fraction (Servais et al. 2005). Amon and Banner (1996) showed that HS support more growth than low molecular weight compounds, and Camper (2004) concluded that the adsorption of HS on the surface of corrosion products enhances bacterial growth, thus bioavailability of HS. This indicates that the adsorption of HS on GAC might also play a role in availability of substrates for growth.

These findings lead to the conclusion that the bacteral communities in GACFs are relatively stable and that the conditions common for all GACFs apparently have a stronger effect than the differences related to the source, pre-treatment and season. The conditions common for GACFs can be summarized as the low concentration of nutrients, large surface available for growth, good protection from predation and effect of adsorptive properties of GAC on availability of natural organic matter. This leads to accumulation of species specialized in attached growth at ultra-oligotrophic conditions as confirmed by the physiological properties of the predominant bacteria in GAC filters.

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SUPPLEMENTAL MATERIAL CHAPTER 4.

Table 4.S1A Number of GAC filters sampled, number of GAC samples and number of T-RFLP patterns retrieved per location. A8-PI is pilot-plant filter from location A7

Location	No. filterbeds (No. of samples)	Filter age (days)	Season
A1	1(8)	90-390	-
A2	1 (7)	90-390	-
A3	3 (3)	150-335	Winter/Summer
A4	4 (5)	760-910	Winter/Summer
A5	3 (3)	35-455	Winter/Summer
A6	3 (4)	45-450	Winter/Summer
A7	3 (3)	365	Winter/Summer
A8-PI	1 (15)	1-365	Winter/Summer

Table 4.S1B Number of GAC filters sampled, number of GAC samples and number of clones retrieved per location

Location	No. filterbeds (No. of samples)	No. Clones retrieved (N)
A1	1(3)	85
A2	1 (1)	12
A3	2 (3)	40
A4	2 (2)	51
A5	1 (2)	10
A6	2 (2)	26
A7	3 (3)	56
A8	1 (1)	13

Table 4.S2 Size and relative frequency of appearance of the predominant T-RFLP fragments in the bacterial communities from GAC filters.

Fragment size group (bp)	Base pare range	Relative frequency of appearance (N=352*)	Present in filterbeds (%)
FS200	196.9-203.7	24%	75
FS340	340.9-342.9	18%	63
FS360	358.8-361.5	9%	50

* A total number of fragments with the relative abundance of peak height >5% in all analyzed communities.

Table 4.S3 Empirical apparent T-RFLP fragment sizes of selected individual clones and isolates.

Taxon	Acession Nr.	Apparent Fragment Size (BP)
Hydrogenophaga (isolate)	EU130958	142
Methyloversatilis (isoalate)	EU180528	198
Polaromonas (isolate)	EU130979	199
Polaromonas (isolate)	EU130980	200
Polaromonas (isolate)	EU130970	200
Polaromonas (clone)	KJ615102	200
Polaromonas (clone)	KJ615087	200
Acidobacteria subgroup9 (clone)	KJ615162	203
Pedomicrobium (clone)	KJ615172	341
Bradyrhizobium (clone)	KJ615097	343
Bradyrhizobium (clone)	KJ615096	343
Hyphomicrobium (clone)	KJ615092	341
Woodsholea (clone)	KJ615353	341
Bradyrhizobium (isolate)	EU180519	343
Unindentified *betaproteobacterium* (clone)	KJ615152	361
TRA3-20 (clone)	KJ615160	361
Unindentified *betaproteobacterium* (clone)	KJ615167	361
Acidobacteria subgroup6 (clone)	KJ615162	372

Table 4.S4 Apparent fragment size and identity of T-RFLP fragments and identity and abundance of clones from the clone libraries of two bacterial communities. GW-OX = community from the GAC filter treating pre-oxidized groundwater; SW-OX = community from the GAC filter treating pre-oxidized surface water

Sample	Closest relatives clone	Clone library N (%)	T-RFLP Fragment size BP (abundance %)	T-RFLP fragment identity
SW-OX	*Bosea* *Hyphomicrobium* *Bradhyrizobium* *Sphingomonadales* *Rhodobacterales*	14(33) 4(9) 2(5) 2(5) 2 (5)	341 (13) 342 (14)	Pedomicrobium Bradhyrizobium Hyphomicrobium
	Polaromonas *Unindetified Comamonadaceae* *Gallionellaceae*	9 (21) 3 (7) 1 (2)	198 (4) 200 (33) 202 (20)	*Polaromonas*
	Gamma proteobacteria	3(7)	337 (2)	
	Cyanobacteria	1(2)	180 (2)	
	Caulobacter	1(2)	99 (2)	
SW-OX	*Rhodospirilales* *Hyphomicrobiaceae* *Rhizobiales* *Unindentified alphaproteo-bacterium*	13(28) 5(11) 5(11) 2 (4)	343(5) 341(2)	*Bradhyrizobium* *Pedomicrobium*
	TRA3-20 *Comamonadaceae* *Nitrosomonadales*	9(20) 4(9) 2(4)	361 (13) 199 (2) 201 (5)	Unindentified *betapro-teobacterium* *Polaromonas*
	Acidobacteria	3(7)	371 (7) 204 (3)	*Acidobacteria* subgroup 6 and 9
	Actinobacteria	1(2)	89 (7)	
	Xantomonadales	1(2)	57(5)	
	Nitrospira	1(2)	58 (4)	

Table 4.S5 Reciprocal Simpson's index (1/D), Shannon Evenness index (E_H) and diversity (H) of T-RFLP patterns from GAC filters from different locations. RSF – rapid sand filters.

	N	Shannon Diversity Index (H)	Shannon Evenness Index (EH)
A7PI	27	3.26±0.65 (1.80-3.92)	0.80±0.05 (0.66-0.85)
A4,A6,A7	16	3.70±0.34 (2.76-4.01)	0.87±0.04 (0.79-0.93)
A3,A5	8	3.29±0.47 (2.34-3.75)	0.82±0.03 (0.77-0.86)
A1	7	2.82±0.57 (2.04-3.70)	0.75±0.03 (0.69-0.80)
A2	7	3.17±0.49 (2.25-3.51)	0.83±0.06 (0.77-0.96)
A2	1	3.63	0.88
A6	1	3.86	0.82
Totaal	67	3.31±0.58 (1.80-4.01)	0.82±0.06 (0.66-0.96)

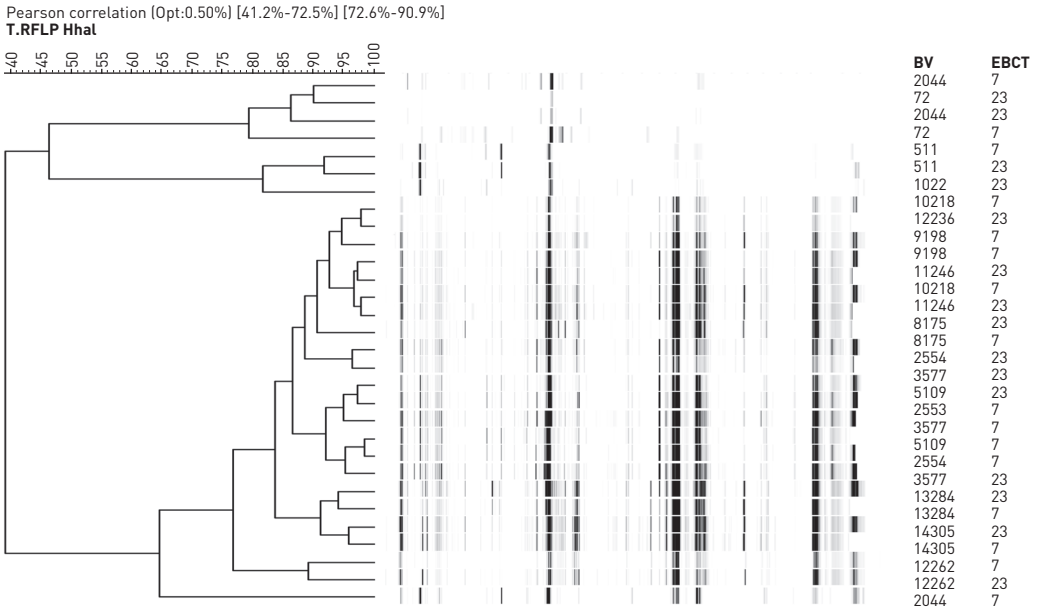
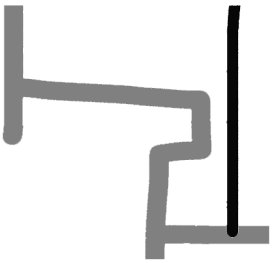


Figure 4.S1 Dendrogram for the T-RFLP patterns of bacterial community in a pilot GAC filter in the period of one year, at different bed volumes (BV) in the top (EBCT 7) and middle layer (EBCT 23) of the filter. The T-RFLP fingerprints were compared with unweighted pair-group method using arithmetic averages (Bionumeric software version 3.0. Applied Maths NV. Sint-Martens-Latem. Belgium). Bedvolumes represent also the runnung time of a filter.



CHAPTER 5

NUTRITIONAL VERSATILITY OF TWO *POLAROMONAS* STRAINS ISOLATED FROM GRANULAR ACTIVATED CARBON FILTERS

This chapter is based on publication:

Magic-Knezev A., and van der Kooij D. (2006). Nutritional versatility of two *Polaromonas*-related bacteria isolated from biological granular activated carbon filters pp. 303-311. In: *Recent progress in slow sand and alternative biofiltration processes*. Eds. Gimbel R., Graham N.J.G., and Collins M. R. I WA Publishing, London, UK.

Abstract

Physiological properties of two bacterial strains isolated from GAC filters and identified as members of the genus *Polaromonas* were investigated in order to characterize the biodegradation processes in GAC filters used in water treatment. The results of batch culture growth experiments with mixed and individual substrates at concentrations typical for drinking water indicate that the two examined *Polaromonas* strains have similar physiological properties. Favorite substrates for growth are carboxylic acids and aromatic acids, while only a few amino acids and carbohydrates are utilized for growth. The maximum growth rate (0.09-0.11 h^{-1}) was reached at low substrate concentrations (10 µg CL^{-1}) of acetate. The saturation constant for growth of strain P-315 on acetate is 0.015µM and 0.039 µM for the growth on benzoate indicating a higher affinity of this strain for acetate. These results indicate that biodegradation of low molecular weight organic compounds in GAC filters is possible at very low concentration of substrate. The obtained results can be used to model the biodegradation in GAC filters for examining the effects of operational parameters on the efficiency of the removal of organic compounds by the combined adsorption and biodegradation in GAC filters.

Key words – **water treatment, GAC, biodegradation, *Polaromonas*, saturation constant, growth rate**

ECN SEI 5.0kV X500 10µm WD13mm

5.1 INTRODUCTION

Biodegradation processes in granular activated carbon (GAC) filters are beneficial for the removal of organic compounds in the process of water treatment. The extent of biodegradation in GAC filters is determined by the concentration of active biomass and by the metabolic properties of this biomass for available substrates. For the optimal exploitation of biodegradation in GAC filtration detailed information is needed about quantitative and qualitative properties of biomass in the GAC filters. Several methods are available for the assessment of the biomass concentration associated with GAC (Magic-Knezev and Van der Kooij 2004; Urfer and Huck 2000; Wang et al. 1995), but information about metabolic properties of bacteria from GAC is scarce. According to the ATP-based method (Magic-Knezev and Van der Kooij 2004), 6 -125 kg of active biomass can be present in a filter bed of 100 m^3 GAC. Further characterisation and optimisation of biodegradation in GAC filters requires information about the identity and physiological properties of predominant bacteria in GAC filters. In a study that was based on cultivation-dependant techniques, the dominant cultivable bacteria in GAC filters used in drinking water treatment were identified as members of the genus *Polaromonas* of the *Comamonadaceae* family (Magic-Knezev et al. 2003). Very little information about the physiological properties of species belonging to this genus is available. Therefore, the aim of this research was to determine the growth characteristics of two strains belonging to the genus *Polaromonas* that were isolated from GAC filters. Experiments were designed to obtain information about:

1. the nature of favourite substrates at concentrations typical for water treatment and
2. the kinetic parameters of growth on selected substrates at concentrations typical for water treatment.

The information about the nature of favourite substrates and kinetic parameters of growth for these two strains, together with the information about the concentration of biomass in a GAC filter contributes to the understanding of the removal of organic matter by the biodegradation processes in a GAC filter.

5.2 METHODS AND MATERIALS

5.2.1 Bacterial strains

Strain P-315 was isolated from a GAC filter treating surface water that was pre-treated by coagulation and sedimentation followed by rapid sand filtration, softening and ozonation. Strain P-305 was isolated from a GAC filter treating ground water pre-treated with rapid sand filtration and softening.

According to the 16S rRNA sequence analysis both strains were identified as members of the genus *Polaromonas*. The closest cultured relative with both strains is *Polaromonas vacuolata* with a sequence similarity >97% (NCBI accession number UAU14585). Although a high level of similarity was observed for 16S rRNA sequence, analysis of genomic DNA with AFPLP fingerprinting (chapter 4) indicated that these two strains belonged to different genotypes.

Inoculum cultures to be used in the growth experiments were prepared by inoculating a 100-ml infusion flask containing basal medium supplemented with 1 mg acetate-C L^{-1} with a

suspension of a fresh culture grown on an R2A agar. Flasks were incubated at 15°C until the maximum colony counts were obtained (4-8 10^6 CFU ml^{-1}). These cultures were kept at 4°C.

5.2.2 Growth on mixed substrates

The growth experiments were performed in 1-l glass-stoppered Erlenmeyer flask heated at 550°C for 4 hours to eliminate traces of organic carbon. The pipettes were cleaned with a 10% solution of $K_2Cr_2O_7$ in concentrated H_2SO_4, followed by 4 hour rinsing with tap water and heating overnight at 250°C. A total of four different types or organic compounds were used as a substrate for growth: I, 20 amino acids; II, 11 aromatic compounds; III, 17 carboxylic acids and IV, 20 carbohydrates. Solutions were prepared in 600 ml of drinking water (AOC concentration $< 5\ \mu g$ acetate-C eq L^{-1}), prepared from dune-infiltrated river water treated with powdered activated carbon, followed by softening, rapid and slow sand filtration. Indigenous bacteria were eliminated by pasteurisation of water at 60°C for 30 minutes. Mixtures of substrates were prepared by adding individual substrates from stock solutions, which had been prepared as described previously (Van der Kooij and Hijnen 1985). The final concentration of each individual substrate in the mixture was 1 μg C L^{-1}. Phosphorus (Na_2HPO_4) and nitrogen were added (NH_4Cl) to avoid growth limitation other than by the carbon source. Flasks were inoculated with the pre-cultured suspension of the strains to reach a start concentration of 100-500 CFU ml^{-1}. The Erlenmeyer flasks were incubated without shaking in the dark at 15 ± 1°C and growth was monitored by periodic determination of viable cells counts on R2A medium (Oxoid) in triplicate after incubation at 25°C during 5-7 days. Parallel to the determination of viable cell counts, concentrations of ATP were measured. Each mixture or concentrations was tested in duplicate flasks.

5.2.3 Growth on individual substrates

For the preparation of 1 mg C L^{-1} solutions autoclaved tap water was supplemented with nitrogen, phosphorous and individual carbon source from the stock solution and inoculated with the 100-1000 CFU of the pre-cultured strain. Growth was carried out in 9-ml test tubes at 15 ± 1°C. The growth was monitored by measurement of the ATP concentration on days 7 and 14 after inoculation. Solutions containing 10 μg CL^{-1} of individual substrates were prepared in the same way as described for the solutions of mixed substrate except only one substrate was added per Erlenmeyer flask. Flasks were inoculated with the pre-cultured suspension of strain to reach a start concentration of 100 to 500 CFU ml^{-1} and monitoring of growth was identical as in the experiments with mixed substrates. For each substrate a duplicate test was performed. The experiments conducted to determine the kinetic parameters of growth (growth yield coefficient (Y), maximal growth rate ($1/G_{min}$) and substrate saturation constant K_s) were carried out in drinking water prepared from aerobic groundwater containing a very low concentration of assimilable organic carbon. The test water was supplemented with nitrogen, phosphorous and the selected carbon source (acetate or benzoate) at concentrations ranging from 1 to 500 μg CL^{-1}. The flasks were incubated at 15 ± 1°C and growth was monitored by periodic determination of viable cell counts on R2A medium (Oxoid) in triplicate after incubation at 25°C during 5-7 days. Parallel to the determination of the viable cell counts ATP concentration was measured.

5.2.4 ATP analysis

The measurement of ATP is based on the production of light in the luciferine-luciferase assay. ATP was released from suspended cells with nucleotide-releasing buffer (NRB, Celsis). The intensity of the emitted light was measured in a luminometer (Celsis Advance™) calibrated with solutions of free ATP (Celsis) in autoclaved tap water following the procedure as given by the manufacturer. The detection limit was 1 ng ATP L^{-1} of sample.

5.2.5 Growth kinetics

The growth yield Y was calculated from the maximum colony counts ($N_{max,}$ CFU/ml) obtained for the applied substrate concentration (dS).

$$Y = dN_{max} / dS \qquad \text{(eq 1.)}$$

The doubling time (G in hours) of the cultures was calculated with the equation:

$$G = \log 2(t'-t)/(\log N_{t'} - \log N_t) \qquad \text{(eq 2.)}$$

where t'- t is the incubation time in number of viable cells (N) increased from N_t to $N_{t'}$.

The saturation constant K_s and the minimum generation time G_{min} were calculated with the Lineweaver-Burk equation:

$$G = G_{min} + G_{min} \cdot K_s / S \qquad \text{(eq 3.)}$$

Where S is the concentration of the added substrate (dS) and the natural substrate concentration (S_n) which was derived from the N_{max} values observed in water without substrate addition. (Van der Kooij and Hijnen 1985).

5.3 RESULTS AND DISCUSSION

5.3.1 Growth on mixed substrates

The growth on mixed substrate at the concentration of individual substrate of 1 μg C L^{-1} is performed for four major groups of organic compounds, viz. 20 amono acids (AA), 11 aromatic acids (AR); 17 carboxylic acids (CA) and 11 carbohydrates (CH). The growth response is compared to the growth in water without added substrate. The strains responded similarly to the substrate mixtures. Both strains showed significant growth in the mixtures of the AA, AR and CA compounds and a low response was observed with CH (Table 5.1). The best response was observed for the AR mixture indicating a high affinity of the strains for these (types of) organic compounds.

Table 5.1 Growth response of two *Polaromonas* strains as the maximum colony forming units (CFU L^{-1}) and the ATP concentration (ng L^{-1}) of the strains (C_m) in the suspensions of mixed substrates and in blank water (C_b). AA = amino acids; CA = carboxylic acids; AR = aromatic compounds; CH = carbohydrates

		AA	CA	AR	CH
Strain P-315	Cm (CFU)	$1.9\ 10^6$	$1.8\ 10^6$	$7.2\ 10^5$	$8.2\ 10^5$
	Cb (CFU)	$2.8\ 10^5$	$2.8\ 10^5$	$5.2\ 10^4$	$3.5\ 10^5$
	Cm (ATP)	228	144	44	60
	Cb (ATP)	25	25	6.4	30
Strain P-305	Cm (CFU)	$1.7\ 10^6$	$7.9\ 10^5$	$3.2\ 10^5$	$4.8\ 10^5$
	Cb (CFU)	$2.1\ 10^5$	$2.1\ 10^5$	$1.5\ 10^4$	$3.1\ 10^5$
	Cm (ATP)	105	67	24	41
	Cb (ATP)	16	16	3.4	22

5.3.2 Growth on individual substrates

The growth of strains on individual carbon sources was tested for two concentrations: 1 mg C L^{-1} and 10 μg C L^{-1}. The results of the growth experiments on a wide range of individual compounds at a concentration of 1 mg C L^{-1} are presented in Table 5.2.

Table 5.2 Utilisation of individual compounds in drinking water by two *Polaromonas* strains at a concentration of 1 mg L^{-1}.

Substrate type	Number of compounds	Number (%) of compounds utilized	
		Strain P-315	*Strain P-305*
Amino acids (AA)	20	6 (30)	3 (15)
Aromatic compounds (AR)	11	6 (55)	6 (55)
Carboxylic acid (CA)	17	17 (100)	15 (88)
Carbohydrates (CH)	21	3 (14)	5 (24)

The strains showed some differences in response to the substrates from four major groups but both strains grew well on the majority of tested carboxylic acids and aromatic compounds. The utilisation of amino acids and especially growth on carbohydrates was limited to a number of compounds from these two groups. However, strains grew better on the mixture of amino acids, indicating that some amino acids serve as a source of energy while other serve as a carbon source. Strain P-305, isolated from the filter treating groundwater, had a slightly lower response in terms of growth with individual substrates than strain P-315 that was isolated from the filter treating surface water. Furthermore, strain P-305 grew slower in the water without substrates added.

A number of compounds was selected for further growth experiments at a concentration of 10 μg C L^{-1}. The best response was observed for acetic acid, tartaric acid and benzoic acid (data not shown).

5.3.3 Growth kinetics of the *Polaromonas* strains for acetic and benzoic acid

Acetic acid and benzoic acid were selected as substrates for the determination of the kinetic parameters of growth. Acetic acid is a by-product of ozonation and contributes to the AOC concentration in drinking water. Benzoic acid was selected to obtain information about the degradation of aromatic compounds which are present in drinking water as a part of natural organic matter (humic and fulvic acid), or as a xenobiotic compounds. Furthermore, kinetic parameters of growth on acetate for *Spirillum sp.* (strain NOX) and *Pseudomonas fluorescens* (strain P17) which are widely applied for the analysis of AOC concentration in treated water, were determined earlier (Van der Kooij 1990).

The kinetic parameters of growth were determined for the concentration range 1-500 μg C L^{-1}. The growth curves observed in water supplied with substrates demonstrated that N_{max} values and the growth rate depend on the substrate concentration. Both substrates promoted the growth rate and increased the colony counts at all concentrations tested (Fig. 5.1.).

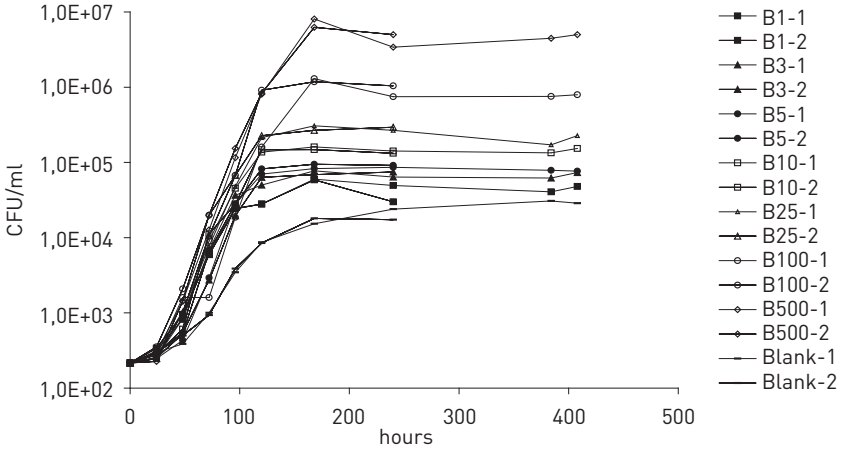


Figure 5.1 Growth of strain P-315 with different concentrations of benzoate at 15ºC.

The growth yield coefficients were calculated with the maximum values of the ATP concentration and the plate count (CFU ml^{-1}) for the applied substrate concentrations. These yield coefficient values differ slightly for the examined substrates and were used to calculate the natural indigenous substrate concentration (S_n) in the blank water (Table 5.3). The S_n concentration calculated from the yield coefficient derived from the ATP measurement were slightly higher than the concentrations calculated with the yield coefficient derived from the plate counts. The average value of the derived indigenous substrate concentration was used for the correction of S (S_n+dS) in the calculation of G_{min} and K_s with the Lineweaver-Burk equation (eq. 3). A plot of the generation time (G) of strain P-315 versus 1/S gave linear function for the growth on acetate and a non-linear function for the growth on benzoate (Fig. 5.2).

Table 5.3 Growth yield of two Polaromonas strains on acetate (Y_{ac}) and benzoate (Y_{ar}) acid and the concentration of indigenous substrate (S_n) derived from the growth yield and the maximum growth in the blank.

Strain	Substrate	Y_{CFU} (CFU µg^{-1} C)	$S_{n(CFU)}$ (µg C L^{-1})	Y_{ATP} (ng ATP µg^{-1} C)	$S_{n(ATP)}$ (µg C L^{-1})
P- 305	Acetate	2.1 10^7±2.3 10^6	1.9±0.3	0.77±0.04	2.1±0.1
	Benzoate	1.7 10^7±2.2 10^6	2.4±0.4	0.75±0.15	2.1±0.3
P- 315	Acetate	1.4 10^7±6.8 10^5	1.7±0.5	0.76±0.01	2.4±0.6
	Benzoate	1.4 10^7±1.8 10^6	1.7±0.4	0.93±0.11	1.9±0.5

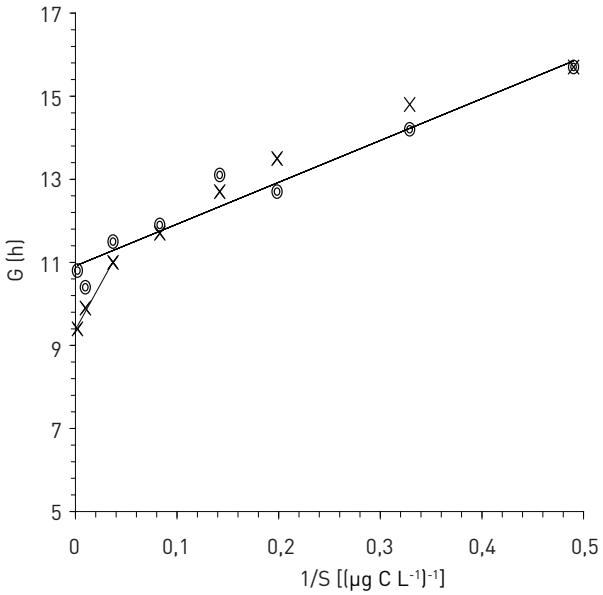


Figure 5.2 Lineweaver-Burk plot of strain P-315 for growth on acetate (◎) and benzoate (x).

The results reveal that S_n and added acetate were utilized simultaneously, while added benzoate is utilized after the depletion of S_n. Due to the preferential utilization of S_n in the solutions with added benzoate, kinetic parameters for growth on benzoate were determined for the concentrations above 10 μg C L^{-1}. The minimum generation time of strain P-315 for both substrates was between 9 and 11 h (0.09-0.11 h^{-1}), but the K_s value for acetate was lower than the K_s value for benzoate (Table 4) indicating a higher affinity of strain P-315 for acetate than for benzoate.

The observed Ks values are low and similar values have been reported for the growth of *Aeromonas hydrophyla* strain M800 on oleate (Van der Kooij and Hijnen 1988) and for the growth of *Flavobacterium sp.* strain S12 on oligosaccharides (Van der Kooij and Hijnen 1985). Furthermore, a similar saturation constant is reported for growth of a *Mycobacterium sp*. on

phenanthrene (Table 6.4). Information about the physiological properties of bacteria belonging to the genus *Polaromonas* is scarce. There are only few species described in this genus and one of the species is isolated from a GAC filter treating polluted groundwater (Coleman et al. 2002). The results of our research indicate that *Polaromonas* strains isolated from GAC filters are able to grow at very low concentration of carboxylic acids, aromatic compounds, and amino acids when present in a mixture (1 μg C L^{-1}).

Carboxylic acids, which are the favourite substrates of *Polaromonas* strains, are formed by the oxidation of natural organic matter and are common constituents of organic matter at the end of the treatment train. Their presence in drinking water may contribute to the biofilm formation in distribution systems. Due to their polar nature, carboxylic acids cannot be removed by adsorption on GAC, and their removal by biodegradation results in the improvement of biological stability of drinking water.

Table 5.4 Saturation constants for growth of some aquatic bacteria on different substrates.

strain	Substrate	K_s (µM)	G_{min} (h)	Reference
Polaromonas P-315	Acetate	0.015	10.9	This research
	Benzoate	0.039	9.4	
Polaromonas	cis-dichloroethene	1.6		Coleman et al.(2002)
Mycobacterium sp.	Phenanthrene	0.026		Miyata et al.(2004)
Aeromonas hydrophyla Strain M800	Oleate	0.01	4.3	Van der Kooij and Hijnen (1988)
	Acetate	0.46	6.7	
Flavobacterium sp.	Maltotetraose	0.019	2.3	Van der Kooij and Hijnen (1985)
	Maltopentaose	0.016	2.2	
	Maltohexaose	0.015	2.2	
Spirillum sp.	Oxalate	0.14	4.1	Van der Kooij (1990)
Pseudomonas fluorescens	Acetate	0.17	5.5	
Pseudomonas spp. GF1TP	Toluene	2.1	2.6	Massol-Deya et al. (1997)
Pseudomonas spp. GF161	Toluene	2.7	3.0	
Ralstonia taiwanensis	Phenol	5.5		Chen et al. (2004)

The removal of organic compounds in GAC filters is a result of adsorption and biodegradation processes. The removal by the adsorption is determined by the adsorption capacity of GAC and the adsorption kinetics of organic compounds in the water, while the removal by the biodegradation is determined by the concentration of biomass and its metabolic potential for the organic compounds that are available as a substrate. The adsorption capacity of GAC for organic compounds can differ significantly, but aromatic compounds are more adsorbable and will therefore occupy the adsorption sites on GAC. When these compounds are utilized by micro-organism the adsorption capacity of GAC will be used more optimally for the target compounds that are non-biodegradable and adsorbable resulting in an improved performance of the GAC filter and improved water quality. The information about the biodegradation kinetics for carboxylic acids by bacteria from GAC will be used for modeling the removal of these compounds in GAC filters, aiming at optimizing the use of this treatment process.

5.5 CONCLUSIONS

Two strains of the genus *Polaromonas*, isolated as predominant cultivable bacteria from GAC filters used in water treatment, have a similar metabolic potential and the following physiological properties of these strains were observed:

1. Preferable substrates for growth are carboxylic acids and aromatic compounds while amino acids are utilized only when present in a mixture. Both strains grow only on a limited number of amino acids and carbohydrates;
2. Growth on benzoate and acetate occurs at very low concentration of these substrates (1 $\mu g\ L^{-1}$) with a relatively low growth rate (0.09 h^{-1});
   The K_s value for growth on benzoate (0.039 μM) and for acetate (0.015 μM) in combination with nearly similar G_{min} values indicate that strain P-315 has a higher affinity for acetate (6) than for benzoate (0.23);
3. The ATP concentration is a suited parameter for the determination of bacterial growth yield and the natural substrate concentration and for determination of kinetic growth parameters at concentrations > 10 $\mu g\ C\ L^{-1}$.

Hence, bacteria in GAC filters used in water treatment are able to utilise biodegradable low molecular weight compounds at very low concentrations, thus contributing to the production of biologically stable drinking water.

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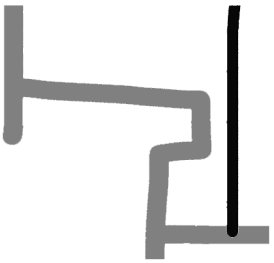

CHAPTER

6

POLAROMONAS AS MODEL FOR ANALYZING THE MICROBIAL UTILIZATION OF NATURAL ORGANIC MATTER IN GRANULAR ACTIVATED CARBON FILTERS IN WATER TREATMENT

Knezev A., van der Aa R. and van der Kooij D.

This chapter has been submitted for publication

Abstract

Bacterial growth and the removal of natural organic matter (NOM) were analyzed in a pilot plant granular activated carbon (GAC) filter that was operated at the surface water treatment pilot plant of Weesperkarspel (Waternet, The Netherlands) during 602 days aiming to assess feasibility of *Polaromonas* as a model for the microbial utilization of NOM in GAC filters (GACFs). Removal of dissolved NOM in a GACF occurred at two rates. In phase I (<20,000 bed volumes BV), with adsorption as the main driving force, NOM is removed approximately three times faster than in phase II with slow adsorption and increased biological activity. The bacterial accumulation rate (BAR) on GAC was higher in the top of the filter (1.6 ng ATP g^{-1} GAC day^{-1}) than in the middle part (1.1 to 1.6 ng ATP g^{-1} GAC day^{-1}), and biomass concentration on GAC (BGAC) reached its maximal level of 315 – 530 ng ATP g^{-1} GAC after approximately 250 days. The low specific growth rate of bacteria on GAC (<0.016 d^{-1}) implies that substrate uptake for maintenance requirements is a significant mechanism in the microbial removal of biodegradable compounds. At a temperature above 15°C bacterial ATP concentration decreased rapidly, but oxygen consumption increased, indicating activity of predating (micro) organisms in the filter. The quotient of oxygen consumption and dissolved organic carbon (DOC) removal exceeded the theoretical value (2.8 mg O_2 mg^{-1} C) for complete NOM oxidation at temperatures >10°C (3.9-11.2 mg O_2 mg^{-1} C) suggesting the utilisation of accumulated living and dead biomass (particulate organic carbon, POC) by predating (micro)organisms. Biomass accumulation and oxygen consumption demonstrate that biodegradation plays a role in NOM removal in the GACF. However, oxygen consumption cannot be used as a quantitative measure for the biodegradation of dissolved NOM because of lack of information about POC removal and oxygen uptake by physicochemical processes on GAC surface. Values of the apparent specific NOM utilization rate (qA, 0.2 – 1.8 µg DOC ng^{-1} ATP day^{-1}) observed in phase II approached the maintenance coefficient of Polaromonas for growth on acetate at lower temperatures and the maximal specific utilization rate at higher temperatures. A quantitative comparison of the apparent specific NOM utilization rate (qA) in the GAC filterbed, with the temperature-adjusted maintenance coefficient and the specific maximal utilization rate of acetate by *Polaromonas* requires more accurate data on BGAC and the contribution of POC removal to microbial activity. The nature of the low molecular weight compounds that may be released by the disruption of humic substance micelles by the adsorption is consistent with the preferred substrates of *Polaromonas spp*. predominating on GAC, but confirmation of this hypothesis requires further research.

Keywords – water treatment, granular activated carbon, natural organic matter, adsorption, biodegradation, ATP, *Polaromonas*,

ECN SEI 5.0kV X500 10µm WD13mm

6.1 INTRODUCTION

Granular activated carbon (GAC) filtration is used in water treatment for the removal of antropogenic contaminants and natural organic matter (NOM) that cause undesirable colour, odour or taste, serve as precursor for the formation of disinfection by products, or support microbial growth in the distribution system. Bacterial activity in GAC filters (GACFs) has been studied with a variety of techniques, and all studies showed a substantial presence of bacteria in GACFs with most activity in the top section and an increasing activity with the age of the filterbed (Camper et al. 1985; Carlson et al. 1996; Servais et al. 1994a,b; Magic-Knezev and van der Kooij 2004; Velten et al. 2007). Changes in NOM composition due to oxidative pretreatment of water enhances microbial activity and extends the operational time of a GACF for the removal of specific organic contaminants and dissolved NOM (Sontheimer et al. 1978; Carlson et al. 1996; Orlandini 1999; van der Aa et al. 2012). Various studies demonstrated enhanced biological activity in GACFs as compared to biofilters with sand, gravel, anthracite or other inert media (van der Kooij, 1983; Nijishima et al. 1992; Wang et al. 1995; Carlson and Amy 1998; LeChevallier et al. 1992; Uhl, 2000). The enhanced biological activity has been attributed to the physical and chemical properties of the GAC surface (Shimp and Pfaender 1982; Chudyk and Snoeyink 1984; Goeddertz et al. 1988; Nishijima et al. 1992; Servais et al. 1994b; Wang et al. 1995), and microbial utilisation of compounds adsorbed onto GAC (Eberhardt 1975; Sontheimer et al. 1978; Weber et al. 1978; Chudyk and Snoeyink 1984; Kim et al. 1997; Summers et al. 2010). However, utilisation of adsorbed compounds has been denied by other investigators (Peel and Benedek 1983; van der Kooij 1983; Sontheimer and Hubele 1986; Kim et al. 1997a; Ypsalski et al. 2008). To date, the mechanisms of the enhanced biological activity in GAC filterbeds remain unresolved.

Bacteria in GACFs utilize organic compounds for growth and maintenance. The extent of the removal due to bacterial utilization is determined by the quantity and metabolic properties of the bacteria in a filter, however, surprisingly little is known about these properties of bacteria in GACFs. Earlier studies, based on cultivation techniques, identified the bacteria isolated from GACFs in water treatment as members of the genera *Pseudomonas, Acinetobacter, Caulobacter, Alcaligenes, Flavobacterium* and *Bacillus* (McElhaney et al. 1978; Wilcox et al. 1983; Stewart et al. 1990). In a more recent study *Polaromonas* spp. and *Hydrogenophaga* spp., both belonging to the *Betaproteobacteria*, were identified by molecular techniques, as the predominant bacteria cultivated from GACFs in water treatment (Magic-Knezev et al. 2009). Also Niemi et al. (2009) reported the predominance of *Betaproteobacteria*, e.g. representatives of the *Burkholderiales*, in GACFs. *Polaromonas* spp. are commonly found in ultra-oligotrophic aquatic environments (Jeon et al. 2004; Page et al. 2004; Loy et al. 2005), indicating that these bacteria are adapted to growth at microgram-per-liter levels. Indeed, the substrate saturation constant (Ks) of two *Polaromonas* strains, isolated from GACFs, is about 1 μg C L^{-1} for acetate and benzoate when growing in batch tests (Magic-Knezev and van der Kooij 2006).

The objective of this investigation was to assess the microbial utilization of NOM in a GACF. For this purpose NOM removal by GAC filtration in a surface water pilot plant was studied for about 1.5 years. Data about oxygen utilization, the concentration of adenosine 5'-triphosphate

(ATP) associated with GAC and the metabolic properties of *Polaromonas* strains isolated from a GACF were used to analyze the microbial utilization of NOM.

6.2 METHODS AND MATERIALS

6.2.1 Pilot plant

A GAC column was operated in a pilot plant at the water treatment plant Weesperkarspel of Waternet, the water cycle company of Amsterdam and surrounding areas. This column received pretreated water from the full-scale treatment plant using groundwater-derived seepage water as source. Pretreatment included coagulation and sedimentation in an open reservoir (lake) followed by rapid sand filtration, ozonation (net ozone dosage 2 mg L^{-1} at temperatures below 12 °C and 1.5 mg L^{-1}, at temperatures above 12 °C) and softening in pellet reactors. This softened water was supplied to the GAC column of the pilot plant starting in July for an operational period of 602 days. The quality characteristics of the influent and the operational parameters of the GAC filterbed are shown in Table 6.1. Backwashing of the GAC filterbed, based on pressure drop, was applied weekly in summer and monthly in winter.

6.2.2 Analytical procedures

The quality of the influent and water at empty bed contact times (EBCT) of 7 minutes (top section, 42 cm; 82 kg GAC), 23 minutes (middle section, 142 cm; 196 kg GAC) and 40 minutes (effluent) (242 cm; 474 kg GAC), was analyzed each 14 to 28 days. Oxygen, DOC, pH, phosphate, nitrate, ammonium and UV absorbance at 254 nm (UVA, m^{-1}) were measured according to standardized methods (Standard Methods 1998). The AOC concentration was determined by measuring the growth of the test strains *Pseudomonas fluorescens* P17 and *Spirillum* sp. strain NOX in pasteurized water samples of 600 ml (van der Kooij 1992). Periodically, approximately 50 g of GAC was sampled from the filterbed with a thoroughly cleaned stainless steel pipe (D = 1.2 cm, L = 35 cm) via the sampling ports, at $EBCT_7$ and $EBCT_{23}$, respectively. High-energy ultrasonic treatment was used for the removal of bacteria from GAC (Magic-Knezev and van der Kooij 2004) The concentration of ATP in the obtained suspension and in water samples was measured as light emission in the luciferine-luciferase assay (Holm-Hansen and Booth 1966) with a luminometer (Advance, Celsis) using a reagent kit for bacterial ATP (Celsis; Brussels, Belgium). The ATP concentration was calculated from the relative light units (RLU) using the coefficient of the linear regression analysis of the calibration curve for free ATP (Celsis) in autoclaved tap water in the range of 1 – 250 ng L^{-1}. From the linear relationship between the bacterial biomass concentration on GAC (B_{GAC}, ng ATP g^{-1}) and time in the growth phase, the biomass accumulation rate (BAR, ng ATP g^{-1} GAC d^{-1}) was calculated.

Bacterial suspensions obtained by sonication of GAC samples (30 ml) were concentrated using a polycarbonate filter (diameter 25 mm, 0.22 μm pore size, type GTTP; Millipore). Subsequently, DNA from bacteria retained on the filter was extracted and purified by the FastDNA Kit for soil (BIO 101. Inc., 1070 Vista, CA, USA) as described by the producer. Extracted DNA was stored at -20°C and used for characterization of the bacterial community with TRFLP-analysis (see Supplemental Material).

Table 6.1 Operational parameters of the GAC filtration and influent water quality characteristics.

Parameter	Value*
Column diameter [m]	0.745
Bed height [m]	2.415
Carbon type [-]	GAC 830P
Total operational period [days]	602
Flow [m^3h^{-1}]	1.6
Surface load [$m\ h^{-1}$]	3.7
Empty bed contact time (EBCT, min]	40
Ozone dosage [$g\ O_3\ m^{-3}$]	1.5
Temp [°C]	3-24
pH [-]	7.6-7.9
DOC [$g\ C\ m^{-3}$]	4.6-6.0
Ultraviolet light (254 nm) absorbance (UVA, m^{-1})	8-11
AOC [10^{-3} g acetate-C eq m^{-3}]	47-88 (68)
Nitrate [$g\ m^{-3}$]	<0.001- 0.20 (0.01)
Phosphate [$g\ m^{-3}$]	0.01-0.09 (0.02)
ATP [$ng\ L^{-1}$]	5-72 (25)

* median value is given between brackets

6.2.3 GAC characteristics

In the GAC column (diameter 74.5 cm), Norit GAC 830P was used with an effective particle size of 0.1 cm (uniformity coefficient 1.7, an apparent density of 0.432 g cm^{-3} (backwashed and drained) and a filter bed porosity of 39%. The specific surface area of the diffusion layer surrounding the GAC particles (85 $cm^2\ g^{-1}$ GAC) was calculated from the median particle size assuming a spherical shape with an average diameter of 0.1 cm, as described in Supplemental Material. The external surface of GAC available for bacterial attachment (700 $cm^2\ g^{-1}$ GAC), including the surface of the pores with a diameter $>1\ \mu$m, was calculated from mercury intrusion analysis for GAC 830, provided by Cabot (Supplemental Material).

6.2.4 Removal of DOC, UVA and oxygen

The removal of DOC, UVA and oxygen was calculated according to equation 1:

$$\Delta X = X_I - X_E \quad (1)$$

where X is the concentration of DOC [mg C L^{-1}], UVA [m^{-1}] or O_2 [mg $O_2\ L^{-1}$], X_I is the concentration in the influent and X_E is the concentration in the effluent.

The specific removal rate of DOC, UVA and oxygen was calculated according to equation 2:

$$X_{SR} = \Delta X \times Q \times (m_{GAC})^{-1} \quad (2)$$

Where Q is the flow (L d^{-1}] , m_{GAC} is the weight of GAC in the column at the defined EBCT [g GAC] and X_{SR} is the specific removal rate of component X [mg C g^{-1} GAC d^{-1}; m^{-1} g^{-1} GAC d^{-1}; mg O_2 g^{-1} GAC d^{-1}].

Specific accumulation of component X (DOC, UVA and oxygen) was calculated according to equation 3:

$$X_{CS}(t) = \sum_{t=0} X_{SR,i,j}(t) \quad (3)$$

Where X_{SR} is the specific removal rate of component X for a given running time (t) [d] and X_{CS} is the specific accumulation of component X [mg C g^{-1} GAC; m^{-1} g^{-1} GAC; mg O_2 g^{-1} GAC].

6.3 RESULTS AND DISCUSSION

6.3.1 NOM removal

The DOC concentration in the influent of the GACF varied between 4.6-6 mg L^{-1}. The concentration drop (c. 15 %) in summer (Fig. 6.1A,C) can be attributed to more effective biological processes in the pretreatment in this period. The DOC removal at EBCT 40 min ($EBCT_{40}$) decreased rapidly from more than 95% at the beginning to approximately 20±5 % after 200 days (7,200 BVs) and approached 10% at the end of the experiment (602 days, 22,000 BVs) (Fig. 6.1A, B). At $EBCT_7$ the 10%-reduction level was reached after about 30,000 BVs (day 150) and approached 5% after about 100,000 BVs (day 500). At $EBCT_{23}$ and $EBCT_{40}$ the DOC removal increased after 350 (Fig. 1A) days when water temperature was > 20°C. The UVA reduction decreased from 98% at the beginning to approx. 26±6 % (at $EBCT_{40}$) after about 200 days (7,200 BVs), increased to 35% at a water temperature > 20°C and subsequently decreased further to about 20% after 500 days (18,000 BVs) (Fig. 6.1D). At $EBCT_7$, the UVA reduction was c. 10% after 100,000 BVs (500 days). The AOC concentration in the influent varied between 47-88 μg C L^{-1} (median 68 μg C l^{-1}) and between 9 - 40 μg C L^{-1} (median 29 μg C l^{-1}) in the effluent ($EBCT_{40}$). The removal dropped from more than 80% in the beginning to 40% at the end of the experiment (data not shown). A significant proportion of the AOC removal occurred at $EBCT_7$ at an average rate of 11.4 ± 3.4 μg AOC g^{-1} GAC d^{-1}. The average removal rate at $EBCT_{40}$ was 3.3 ± 0. 9 μg AOC g^{-1} GAC d^{-1}.

Two phases in NOM removal can be distinguished at $EBCT_7$, less clearly in the $EBCT_{7-23}$ section and and not in the $EBCT_{23-40}$ section (Fig. 6.2). Phase I ended after an operational period of 100 days (20,000 BVs) and a cumulative specific DOC removal (DOC_{CS}) of approx. 122 mg g^{-1} GAC. The average specific DOC removal rate (DOC_{RS}) in the top layer in phase II was 21% of DOC_{RS} in phase I and remained fairly constant (RSE = 2%, Table 6.2). UVA_{RS} in phase II at $EBCT_7$ was 30% of UVA_{RS} in phase I (RSE 1%). The decreased removal rates of DOC and UVA in phase II at $EBCT_7$ (Fig. 6.2, Table 6.2) show that adsorption of NOM became much less efficient when

a certain apparent solid phase concentration had been reached. The phase with reduced NOM removal has been defined as the (pseudo) steady-state phase based on two assumptions: (i) the adsorption capacity of GAC is exhausted and (ii) NOM is removed primarily by biodegradation (Sontheimer et al. 1978; Servais et al. 1991; Billen et al. 1992; Lechevallier et al. 1992; Carlson et al. 1996). However, Peel and Benedek (1980; 1983) developed a dual-rate adsorption kinetic model and concluded that slow diffusion into the micropores causes a continuing adsorptive removal of NOM. Summers and Roberts (1988) attributed the continuing NOM adsorption at extended running times by changes in the orientation of adsorbed NOM at high solid phase concentrations. We observed an increased NOM removal, most pronounced with UVA, after 350 days at an increasing temperature (Fig. 6.1B, D). Schreiber et al. (2005) also observed an increased adsorption of NOM at elevated water temperatures and attributed the effect of temperature to an entropic process controlled by hydrophobic interactions with the GAC surface, resulting in a modified orientation of adsorbed NOM. Nevertheless, an increasing number of bed volumes will result in a (slow) decrease of the adsorption capacity. The continuing decline of NOM removal after approximately 400 days (Fig. 6.1 B, D) indicates that high BV numbers are required to achieve a steady-state removal in which the contribution of adsorption has become negligible. A continuing decrease in the removal of NOM after more than 50,000 BV has also been observed in other studies (Bonné et al. 2002; Chowdhury et al. 2013). However, the effective removal of pesticides added to the influent after several years of operation demonstrated a significant residual adsorption capacity in GACFs (Bonné et al. 2002).

Table 6.2 The average specific removal of DOC and UVA (DOC_{RS}, UVA_{RS}) with standard error of the regression coefficient during two phases in the top (0-7), middle (7-23) and bottom section (23-40) and the whole column (0-40).

Phase	EBCT (min)*	DOC_{RS}	UVA_{RS}	O_{RS}
		(mg g^{-1} GAC d^{-1})	(m^{-1} g^{-1} GAC d^{-1})	(mg g^{-1} GAC d^{-1})
I	0-7	1.10 ± 0.06	1.77 ± 0.15	0.37 ± 0.02
	7-23	0.29 ± 0.02	0.48 ± 0.02	0.30 ± 0.02
	23-40	0.06 ± 0.01	0.11 ± 0.02	0.25 ± 0.01
	0-40	0.34 ± 0.01	0.56 ± 0.02	0.29 ± 0.02
II	0-7	0.23 ± 0.004	0.53 ± 0.006	0.67 ± 0.02
	7-23	0.08 ± 0.005	0.15 ± 0.004	0.30 ± 0.02
	23-40	0.07 ± 0.001	0.09 ± 0.002	0.33 ± 0.02
	0-40	0.10 ± 0.002	0.19 ± 0.003	0.38 ± 0.02

* corresponds with filterbed section

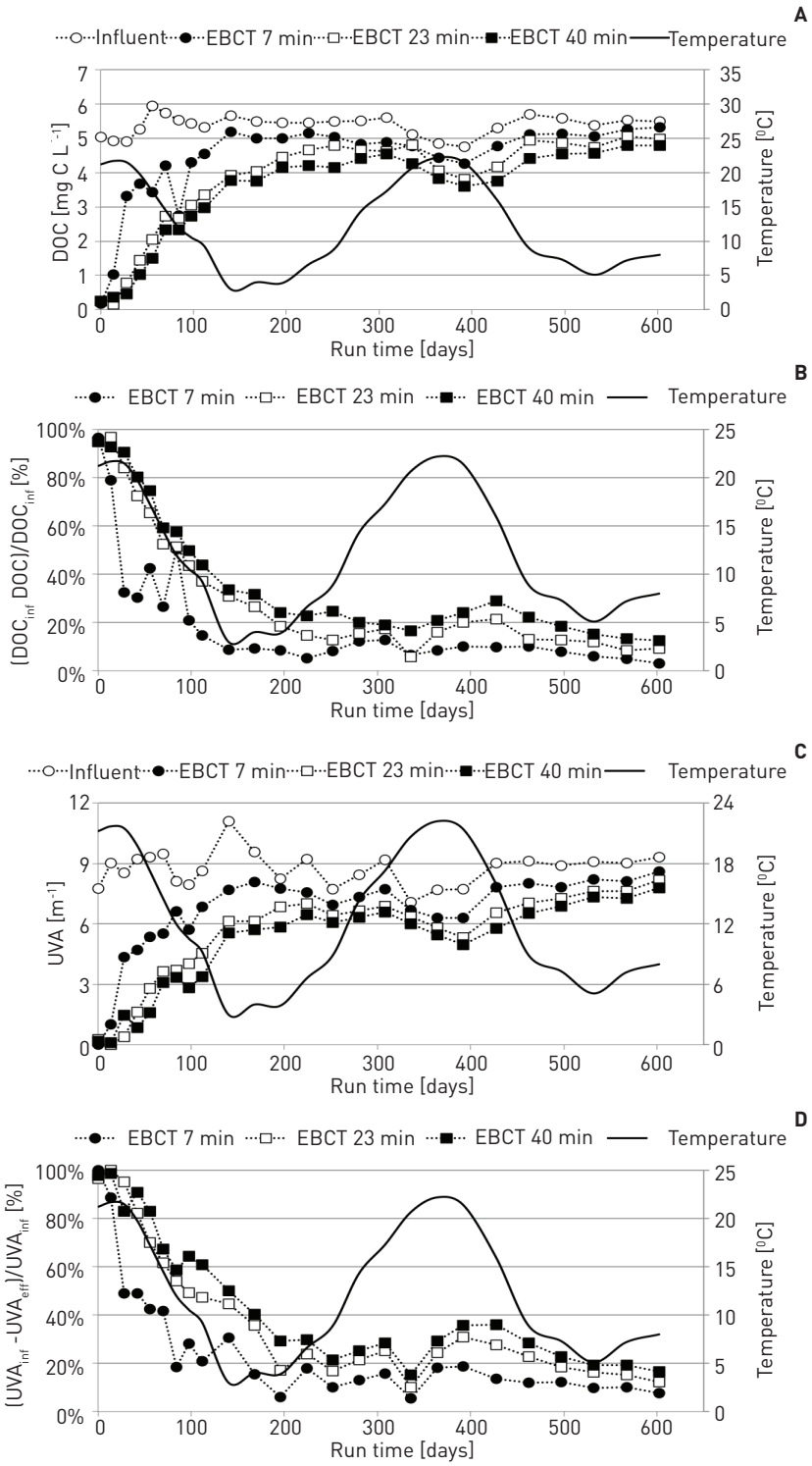

Figure 6.1 Concentration and the percentual removal of DOC (A,B) and UVA (C, D) in relation to the operational period and water temperature. EBCT, empty bed contact time.

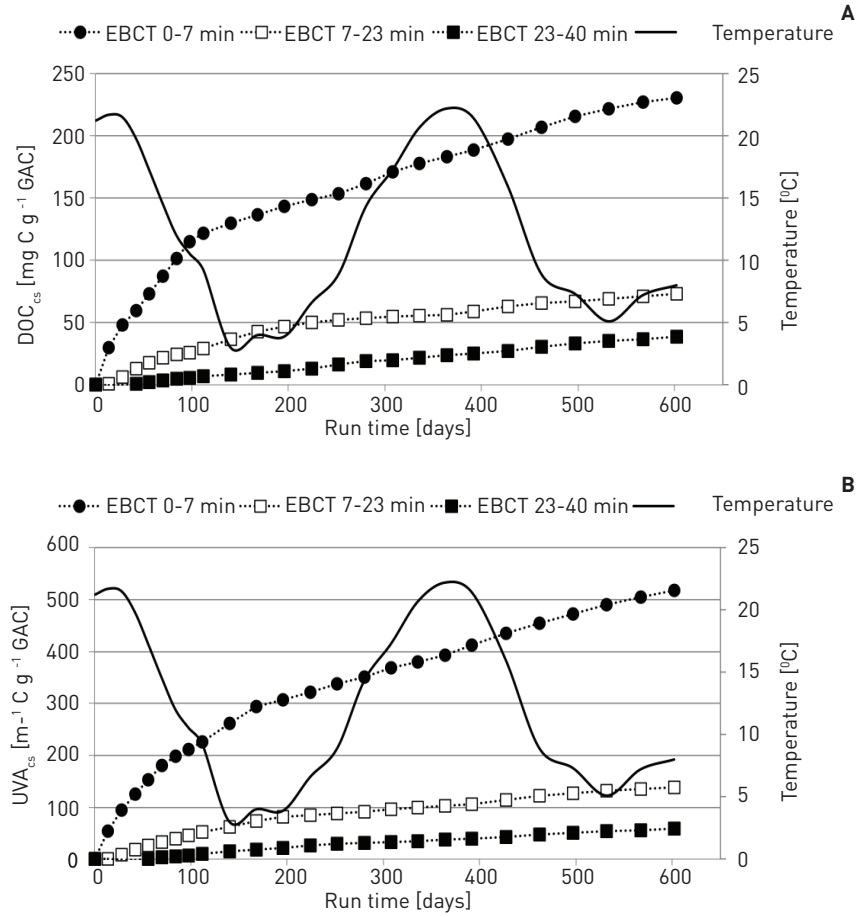

Figure 6.2 The cumulative specific DOC removal (DOC_{CS}) (A) and the cumulative specific UVA removal (UVA_{CS}) (B) in the top ($EBCT_{0-7}$ min), middle ($EBCT_{7-23}$ min) and the bottom section ($EBCT_{23-40}$ min) of the filterbed.

The NOM removal percentages reported for (pseudo) steady-state GACFs vary from 10 to 45% (Table 2, Supplemental Material). Relatively high values in some studies most likely are affected by the contribution of adsorption to the removal of NOM at a short running time and/or a relatively high percentage of biodegradable compounds in the influent. Investigations with filter beds of GAC operated in parallel with inert materials clearly demonstrated a higher level of biological activity (e.g. oxygen consumption, number of bacteria) and a better NOM removal in GACFs (van der Kooij 1983; Nijishima et al. 1992; Wang et al. 1995; Carlson and Amy 1998; LeChevallier et al. 1992; Uhl 2000). Obviously, certain GAC properties enhance microbial activity thus affecting NOM removal by biodegradation.

6.3.2 Biomass accumulation

The concentration of bacterial biomass on GAC (B_{GAC}) increased immediately after the start (Fig. 6.3). The ATP concentration in the influent ranged from 5-72 ng L^{-1} and was slightly lower in the effluent (2 – 56 ng L^{-1}; Table 6.1). From these data an average rate of ATP retention in the GACF of 0.0013 ± 0.0011 ng g^{-1} GAC d^{-1} was calculated. The bacterial biomass ac-

cumulation rate (BAR), calculated from the increase of B_{GAC} was 1.6 ± 0.3 ng ATP g^{-1} GAC d^{-1} at $EBCT_7$ and 1.1 ± 0.3 ng ATP g^{-1} GAC d^{-1} at $EBCT_{23}$. After reaching a maximal value (530 ng ATP g^{-1} GAC at $EBCT_7$ and 360 ng ATP g^{-1} GAC at $EBCT_{23}$), B_{GAC} started to decline at a water temperature of 15 °C. When temperature declined B_{GAC} increased again, but at a lower rate than in the phase I (1.1 ng ATPg^{-1} GAC d^{-1} in the top section and 0.4 ng ATP g^{-1} GAC d^{-1} in the middle section) (Fig. 6.3). The BAR/B_{GAC} ratio, which represents the net specific growth rate, declined from 0.016 d^{-1} (at 100 ng ATP g^{-1} GAC and BAR =1.6 ± 0.3 ng ATP g^{-1} GAC d^{-1}) to 0.003 d^{-1} at the maximal B_{GAC} concentration of 530 ng ATP g^{-1} GAC. Hence, the growth rate on GAC is very low.

The maximal B_{GAC} concentration in the GACF is within the range of concentrations observed in full-scale GAC filterbeds in the Netherlands (Magic-Knezev and van der Kooij 2004) and is similar to values reported by Velten et al. (2007). Conversion of B_{GAC} to biomass carbon (C = 250 x ATP; Karl 1980) yields concentrations of 11 μg C cm^{-3} GAC at 100 ng ATP g^{-1} GAC to 58 μg C cm^{-3} GAC, at 530 ng ATP g^{-1} GAC, respectively. The maximal values are similar to the maximal adsorption capacity of GAC for bacteria (40 μg C cm^{-3}, Billen et al. 1992). The values in our study exceeded the biomass concentrations (< 10 μg C cm^{-3}) reported by Servais et al. (1991) for GAC at a full-scale water treatment plant, which may be explained by an underestimation of the biomass concentration on GAC by the $^{14}CO_2$ production from radioactively labeled glucose caused by the poor glucose affinity of the bacteria predominating in GACFs (Magic-Knezev and van der Kooij 2006).

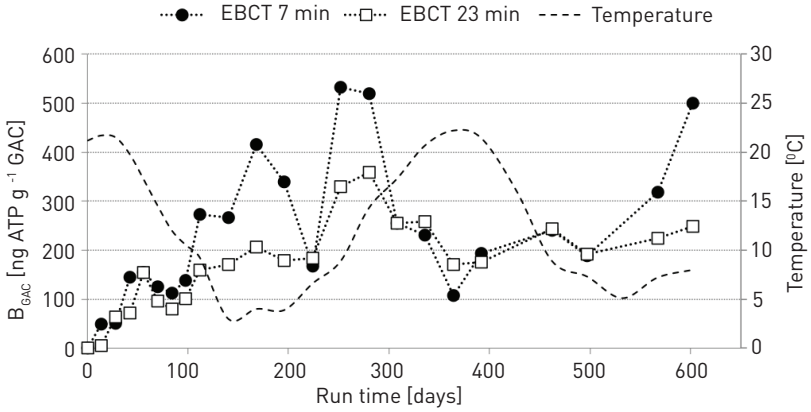

Figure 6.3 Concentration of ATP on GAC (B_{GAC}) in the top ($EBCT_7$ min) and middle section ($EBCT_{23}$ min).

The BAR values converted to the surface area of the diffusion layer surrounding the GAC particles (13 – 19 pg ATP cm^{-2} d^{-1}) are in the range of the biofilm formation rates (BFR) observed in drinking water in the Netherlands (1-100 pg ATP cm^{-2} d^{-1}, median 7.4 pg ATP cm^{-2} d^{-1}) (van der Kooij and Veenendaal 2014). Dosage of 10 μg of acetate-C L^{-1} to a biofilm monitor gave a BFR of 360 pg ATP cm^{-2} day^{-1} (van der Kooij et al. 1995). The low BAR values in the GAC column at influent AOC concentrations > 10 μg of acetate-C eq. L^{-1} (Table 6.1) therefore indicate that compounds contributing to the AOC in the influent are less readily available than acetate. Furthermore, from the average AOC removal rates (3.4 and 10.4 μg C g^{-1} GAC d^{-1}) and the growth

yield of *Polaromonas* on acetate (0.8 ng ATP μg^{-1} C; Magic-Knezev and van der Kooij 2006), it can be derived that biological AOC removal should result in BAR values on GAC exceeding 40 – 122 pg ATP $cm^{-2} d^{-1}$. The lower BAR values observed on GAC (13 – 19 pg ATP $cm^{-2} d^{-1}$) may be attributed to : (i) an adsorption-dominated AOC removal and/or (ii) a low yield of bacterial biomass formation due to substrate uptake for maintenance requirements at the low specific growth rate (< 0.016 d^{-1}).

We observed a substantial decrease of the B_{GAC} at temperatures above 15°C (Fig. 6.3), however, the oxygen utilization rate increased with increasing temperature (Fig. 6.4A). A season-related presence and activity of protozoa and invertebrates in biological filters, including GACFs, has been observed in many studies (Husmann 1982; van der Kooij 1983 ; Schreiber et al. 1997; Wang et al. 2014). The B_{GAC} decrease in association with an increase of the oxygen utilization rate can therefore be explained by an increased consumption of bacterial biomass by protozoa and invertebrates at elevated temperatures. The increase of the endogenous decay rate with temperature (Tijhuis et al. 1993) may also lead to a B_{GAC} decrease.

6.3.3 Oxygen uptake

Biological activity in GACFs is associated with oxygen uptake for the conversion of biodegradable compounds into energy and biomass. After a high initial specific oxygen removal (O_{RS}, Fig. 6.4) at a low B_{GAC} concentration (Fig. 6.3), that may have been caused by adsorption or chemisorption of oxygen onto GAC (Probes et al. 1975; Philips et al. 1998), the O_{RS} declined to < 0.2 mg O_2 g^{-1} GAC. After approx. 100 days O_{RS} started to increase, reached a maximal level of about 1.3 mg O_2 g^{-1} GAC at $EBCT_7$ at 15 °C and decreased after 400 days when temperature declined (Fig. 6.4). The O_{RS} in the middle and bottom sections were similar and lower than in the top section. From the average NOM composition (60% C, 5% H and 32% O; Malcolm and MacCarthy 1986), it can be derived that 2.8 mg O_2 mg^{-1} C is needed for oxidation to CO_2 and H_2O. The $dO_2/dDOC$ ratio at $EBCT_{40}$ exceeded the level of 2.8 mg mg^{-1} after an operational period of 100 days and also at $EBCT_7$ and $EBCT_{23}$ at a water temperature > 10°C (Fig. 6.5A). A maximal level of 12 mg mg^{-1} coincided with the maximal water temperature (Fig. 6.5A). Both physicochemical and biological processes may have contributed to the excess level of oxygen utilization ($dO_2/dDOC$ > 2.8). The physicochemical processes include (i) adsorption and/or chemisorption of oxygen and (ii) oxygen utilization by polymerization of phenolic NOM components (Vidic and Suidan 1991), but quantitative data about oxygen uptake by these processes are not available. Biological processes leading to excess oxygen utilization are (i) the activity of predating organisms (protozoa, invertebrates) in the GACF resulting in the turnover of biomass and/or (ii) utilization of previously adsorbed NOM. Predating organisms consume living and dead biomass (detritus), which implies that also particulate organic carbon (POC) accumulating in the filter bed can affect oxygen consumption. Backwashing of GAC filterbeds is applied to limit the release of organisms into the treated water (Schreiber et al. 1997, Wang et al. 2014). The loss of bacterial biomass by backwashing, with a mild regime necessary to prevent GAC loss, most likely is limited because bacteria are strongly attached to the surface of GAC (Camper et al. 1985, Magic-Knezev and van der Kooij 2004). However, data about the total amount of predating organisms and the oxygen consumption by these organism are not available.

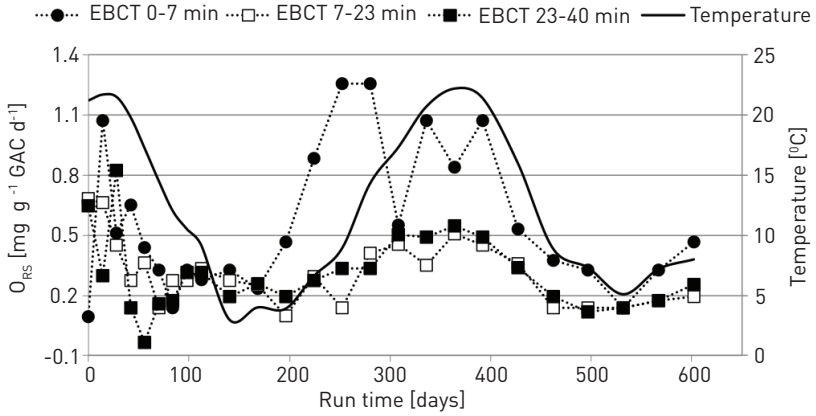

Figure 6.4 Specific oxygen consumption rate(O_{RS}) in the top section ($EBCT_{0-7}$ min), middle section ($EBCT_{7-23}$ min) and bottom section ($EBCT_{23-40}$ min) of the filter bed.

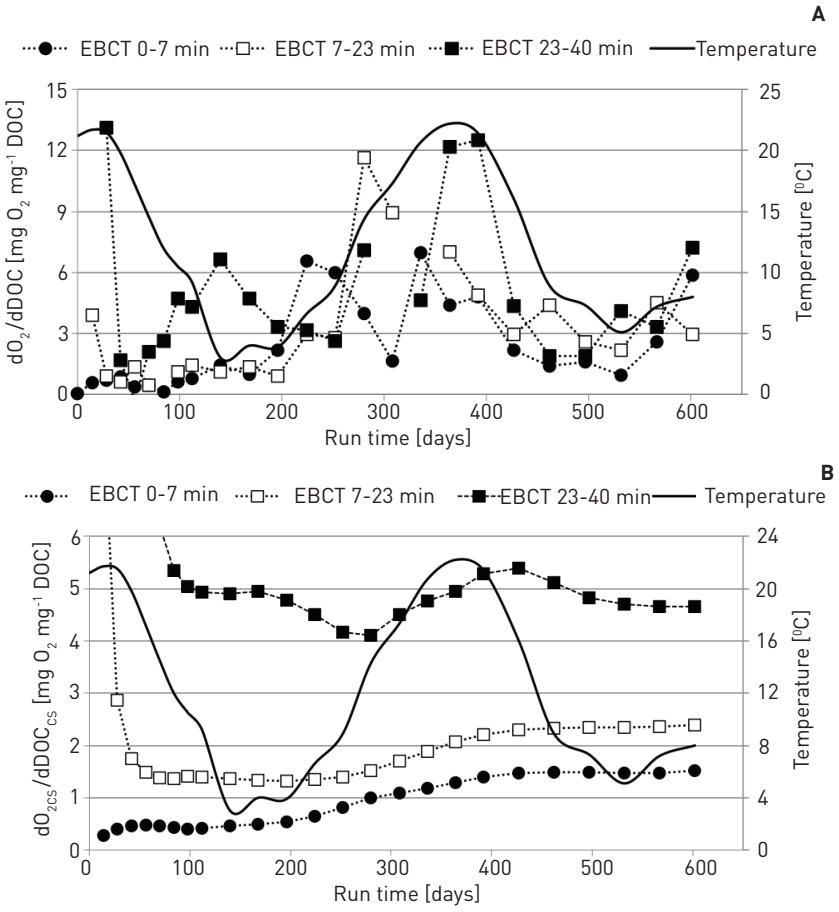

Figure 6.5 Ratio of the oxygen consumption (dO_2) and DOC removal (dDOC) (A) and the ratio of the cumulative specific oxygen consumption (O_{CS}) and the cumulative specific DOC removal (DOC_{CS}) (B) in the top ($EBCT_{0-7}$ min), middle ($EBCT_{7-23}$ min) and bottom section ($EBCT_{23-40}$ min) of the filter bed.

The POC concentration (difference between the concentration of TOC and DOC) in the GAC influent averaged about 0.2 mg L^{-1} (Supplemental material). After 250 days, a POC removal of 0.2 mg L^{-1} at $EBCT_7$ would result in an accumulation of c. 9 mg C g^{-1} GAC at a cumulative DOC removal of c. 150 mg C g^{-1} GAC (Fig. 6.2A) and an O_{RS} increasing to 1.3 mg O_2 g^{-1} GAC d^{-1} (Fig. 6.4). The O_{RS} is relatively low as compared to the amount of accumulated DOC (0.009 mg O_2 mg^{-1} C d^{-1}) and POC (0.14 mg O_2 mg^{-1} C d^{-1}). Accumulated POC may constitute a larger amount of biodegradable compounds available to (micro)organisms than the adsorbed DOC, thereby contributing signifinantly to the O_{RS} increase at increasing temperature. Data indicate (Supplemental material) that GACFs contain similar POC concentration in the effluent. However, the POC in the influent may be represented by adifferent type of carbon than the POC in the effluent. It is wel known that GACFs release GAC fines (Camper et al. 1986). Hence, qualitative POC data for a mass balance calculation are not available.

The ratio of the cumulative specific removal of oxygen and DOC (O_{CS}/DOC_{CS}), in the top and middle section of the filter bed increased in time and reached maximal levels after about 400 days (Fig. 6.5B). The maximal ratio of c. 1.5 mg mg^{-1} at $EBCT_{0-7}$ corresponds with c. 50 % oxidation of the removed DOC to CO_2 and H_2O and the level of 2.5 mg mg^{-1} at $EBCT_{7-23}$ complete oxidation. These O_{CS}/DOC_{CS} ratios may have been affected by, the accumulation of POC, some variation in the oxidative state of NOM and/or O_2 utilization by physicochemical processes, e.g. the oxidation of adsorbed phenolic NOM components (Vidic and Suidan 1991) and/or uptake of oxygen by GAC (Prober et al. 1975; Philips et al. 1998). The O_{CS}/DOC_{CS} ratio was highest in the bottom section of the filter bed and stabilized after the initial period to values between 4.1 and 5.4 mg O_2 mg^{-1} DOC. However, the similarity of the O_{RS} in the $EBCT_{7-23}$ and the EBCT23-40 sections (Fig. 6.4) indicate that the accumulated DOC and/or POC did not affect the oxygen comsumption in these sections. Therefore, the O_{RS} ranging from approx. 0.2 to 0.5 mg O_2 g^{-1} CAC d^{-1} may have been due to physicochemical processes mentioned above. Overall, the O_{CS}/DOC_{CS} ratio cannot be used as a measure for the biodegradation of adsorbed compounds.

6.3.4 *Polaromonas* as model for bacterial activity in GACFs

The spatial and temporal stability of the bacterial community (Fig. 6. S1) is consistent with other studies showing that only a few bacterial species, mainly members of the *Betaproteobacteria*, e.g. *Polaromonas*, *Hydrogenophaga* spp., predominate in GACFs operating under different conditions, and that the presence of these bacteria is characteristic for GACFs in water treatment (Magic Knezev et al. 2009; Niemi et al. 2009; Chapter 4). *Polaromonas* spp. are specialized in the utilization of carboxylic and aromatic acids with a growth yield (Y_G) for acetate and benzoate of 0.8 and 1.0 ng ATP μg^{-1} C, respectively, and a maximal specific growth rate (μ_{max}) at 15°C of 1.52 d^{-1} for acetate and 1.78 d^{-1} for benzoate (Magic-Knezev and van der Kooij 2006). From these values, the maximal specific utilization rate ($q_{max} = \mu_{max}/Y_G$) of acetate and benzoate at 15°C can be derived, viz. 1.9 μg C ng^{-1} ATP d^{-1} and 2.2 μg C ng^{-1} ATP d^{-1}, respectively. The apparent growth yield of the bacteria in the GAC filterbed (BAR/DOC_{RS}) of 0.7-34 ng ATP mg^{-1} C is only 0.1 - 4% of the yield of the *Polaromonas* strains on acetate (1 ng ATP μg^{-1} C) and benzoate (0.8 ng ATP μg^{-1} C) in batch experiments (Magic-Knezev and van der Kooij 2006). This low yield can be explained by (i) the adsorptive removal of most DOC, (ii) substrate utilization for maintenance requirements and/or (iii) protistan grazing. The low ATP

concentration per cell in GACFs as compared to RSFs (Magic-Knezev and van der Kooij 2004) is consistent with the notion that maintenance is the activity mode of most bacteria in GACFs. At a low growth rate, the growth yield is reduced by the use of the energy source(s) for maintenance of the produced biomass. According to Pirt (1982):

$$q = \mu/Y_G + m \quad (1)$$

where q is the specific utilization rate of the energy source per unit of biomass and time, Y_G is the maximal growth yield, μ is specific growth rate (d^{-1}), and m is the maintenance energy coefficient, which can be derived from:

$$m = \mu_E/Y_G \quad (2)$$

where μ_E (d^{-1}) is the endogenous decay rate. The maintenance energy coefficient of *Polaromonas* for acetate can be obtained from equation 2 by using Y_G for growth on acetate and μ_E calculated from the minimal maintenance Gibbs energy requirement and the Gibbs energy dissipation associated with growth on a specific organic compound (Tijhuis et al. 1993). Fig. 6.6 depicts q_{max} and m of *Polaromonas* for acetate in relation to temperature, based on one doubling of μ_E at 8°C increase and reveals that m is about 30 times lower than q_{max}. The DOC_{RS}/B_{GAC} ratio, representing the apparent specific substrate utilization rate (q_A), exceeds the q_{max} of *Polaromonas* for acetate in phase I, which is consistent with the adsorptive removal of NOM. Moreover, in the top section the q_A values are lower than calculated because the average B_{GAC} will be higher, e.g. 2 times, than measured at 42 cm ($EBCT_7$). B_{GAC} for the middle section is calculated from the average of at $EBCT_7$ and $EBCT_{23}$. On the other hand, POC utilization will increase the B_{GAC}, leading to an underestimation of q_A. In phase II, q_A ranges between the val-

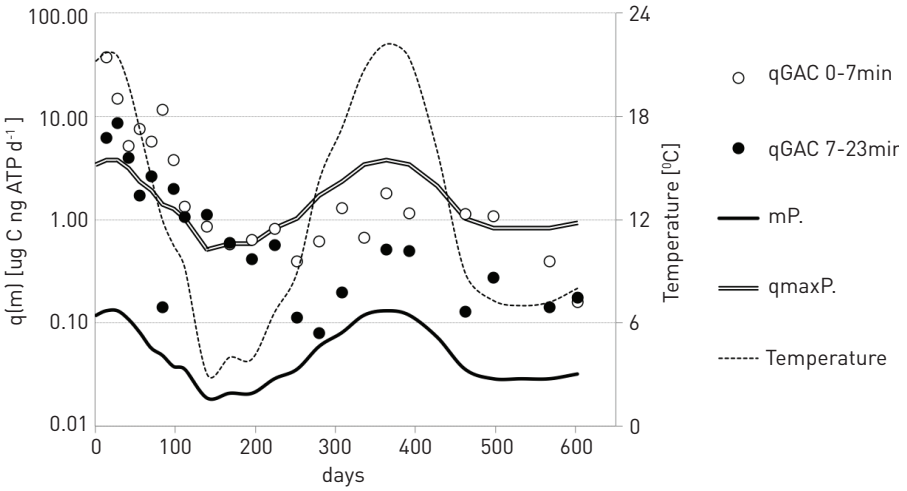

Figure 6.6 The apparent specific utilization rate q (µg DOC ng^{-1} ATP d^{-1}] in the top section ($EBCT_7$ min), and the middle section (EBCT 7-23 min/) of GAC and the temperature-adjusted maintenance coefficient of *Polaromonas* for acetate (m P.) and the maximal specific utilisation rate of *Polaromonas* for acetate (q_{max}p.) in the opertional period.

ues of m and q_{max} of *Polaromonas* for acetate. The increased q_A at the low B_{GAC} can be explained by an elevated growth rate of *Polaromonas* and other bacteria induced by protistan grazing at elevated temperatures (Pernthaler 2005). Overall, the *Polaromonas* model is qualitatively consistent with the impact of adsorption, maintenance and growth on DOC removal, but quantitative interpretation requires more detailed information about B_{GAC} levels, and biomass turnover processes in the GACF.

6.3.5 Effect of adsorptive properties of GAC on attachment and microbial activity

Attachment of bacteria, as well as the adsorption of organic compounds, onto the charged and hydrophobic GAC (graphite) surface (Bjelopavlic et al. 1999) results in a decrease of the surface free energy by the hydrophobic interactions between GAC and bacteria and organic compounds, respectively. The hydrophobic interactions may select for the attachment of microorganisms with hydrophobic properties. Consequently, it is much more difficult to remove bacteria from GAC than from sand (Magic-Knezev and van der Kooij 2004), making backwashing in GAC filterbeds less effective than in sand filters. The strong attachment (Camper et al.1985; Magic-Knezve and van der Kooij 2004; Bilen et al. 1992), the small cell size (Magic-Knezev and van der Kooij 2004) and lower accessibility of attached bacteria on the highly porous GAC surface (Wright et al. 1995) hampers protistan grazing of these bacteria. Still, the reduced B_{GAC} concentration in combination with excess oxygen utilization at elevated temperatures (Fig. 6.5A) indicates the impact of grazing by protists and invertebrates in the GACF, and implies that a significant proportion of the microbial activity is associated with organic aggregates accumulating on and between the GAC particles. As mentioned above, these organic aggregates can be the result of the accumulation of POC present in the influent, but are also generated in the filterbed by the utilization of biodegradable compounds. As the majority (>90%) of bacterial activity measured as ATP concentration in GACFs occurs on the surface of GAC particles (Magic-Knezev and van der Kooij 2004), the activity associated with organic aggregates accumulating between the GAC particles is probably related with the presence of protists and invertebrates in GACFs. The applied mild backwashing regime in GACFs may result in more accumulation of organic aggregates than in filterbeds of sand or anthracite, thus enhancing biological activity in GACFs.

Humic substances (HS), the major component of NOM, generally are considered as recalcitrant, but Amon and Benner (1996) reported that HS supported more bacterial growth in water than low molecular weight compounds. The proportion of the biodegradable organic carbon (BDOC) to the DOC concentration in aquatic environments (Servais et al. 2005) also indicates that bacteria utilize HS compounds. Camper et al. (2004) concluded that HS utilization is responsible for the enhanced microbial growth on corroding iron surfaces. Microbial activity on the organic aggregates may contribute to biodegradation of HS, but the question remains whether or not adsorption-enhanced utilization of HS occurs in GACFs. First of all, more than 99.9 % of HS adsorption will occur in pores < 50 nm (Bjelopavlic et al. 1999), which are too small for bacteria. Degradation of HS has been attributed to the activity of extracellular enzymes (Billen 1991, Billen et al. 1992), but enzymes excreted by bacteria probably will be inactivated by adsorption on GAC before reaching the adsorbed compounds. The recently developed supramolecular concept of the HS structure may provide a hypothesis for

an adsorption-enhanced utilization of NOM. This concept is based on observations that HS includes a large variety of relatively small molecular organic components (200 - 2500 Da) held together by weak chemical interactions such as hydrophobic associations, charge interactions, metal bridging and hydrogen bonds (Conte and Piccolo 1999; Sutton and Sposito 2005). Consequently, the micelle-like HS aggregates are more susceptible to structural changes than the previously assumed polymeric structure. The change in orientation of adsorbed HS as proposed by Summers and Robbert (1988) and Schreiber et al. (2005), controlled by hydrophobic interactions, may be associated with a release of low-molecular-weight biodegradable components from HS. This release depends on temperature and the concentration of adsorbed NOM. Aromatic ring structures with carboxylic and phenolic substituents appear to be major constituents of HS (Sonnenberg et al. 1988; Buuan et al. 2007). Certain aromatic compounds (e.g. benzoic, *para*-hydroxy benzoic, phthalic and vanillic acid) and (hydroxy) carboxylic acids are preferred substrates for *Polaromonas* spp. (Mattes et al. 2008), including strains isolated from GAC (Magic-Knezev and van der Kooij 2006). A strong attachment of *Polaromonas* to the GAC surface and the high substrate affinity may enable the utilization of released compounds at low concentrations (Magic-Knezev and van der Kooij 2006, Chapter 4). However, the hypothesis that adsorbed HS aggregates contribute to the enhanced biodegradation in GACFs implies that a decrease of the adsorptive DOC removal to a low percentage after passage of more than 20,000 bed volumes (Fig. 6.2; Bonne et al. 2002; Chowdhury et al. 2013) may be associated with a decrease in biodegradation.

6.4 CONCLUSIONS

1. Removal of dissolved NOM in a GACF occurred at two rates. In phase I (<20,000 BV), with adsorption as the main driving force, NOM is removed approximately 3 to 5 times faster than in phase II with slow adsorption and increased biological activity.
2. Biomass accumulation and oxygen consumption demonstrate that biodegradation plays a role in NOM removal in the GACF. However, oxygen consumption cannot be used as a quantitative measure for the biodegradation of dissolved NOM because of lack of information about POC removal and oxygen uptake by physicochemical processes on GAC surface.
3. The low specific growth rate of bacteria on GAC implies that substrate uptake for maintenance requirements is a significant mechanism in the microbial removal of biodegradable compounds.
4. The elevated specific oxygen consumption rate (O_{RS}) in the top section at water temperatures >15 °C can be attributed to the activity of predating (micro)organisms utilizing the accumulated living and dead (POC) biomass.
5. The similarity of the O_{RS} values in the middle and bottom sections at a different cumulative specific DOC removal (DOC_{CS}) indicates that oxygen consumption is not related to DOC accumulation.
6. A quantitative comparison of the apparent specific DOC utilization rate (q_A, mg C ng^{-1} ATP d^{-1}) in the GAC filterbed, with the temperature-adjusted maintenance coefficient and the specific maximal utilization rate of acetate by *Polaromonas* requires more accurate data on B_{GAC} and the contribution of POC removal to microbial activity.
7. The nature of the low molecular weight compounds that may be released by the disruption of

HS micelles is consistent with the preferred substrates of *Polaromonas* spp. predominating on GAC, but confirmation of the hypothesis requires further research.

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SUPPLEMENTAL MATERIAL TO CHAPTER 6

1 Terminal restriction fragment polymorphism (T-RFLP) Method.

T-RFLP was performed as described by Dunbar et al. (2001). In brief, the 16S rRNA gene of bacteria harvested from GAC was amplified with Platinum Taq polymerase (Hot-Start Taq polymerase, Invitrogen, Life Technology Corporation, California, USA) using 8F-FAM (5'-AGAGTTTGATC (A/C)TGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGTACA-3') primers by the iCycler IQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). The following thermal cycle was applied: 30 cycles 95°C for 45 s. 57° C for 60 s and 72 for 2 min; and a final extension at 72°C for 7 minutes. PCR products were purified (DNA Clean & Concentrator-5 Kit van Zymo Research Corporation, Orange, U.S.A) and digested with restriction enzymes HhaI (Promega Corporation, USA) at 37°C for 4-6 hours. Digestion products were purified and after addition of the internal standard (Genescan –500 ABI 401734, Life Technology Corporation, California, USA) and loading buffer (Hi-Di formamide) DNA was denaturized during 3 minutes at 95 °C and transferred directly to ice for minimum of 13 minutes. Electrophoresis was performed in the short capillary/POP polymer in the Run module "GS STR pop 41 ml D 28 min" on an ABI 310 automated DNA Sequencer (AME Bioscience A/S., Toroed, Norway) with extended running time to 40 minutes. T-RFLP was generated for filter GAC1 during a period of one year for the layers of 7 min EBCT and 23 min EBCT. The T-RFLP fingerprints were compared using Pearson correlation. A dendrogram was derived by the unweight pair-group method using arithmetic averages (Bionumeric software version 3.0. Applied Maths NV, Sint-Martens-Latem, Belgium).

Results

The T-RFLP fingerprints, representing the bacterial community on GAC, grouped in two major clusters (Fig. 6.1) which showed an increase in diversity. Cluster A comprised the communities during the first two months (72 – 2044 BV) of operation and cluster B comprised the communities during the next 10 months. The fingerprints from the top and middle section of the filter bed in cluster B were highly similar (> 80 %) and randomly distributed.

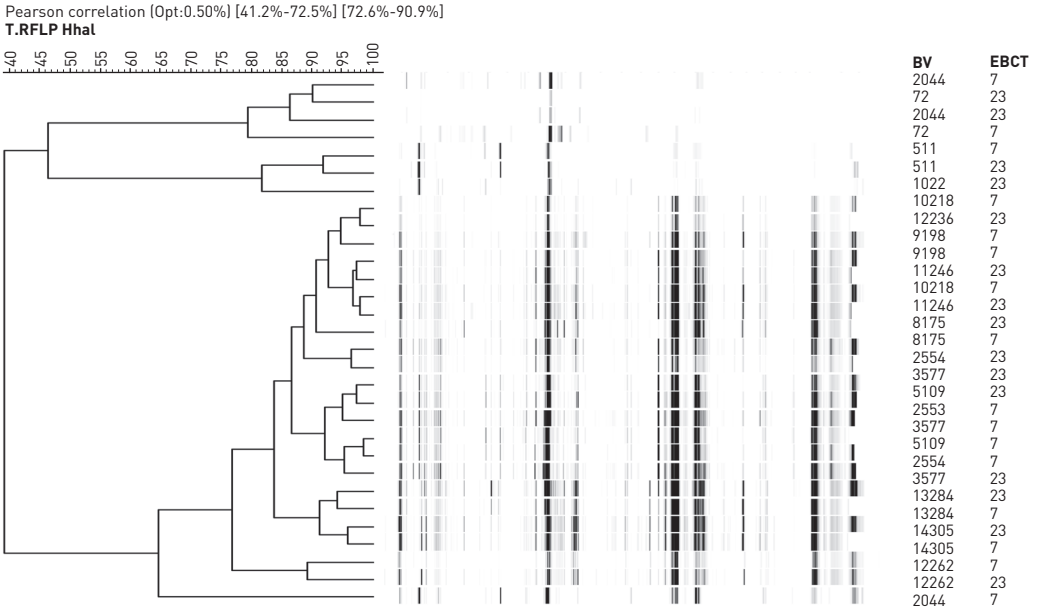


Figure 6.S1 Dendrogram for the T-RFLP patterns of bacterial community in a pilot GACF in the period of one year, at different bed volumes (BV) in the top (EBCT 7) and middle layer (EBCT 23) of the filter. The T-RFLP fingerprints were compared with unweighted pair-group method using arithmetic averages (Bionumeric software version 3.0. Applied Maths NV. Sint-Martens-Latem. Belgium).

2 Specific surface of GAC

The surface area of the diffusion layer (A_{diff}) was calculated by assuming spherical-shaped GAC particles. The surface (A_p) and volume (V_p) of a single particle of the radius r= 0,05 cm was calculated as follow:

$A_p = 4\pi r^2$
$V_p = 4/3\ r^3\ \pi$

For a single spherical GAC particle of the radius r= 0,05 cm

$A_p = 3.14\ 10^{-6}\ m^2$
$V_p = 5.2\ 10^{-10}\ m^3$

Total number of particles (N) with radius r = 0.005 cm in a unit of volume of GAC with the filterbed porosity ($\varepsilon_p = 0.39$) was calculated as:
$N = (1- \varepsilon_p)/ V_p$

Number of particles in $1m^3$ of GAC is $N= 1.2\ 10^9$
Surface area of the diffusion layer (A_{diff}) in one gram of GAC was calculated as follow:
$A_{diff} = N \times Ap \times \rho_m^{-1}$

Where ρ_m is density of GAC (0.432 g cm^{-3})

A_{diff} = 8.5 m^2 kg^{-1} GAC (85 cm^2 g^{-1}GAC)

GAC properties, such as effective particle size (1 mm) and bed density (0.432 g cm^{-3}) were obtained from CABOT product information sheet No. 2213 (JUN 2103) for NORIT® GAC 830.

Table 6.S1 The surface area for all pores derived from BET analysis and the surface area of micro-, meso- and macropores of GAC 830 derived from mercury intrusion analysis as provided by Cabot.

Pore radius [nm]	Pore volume [cm^3 g^{-1} GAC]	Surface area [m^2 g^{-1} GAC] (% of BET)
All pores	0.5032	924 (100)
>2 (mesopores)	0.42068	83 (9%)
2-50	0.24599	81 (9%)
>50 (macropores)	0.17469	1.7 (0.2%)
>500	0.0784	0.070 (0.008%)

3 NOM removal in biological filters

Table 6.S2 Rlative removal of NOM in the steady state phase in biological filters.

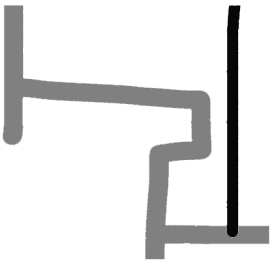
Removal	Organic carbon	Filter type	Run time	EBCT (min)	T°C	Reference
20-40%	DOC	GAC	750 days			Sontheimer and Hubele 1986
10-20%		Sand				
45%	TOC	GAC *	**		4-26	LeChevallier et al. 1992
17%		Dual media				
10%	refractory DOC	GAC	200 days	10	20	Servais et al. 1994a
30-40%	BDOC					
21-27%	TOC	GAC	1 year	9.2	13-27	Wang et al. 1995
16-20%	TOC	Sand, Anthracite				
10%	DOC	Anthracite		10	10	Carlson and Amy 1998
27%	TOC	GAC *	**	20	5-16	Uhl 2000
5-15%	DOC	Glass/ Pumice/ Sand				
10-15%	DOC	Sand	**	13-17	12-22	Fonseca et al. 2001
20-30%	DOC+O_3	Sand				
10%	DOC	GAC	4 years	20	4-20	Bonne et al. 2002
10%	TOC	GAC	2 years			Chowdhury et al. 2013

* Exhausted GAC, ** steady state (running time not reported).

4 POC in GACF

Table 6.S3 Data POC Influent and effluent GACF at the full scale treatment plant Weesperkarspel

Datum	Influent	Effluent
	[µg C/l]	
16/01/07	280	175
13/02/07	97	141
13/03/07	217	216
10/04/07	283	669
08/05/07	324	289
05/06/07	109	328
03/07/07	215	177
31/07/07	329	8
28/08/07	-60	29
25/09/07	541	164
23/10/07	-39	68
20/11/07	122	162
18/12/07	108	135
Average	194.34	196.95
St. dev	45.29	46.66
n	13	13



CHAPTER 7

GENERAL DISCUSSION

7.1 STUDY OBJECTIVES

The investigations described in the previous chapters were carried out to analyse the microbiological processes in relation to the GAC characteristics and the removal of natural organic matter (NOM) in Granular Activated Carbon filters (GACFs) in water treatment. The main goal of the study was to obtain a qualitative description of the interactions between microbial activity and the surface characteristics of GAC. For this purpose the concentration of active bacterial biomass in GACFs, the identity and properties of the predominating bacteria and the effects of the adsorptive properties of GAC on biodegradation were studied. These issues were addressed by using ATP as a parameter for the concentration of active bacterial biomass. Classical cultivation-based and molecular methods were used to quantify and characterize the microbial communities in various full-scale filters at six surface-water treatment plants, three groundwater-treatment plants and two pilot plants in the Netherlands. The selected water supplies varied in type and concentration of NOM in the raw water, pre-treatment and the operational conditions of the GACFs. Furthermore, the growth kinetics of two *Polaromonas* strains, the predominating genus in GACFs, was studied in batch experiments. The pilot plant studies, at a groundwater-treatment plant and at a surface-water treatment plant, were carried out to assess the microbial activity in GACFs under controlled conditions. In this discussion, the results described in the previous chapters are further analysed, in combination with data available in the literature, with focus on:

1. The effect of GAC surface characteristics on the microbial activity and biodegradation in GACFs.
2. The mechanisms behind the enhanced performance of GACFs.
3. The significance of insights obtained in this study for drinking water treatment practice.

7.2 CHARACTERISTICS OF GAC FILTRATION IN WATER TREATMENT

7.2.1 Comparison of GACFs with rapid sand filters (RSFs)

The removal of biodegradable compounds in biological filtration processes is affected by (i) contact time, (ii) nature of biomass carrier (specific surface area), (iii) concentration and nature of biodegradable compounds in the influent, and (iv) temperature. In the Netherlands, contact times of biological processes applied in water treatment range from several months to several years (dune passage and river bank filtration), several hours (SSF), and less than one hour (RSF, GACF). GACFs and SSFs generally are operated at the end of the treatment train, whereas RSFs are mainly used for the removal of particles, from coagulation /sedimentation processes in pre-treatment of surface-water or from the oxidation of iron and manganese in groundwater treatment. Consequently, RSFs are backwashed more frequently than GACFs (see also Chapter 1). Typical operational characteristic of the different biological filtration processes applied in water treatment in the Netherlands are presented in Table 7.1. Many studies have shown that GACFs support more biological activity than filter beds of inert media (see Chapter 1 and Chapter 6). Given the similarities in process conditions in GACFs and RSFs, or sand-anthracite (dual-media) filters, a comparison of GAC filtration

Table 7.1 Operational conditions and performance indicators for investigated full-scale (FS) GACFs, RSFs and SSFs (Chapter 2) and for the pilot plant GACF, respectively (Chapter 6). HLR, hydraulic loading rate ($m^3m^{-2}h^{-1}$); OLR, organic loading rate ($g\ m^{-2}h^{-1}$); EBCT, empty bed contact time; D = diameter of filter material; ATP, concentration of bacteria in filter bed ($ng\ cm^{-3}$ filter material); dDOC and dO_2 are the relative removal of DOC and oxygen, respectively, related to the influent concentration; n, number of filters investigated.

Filter type		HLR	OLR	EBCT	D	ATP	dDOC	dO_2
		[$m^3m^{-2}h^{-1}$]	[$gm^{-2}h^{-1}$]	[min]	[mm]	[$\mu g\ m^{-3}$]	%	%
GACF FS	Min	2.7	6.2	10	0.8	24	13*	4*
(n=30)	Max	26	55	45	1.7	5067	58*	42*
	P50	8	26.5	16	1.0	326	30*	20*
RSF(DMF) FS	Min	3.5	7.4	5	0.7	16	0	1
(n=9)	Max	11	23	25	3.5	2592	47	33
	P50	5	8	12	1.8	319	15	13
GACF PP**	Min	1.6	9.6	35	0.8#	110	13	14
(n=1)	Max				1.7	532	29	80
	P50				1.0	267	20	32
SSF FS	Min	0.2	0.5	30	0.3	18	n.a.	<1
(n=3)	Max	0.5	3	240	1.2	93	n.a	<1
	P50				0.7		n.a	<1

* Calculated from values in mixed effluent of full-scale GAC filters; ** calculated from data of one pilot plant GAC in the pseudo-steady state" (Chapter 6); n.a., data not available. # values for the material in one GACF.

with RS filtration is relevant for analysing the effects of specific GAC characteristics on the biological activity.

The contact time and loading rates in GACFs and RFSs are in the same range, but at some locations GACFs are operated at a higher loading rate and/or a longer contact time than RSFs. The ranges of the bacterial biomass concentrations (ATP) in GACFs and RSFs do not differ from each other ($P = 0.158$; Chapter 2). The similarity in size of the filter material provides a similar surface of the diffusion layer in both types of filters, 21 – 52 $cm^2\ cm^{-3}$ at D values between 1.8 and 0.7 mm, respectively. However, the highly porous structure of the GAC particles provides a larger surface area (e.g. c. 300 $cm^2\ cm^{-3}$ in Norit 830; Table 7.2) for attachment of bacteria (pores $D > 1000$ nm) and a total intra-particle area available for adsorption of organic contaminants and NOM (c. 400 $m^2\ cm^{-3}$)

Table 7.2 The area for surface diffusion of a GAC or a sand particle with a diameter of 1 mm, the surface area of all intraparticle pores in GAC 830 derived from BET analysis and the surface area of micro-, meso- and macropores of GAC 830 derived from mercury intrusion analysis as provided by Cabot. *Sheet No. 2213 (JUN 2103) for NORIT® GAC 830.*

Pore radius [nm]	Pore volume [cm^3 cm^{-3} GAC]	Surface area [m^2 cm^{-3} GAC] (% of BET)
All pores	0.2174	400 (100)
>2 (mesopores)	0.1817	36 (9)
2-50	0.1063	35 (9)
>50 (macropores)	0.0755	0.7 (0.2)
>500 (accessible for bacteria)	0.0339	0.030 (0.008)
Surface diffusion layer	n.a.	0.0037 (0.0009)

n.a. not applicable.

The comparison of the efficiency of NOM removal by biological activity in GACFs and in filters with inert materials is complicated by the adsorptive removal in GACFs. Furthermore the NOM removal in GACFs and in RSFs is affected by the position of these filters in water treatment. For example, the application of ozonation in pre-treatment increases the concentration of easily biodegradable organic compounds (e.g. van der Kooij and Hijnen 1984), thereby increasing the contribution of biological activity to the DOC removal by GAC filtration (Sontheimer and Hubele 1987).

The adsorptive removal rate in GACFs generally decreases after approximately 10,000 to 20,000 BVs (Chapter 6; Peel and Benedek 1983), and the slow removal rate phase has been defined as *"(pseudo) steady state"* phase. In this phase the removal is relatively stable (Chapter 6, Fig. 1B,D) and it has been suggested that in this phase, NOM is primarily removed by biodegradation (Servais et al. 1994, Wang et al. 1995, Velten et al. 2011). However, Peel and Benedek (1980, 1983) demonstrated that in this phase the slow adsorption is the consequence of the slow diffusion of low-molecular-weight compounds in the micropores.

Although the continiung NOM removal in GACFs in the steady state phase has been attributed to slow adsorption, research consistently provided evidence for an enhanced microbial activity in GACFs (Eberhardt 1975; Sontheimer et al. 1978; van der Kooij 1983; Chudyk and Snoeyink 1984; Nishijima et al. 1992, Servais et al. 1994, Wang et al. 1995, Jones et al. 1998; Uhl 2000; Klimenko et al. 2004; Aktas and Cecen 2007). Factors enhancing microbial activity and the consequences of microbial activity in GACFs will be analysed in the next paragraph.

7.3 FACTORS ENHANCING THE MICROBIAL ACTIVITY IN GAC FILTER BEDS

7.3.1 Operational conditions and GAC characteristics

In GAC filters (GACFs) organic compounds are removed by adsorption and biodegradation and many studies have shown that microbial activity in GAC filter bed is enhanced as compared to filtration by filter beds of sand or anthracite. However, the question is: does adsorption of organic compounds enhance microbial activity, or is the enhanced microbial activity in GACFs caused by certain conditions in the GACF?. The operational conditions (HLR, OLR) of GACFs resemble those of RSFs, whereas SSFs are operated at a much lower loading rates and a much longer contact time (several hours, e.g. 240 minutes) (Table 7.1). The flow velocity in GACFs and RSF fall in the same range and also is not a rate-limiting factor in biological processes (Huck et al. 2013). However, frequent backwashing of RSFs is applied to prevent filter bed clogging. Backwashing of GACFs is applied less frequently, as the pressure drop occurs less quickly than in RSFs. Furthermore, GACFs are backwashed less vigorously than RSFs because of the lower specific weight of GAC (0.4 – 0.5 g cm^{-3}) as compared to sand (2.6 g cm^{-3}) and anthracite (1.5 -1.8 g cm^{-3}). Hence, the enhanced microbial activity in GACFs may be related to a less efficient backwashing and/or specific GAC characteristics. Huck and Sozansky (2008) and Uhl (2000) showed that contact time and the specific surface area are the most important factors affecting the NOM removal in biological filters. As contact times in RSFs and GACFs are within the same range (Table 7.1), the focus will be on the nature and size of surface of the filter material.

GAC differs from sand, and also from anthracite, by the surface geometry affecting the surface area per unit of bed volume available for bacterial attachment and its physicochemical properties. The grain size considerably affects the specific surface area in a biofilter, but the roughness (porosity) of the material also plays an important role in this respect. The external surface for diffusion of organic compounds, oxygen and inorganic nutrients to the filter bed material can be derived by assuming a spherical shape of the grains and is estimated at c. 35 cm^2 cm^{-3} for particles of 1 mm diameter and lower for particles with a larger diameter, typical for sand and anthracite in RSFs (Table 7.1). The intraparticle porosity of GAC (0.2174 cm^3 cm^{-3}, Table 7.2) is much higher than the negligible intraparticle porosity of quartz-based sand (Brusseau 1993). The high intraparticle porosity of GAC is associated with a large specific surface area (c. 400 m^2 cm^{-3}) as derived from nitrogen adsorption, and c. 36 m^2 cm^{-3} for the surface of the pores with a diameter > 4 nm (Table 7.2). The specific surface of the pores with a diameter > 1 μm, which are accessible for bacteria, represents only 0.0302 m^2 cm^{-3} (c. 300 cm^2 cm^{-3}), which is <0.1% of the surface in the pores > 4 nm diameter. Therefore, GAC provides a 10 times larger area for attachment of bacteria as compared to sand or anthracite with the same size (c. 35 cm^2 cm^{-3}). Furthermore, the pores < 10 μm may provide protection to grazing protozoa (Wright et al. 1995) and invertebrates and to shear forces during backwashing.

The surface chemistry of GAC also differs from the surface chemistry of sand. The graphite-based GAC particles have a hydrophobic surface, whereas the surface of the silicate-based sand particles is negatively charged. In both materials, metal ions and functional groups containing

oxygen are present at the surface, increasing its polarity and charge. Although the majority of the GAC surface is hydrophobic, certain parts of the GAC surface are charged due to the presence of surface oxides, metals and functional groups. The charge of the GAC surface, which depends on the ionic strength and pH, will be negative for most GAC applications in drinking water treatment (Prober et al. 1975, Sontheimer et. al., 1988; Snoeyink and Summers 1999; Ypsalsky et al. 2009). Attachment to a hydrophobic surface results in a decrease of the surface-free energy (van Loosdrecht et al. 1989, Bos et al. 1999), and the hydrophobic nature of GAC facilitates the adsorption of hydrophobic components. The surface charge facilitates interactions with polar components, enhancing thereby the adsorption of NOM and the bacterial attachment and making the GAC more favourable for bacterial attachment than sand. The initially reversible attachment is transformed to irreversible attachment by the hydrophobic and electrostatic interactions between the bacteria and the GAC surface.

7.3.2 Effect of GAC surface properties on microbial activity and community composition

The total direct cell counts (TDC) of 30 GACFs in 9 full-scale treatment plants ranged from 1 x 10^9 cells cm^{-3} to 4 x 10^{10} cells cm^{-3} (Chapter 2, Table 2.2). With a surface area of c. 300 cm^2 cm^{-3} accessible to bacteria, these concentrations range from 3 x 10^6 to 1.3 x 10^8 cells cm^{-2}, indicating that the average surface coverage ranged from less than 1.5% to c. 65%, when assuming complete coverage at 2 x 10^8 cells cm^{-2} (for cells of 0.5 x 1.0 μm). These percentages may be lower because bacteria may be smaller than 0.5 x 1.0 μm and are also present on on particulate organic carbon (POC) in the spaces between the GAC particles (Chapter 2, Camper et al. 1986). The TDCs on sand in rapid sand filters ranged from 2 x 10^7 to 2 x 10^{10} cells cm^{-3}, corresponding with 5.7 x 10^5 to 5.7 x 10^8 cells cm^{-2} at a specific surface area of c. 35 cm^2 cm^{-3}. These data suggest that the concentration of attached bacteria on GACF and RSF does not clearly differ from each other. However, the interpretation of these data is complicated by the effect of the operational conditions of the involved GACFs and RSFs, e.g. the GACFs are positioned at the end of the treatment train, whereas RSFs are part of pre-treatment. Furthermore, at a high concentration, when backwashing is required, the surface coverage of sand exceeds 100% implying that many bacteria are not directly attached to the surface, but associated with other bacteria, making backwashing more effective.

TDC concentrations reported for drinking water exposed surfaces in model distribution systems ranged from about 4 x 10^6 cells cm^{-2} to 7 x 10^6 cells cm^{-2} (Mathieu et al. 1993) and were 2.6 x 10^6 cells cm^{-2} in a system supplied with treated groundwater (AOC 6 μg C L^{-1}) (Boe-Hansen et al. 2002). In a biofilm monitor supplied with treated groundwater (DOC = 2.4 mg L^{-1}, AOC = 5 μg C L^{-1}) at a flow rate of 0.2 m s^{-1}, a TDC concentration of 10^7 cells cm^{-2} was observed (van der Kooij et al. 2003). Hence, the TDC values on sand (RSF) and GAC may attain a level that is one order of magnitude higher than the concentration on surfaces exposed to drinking water with a low growth potential. However, cells may differ in size and activity and therefore, the ATP concentration is a better parameter for comparing biofilm concentrations than TDC values. Table 2.2 in Chapter 2 shows that the ATP concentrations in GACFs and RSF ranged from about 25 ng ATP cm^{-3} to 5000 ng ATP cm^{-3} and did not differ significantly. However, the median value of the amount of ATP $cell^{-1}$ in RSFs (0.36 fg) was more than 10 times higher than in the GACFs (0.021 fg). Obviously, bacterial cells in GACFs are smaller and/or less active

than those in RSFs. This difference can be attributed to a difference in growth rate, viz. slow growth in GACFs (net specific growth rate < 0.016 d^{-1}, derived from the bacterial biomass accumulation rate (Chapter 6) and rapid growth in RSFs due to weak attachment in combination with the effect of the frequent backwashing of the filter beds. The observations on the rate of growth on GAC also confirm earlier reports on slow microbial growth in GACFs (Bancroft et al. 1983) and are consistent with calculations of the growth rate in biofilms in model distribution systems (Mathieu et al. 1993; Boe Hansen et al. 2002). Furthermore, the suggested relationship between growth rate and ATP level $cell^{-1}$ is consistent with recently published observations demonstrating an ATP level < 0.1 fg ATP $cell^{-1}$ in bacteria in biofilms at a low growth rate, whereas levels of 0.2 to 0.5 fg ATP $cell^{-1}$ were observed in bacteria in rapidly growing biofilms (Sack et al. 2014). Hence, microbial growth in GACFs is characterized by the presence of small cells, a limited surface coverage and slow growth, indicating that growth is limited by the supply of biodegradable compounds.

The impact of the GAC surface on bacterial attachment is clearly demonstrated by the intensity and duration of ultrasonic treatment required for the removal (detachment) of bacterial biomass from GAC (240 Watt) as compared to sand (80-120 Watt) (Chapter 2). Billen et al. (1992) also showed an increased attachment rate (2 times) and a decrease detachment rate (5 times) with GAC as compared to sand. The high similarity between GAC communities from different locations (Chapter 4) suggests that the surface characteristics of GAC have a strong effect on the bacterial community composition. The temporal stability of the bacterial community composition in a pilot plant pilot GACF (Chapter 4, 6) shows that the first colonizers of the GAC surface remain predominant throughout the operation of a filter in spite of seasonal changes in water quality and changes in apparent DOC concentration accumulated on GAC. Furthermore, the composition of the bacterial community on GAC differs from the composition of bacterial communities in other fresh-water environments, e.g. T-RFLP patterns of GACFs and RFSs from same location showed less similarity than those of GAC communities from different locations (Chapter 4). Furthermore, the genus *Polaromonas* predominates in GAC filters (Chapter 3, 4) but is not frequently reported as important constituent of aquatic bacterial communities, except in bottled drinking water (Loy et al. 2005) and in a system distributing drinking water treated by ion exchange (IEX) (Liu et al. 2014). Experiments with *Polaromonas* strains isolated from GAC filters demonstrated that these bacteria are specialized in the utilization of carboxylic and aromatic compounds, whereas carbohydrates are very poorly utilized (Chapters 4, 5). Furthermore, two *Polaromonas* strains, isolated from GACFs, exhibited low half-saturation constant (K_s) values for acetate and benzoate, 0.015 μM and 0.04 μM, respectively. These properties are consistent with other studies on this genus (Mattes et al. 2008) and observations of these bacteria in ultra-oligotrophic environments (Niemi et al. 2009, Loy et al. 2005, Liu et al. 2014). The specific nature of the bacterial community in GACFs in water treatment is also demonstrated by the presence of another predominating bacterial group detected with molecular methods and not by culture in the GACFs. These organisms are most closely related to members of the candidate order TRA3-20 (Edwards et al. 1999) of the *Betaproteobacteria* at the approximate genus-level (≥95% sequence similarity). Currently, no information is available about physiological characteristics of these bacteria, nor about their preferred habitat or niche because of the limited number of observations of this organism in the environment. The presence of representatives of species from the order *Rhizobiales* indi-

cates the availability of amino acids and/or NH_3 in the GACFs. Amino acids and ammonium are efficiently removed by biological processes in pre-treatment, but may be present in GACFs as a result of biomass turn-over processes. Protozoa grazing on bacteria release relatively large amounts of these compounds (Sherr and Sherr 2002). The intensity of protozoan grazing depends on the concentration of prey bacteria and water temperature (Schreiber et al. 1997, Wang et al. 2014, Pernthaler 2005).

7.3.3 Effect of adsorptive properties of GAC on the availability of growth substrates

The increasing bacterial activity in the initial phase of GAC filtration (Chapter 6, Fig. 3) indicates that sufficient substrate is available for maintenance and growth in virgin GAC filters and is consistent with other reports (Servais et al. 1991, 1994, Wang et al. 1995, Velten et al. 2007). At the beginning of the operational period, organic compounds and bacteria are simultaneously adsorbed to the GAC surface and the adsorptive processes decrease the NOM concentration in the bulk phase by more than 50% (Chapter 6). Hence, in the initial period, substrates available for growth of the attached bacteria mainly are non-adsorbable organic compounds. In this phase, adsorption of NOM on GAC may enhance transportation of biodegradable compounds to the bacteria in the GAC pores. The stability of the bacterial community composition, as described in Chapters 4 and 6, suggests that the nature of the substrates used for bacterial growth and maintenance does not change in the operational period or that the first colonizers are able to adapt to changes in the composition of the available substrate. However, the question to be answered is whether or not adsorbed organic compounds are available for bacteria.

One of the most frequently used explanations for the enhanced microbial activity in GACFs is the utilisation of adsorbed compounds (e.g. Eberhardt 1975, Sontheimer et al. 1978, Speitel et al. 1989, Nishijima et al. 1992, Jones et al. 1998, Aktas and Cecen 2007). However, this notion was and is not generally accepted (e.g. van der Kooij 1983, Peel and Benedek 1983; Sontheimer and Hubele 1987; Huck et al. 2014). Two difficulties are related to the hypothesis of an adsorption-enhanced biodegradation: (i) the majority of the adsorbed compounds is located in micropores which are not accessible to bacteria, and (ii) adsorbed compounds can only be utilized after desorption.

The molecular weight of NOM components varies between <500 - > 300,000 g mol^{-1}, with the majority (c. 90%) < 10,000 g mol^{-1} (Thurman et al. 1982; Newcombe et al. 1997; Kilduff et al. 1996; Perminova et al. 2003). The size of molecules from different molecular-weight fractions ranges between 0.5 - 3 nm (Thurman et al. 1982; Perminova et al. 2003 Newcombe et al. 1997) indicating that the majority of the NOM components is adsorbed in pores with D < 5 nm. The surface of pores accessible to bacteria (D > 500 nm), represents less than 0.08% of the total GAC surface available for the NOM adsorption. Hence, most NOM components (>> 99%) are adsorbed on surfaces not accessible to bacteria.

Bacterial uptake of compounds requires mobility of the substrate, and adsorbed substrates can only become available for bacteria after desorption. Desorption of an adsorbed compound may occur at a reversed concentration gradient or by competitive adsorption. The utilization

of adsorbed substrates has been demonstrated experimentally for single compounds (e.g. phenol) at high bulk phase concentrations (200 ppm) irrelevant for drinking water treatment and conditions creating a reverse concentration gradient (Andrews and Chi Tien 1981, Chudyk and Snoeyink 1984, Goeddertz et al. 1988, Speitel et al. 1989, Hutchinson and Robinson 1990). For humic substances (HS), which are the major fractions of organic carbon in the GACFs influents, a reverse gradient is unlikely to occur at the relatively constant NOM concentration in the feed water. However, competitive adsorption may play a role, and compounds with a high absorbability can potentially replace adsorbed compounds with a lower absorbability. Observations that smaller NOM components are better absorbed by GAC (Summers and Roberts 1986) have been confirmed by Kilduff et al. (1996) who demonstrated competition between NOM components and an adsorptive fractionation based on molecular size. Summers and Robberts (1988) hypothesized that adsorbed HS undergo a conformational change when a certain solid phase concentration on GAC has been reached, and that in this process utilizable components might become available to the attached microorganisms. An increase in bioavailability of humic compounds has also been observed after the adsorption to iron oxides in the soil and aquatic environments (Camper 2004). Stumm and Morgan (1981) suggested that the adsorption on iron oxide surfaces forces the humic molecules to collapse, allowing for maximal points of interaction between their oxygen-containing functional groups and iron-oxide surface sites through ligand exchange or H-binding mechanisms. The collapse of the molecule may cause it to uncoil and expand, exposing the previously hidden usable functional groups (Gu et al. 1994), making them available for microbial attack (Camper, 2004). However, such utilization would require adsorption onto a surface accessible to bacteria, which is not the case with GAC. Furthermore, the recently developed supramolecular concept of the HS structure implies that HS are not high-molecular-weight polymeric compounds, thus making the utilization of uncoiled molecular structures unlikely. The supramolecular concept, however, may provide a hypothesis for an adsorption-enhanced utilization of NOM. This concept is based on observations that HS include a large variety of relatively small molecular organic components (200 - 2500 Da) held together by weak chemical interactions such as hydrophobic associations, charge interactions, metal bridging and hydrogen bonds (Conte and Piccolo 1999; Sutton and Sposito 2005). Consequently, the micelle-like HS aggregates are more susceptible to structural changes than the previously assumed polymeric structure, and adsorption to the hydrophobic or charged GAC surface may lead to micelle disruption and release of low molecular weight compounds, as has been observed by the addition of specific chemicals to HS solutions (Sutton and Sposito 2005).

Overall, two mechanisms with the potential to increase the bioavailability of NOM components absorbed in GACFs exist: (i) adsorptive fractionation based on molecular size (competition) and (ii) adsorption induced release of biodegradable components during the process of reorientation of supramolecular structures (micelles) of HS on the GAC surface. These mechanisms should occur in pores < 500 nm, because the surface of the pores accessible to bacteria is too small (<0.1 %) to play a significant role.

In the pilot plant GACF, after c. 100 days, the removal rate decreased, and at that point on average c. 120 mg of DOC was removed per g GAC in the top layer (Chapter 6. Fig. 6.2A). Assuming that all the removed NOM is adsorbed and evenly distributed on the GAC surface

available for NOM adsorption in the pores > 1.0 nm (83 $m^2\ g^{-1}$), the average concentration would correspond to 0.14 μg C cm^{-2}. A B_{GAC} concentration of 300 ng ATP g^{-1} GAC (Chapter 6, Fig. 6.3) and a maintenance coefficient of 0.065 μg C ng^{-1} ATP d^{-1} (at 15 °C) would correspond to an uptake of 20 μg C $g^{-1}\ d^{-1}$. At an accessible surface area of 300 $cm^2\ g^{-1}$, an apparent amount of 42 μg C (300 x 0.14 μg C) is present. This estimation confirms that the compounds adsorbed onto the GAC surface accessible to bacteria cannot fulfil the maintenance energy requirements of the microbial community. Hence, desorption of substrates from the surface not accessible to bacteria (83 $m^2\ g^{-1}$) is needed, but data about the rate of desorption, related to the reassembling of adsorbed HS are not available. However, in the pilot plant GACF the specific oxygen consumption in the middle and bottom segments did not differ from each other (Chapter 6, Fig. 6.4) despite the difference in the specific DOC removal. Therefore it can be concluded that the contribution of desorbing compounds to the microbial activity is very limited.

From the considerations and calculations described above, it can be concluded that the enhanced microbiological activity in GACFs is related to the strong attachment of bacteria onto a relatively large surface in pores providing shelter against predation and shear forces, and that adsorbed NOM plays a negligible role in the biological processes in GACF.

7.4 PRACTICAL SIGNIFICANCE AND FUTURE PERSPECTIVES

The microbial activity in GACFs in water treatment is usually regarded as beneficial due to the additional removal of NOM from the influent by biodegradation processes that result in an extension of the periods between the regenerations. However, the strong attachment of bacteria onto GAC and the poor backwashing efficiency cause an accumulation of living and dead biomass in GACFs. The disproportion between oxygen utilisation and DOC removal indicates a biomass turnover associated with protozoan and invertebrate activity. The release of these organisms and biomass aggregates (detritus) in the form of particulate organic carbon (POC) will affect the effluent water quality and may impair the biological stability. The most important factors that affect the accumulation and turnover of biomass in GAC filters are GAC properties (porosity, hydrophobicity and functional groups), influent quality (NOM), and backwashing regime. The control of microbial activity in GACF and the emission of the POC from the GACFs therefore can be improved by (i) decreasing the concentration of growth-promoting compounds by adapting pre-treatment, (ii) improving the backwashing of GACFs, (iii) appropriate post-treatment and (iv) selection of GAC with more appropriate surface properties. GAC with more macropores on the outer surface can provide more hiding places for bacteria and hamper protozoan activity reducing thereby biomass turnover and the emission of POC. Another option is to reduce the microbial activity in GACFs by using GAC without pores accessible to bacteria. Also removal of most easily biodegradable compounds from the influent by pre-treatment with RSF (or DMF) can reduce the biological activity in GACFs. From the water quality parameters, the nature and concentration of NOM are the most important aspects affecting the biological processes in GACFs. Alternative pre-treatment for the removal of NOM, e.g. by ion exchange preceding GAC filtration might improve the efficiency of GAC filtration in the removal of micro-pollutants. To limit accumulation and release of biomass (aggregates) in

the effluent an adequate pretreatment (rapid-sand filtration or dual-media filtration) or post-treatment (slow sand filtration or ultrafiltration) may further improve the benefits of biological processes in GAC filters.

A number of issues require further research:

- The presence of POC in the influent and its effect on the biological processes has attained insufficient attention in the studies on GAC filtration in water treatment;
- Similarly, the presence of POC in the GAC filtrate and its effect on fouling and microbial regrowth in distribution systems require more investigations;
- The re-evaluation of the threshold levels for NOM in relation to regrowth and biological stability in distribution networks could help to optimize GAC filtration and limit the release of POC and dissolved organic carbon produced by the biomass.
- The appropriate pre-treatment, post-treatment and backwashing procedure to minimize the release of POC from GACFs.

The investigations described in this thesis provided a simple method for assessing the bacterial activity (as ATP) in GACFs. Application of this method in GACFs and other biological filtration processes can provide quantitative information about the level of biological activity and support measures aiming at reducing or promoting such activity. Furthermore, the use of ATP as parameter for bacterial biomass facilitates comparison between biological processes in a wide range of aquatic habitats, including drinking water distribution systems.

Information on nutritional properties and growth parameters of *Polaromonas* (Chapter 3, 4, 5) are useful for modelling the removal of biodegradable organic compounds. However, the nature and concentration of compounds promoting bacterial growth in GACFs are still unresolved. Information about the identity of the predominating bacteria and their metabolic properties can be used to elucidate the nature of such compounds. This approach requires the isolation and further study of the predominant species in GACFs, including bacteria classified as TRA3-20 (Chapter 4).

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SUPPLEMENTS

SUMMARY

SAMENVATTING

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ABOUT THE AUTHOR

LIST OF PUBLICATIONTS

DIPLOMA FOR SPECIALISED PhD TRAINING

SUMMARY

This thesis focuses on the contribution of the bacterial community in granular activated carbon filters (GACFs) in water treatment to the removal of natural organic matter (NOM). Observations in full-scale treatment plants, laboratory experiments and pilot-plant studies were conducted with the aim of describing the interactions between the adsorption processes and microbial activity in GACFs. For this purpose (i) a method for measuring the abundance of active bacteria on GAC was developed and evaluated; (ii) the bacteria predominating in the bacterial community in GACFs were isolated and identified, with cultivation-dependent and molecular approaches (iii) the physiological properties of a predominant species were elucidated and (iv) the microbial activity and adsorption of NOM in a pilot plant GACF were quantified and their relationships were analysed.

A method for measuring the concentration of active bacterial biomass in GACFs was developed as described in **Chapter 2**, using high-energy sonication at a power input of 40 W for the removal of attached microorganisms and subsequent quantification of adenosine triphosphate (ATP). A series of six to eight sonication treatments of two minutes each was needed to yield more than 90% of the attached active bacteria, thereby demonstrating strong attachment of the bacteria to the GAC surface. The ATP concentrations in 30 full-scale GACFs and nine rapid sand filters (RSFs) at nine treatment plants in the Netherlands ranged from 25 to 5000 ng ATP cm^{-3} GAC, with the highest concentrations at long filter run times and receiving water treated with ozone. Although RSFs and GACFs exhibited similar activity, the median value of the ATP content per bacterial cell in the RSFs ($3.6x10^{-7}$ ng ATP/cell) was approximately 17 times higher compared to bacteria in GACFs ($2.1x10^{-8}$ ng ATP/cell); indicating that most bacteria in the GACFs were in the stationary growth phase, whereas bacteria in the RSFs were in the exponential growth phase.

As described in **Chapter 3**, genomic fingerprinting and 16S rRNA gene sequence analysis of the predominant cultivable bacteria from 21 GACFs in nine water treatment plants in the Netherlands revealed that most of the isolates from GAC belonged to the *Betaproteobacteria* (68%) or *Alphaproteobacteria* (25%). *Polaromonas* was almost always present in the GACFs (86%), whereas members of the genera *Hydrogenophaga*, *Sphingomonas* and *Afipia* were isolated from 43%, 33% and 29% of the filter beds, respectively. The predominant genus *Polaromonas*, which has mainly been observed in oligotrophic freshwater environments, included a total of 23 different genotypes, as revealed by AFLP analysis.

Cultivation independent assessment, based on the sequence and T-RFLP (terminal restriction length polymorfism) fingerprint analysis of bacterial 16S rRNA genes, identified a total of 135 operational taxonomic units (OTUs) in both full-scale and pilot-plant GACFs at five drinking water treatment plants (**Chapter 4**). In concordance with the cultivation-based study, the most abundant OTUs from the GACFs belonged to the *Betaproteobacteria* (43% of all sequences) and *Alphaproteobacteria* (38%), with representatives from the genera *Polaromonas (11%)*, *Afipia, Sphingomonas, Variovorax* and unclassified *Commamonadaceae*. Furthermore, the cultivation-independent approach additionally revealed the presence of the TRA3-20 group of yet uncultured *Betaproteobacteria* (14.5%), *Acidobacteria* (7%), and the alphaproteobacterial families *Bradyrhizobiaceae* (9%) and *Hyphomicrobiaceae* (7%) from the order Rhizobiales.

The assessment of bacterial diversity by T-RFLP analysis revealed a relatively high similarity (>60%) between the communities in the GACFs at different locations. High temporal as well as spatial stability was observed in the community in a pilot-plant filter over a period of 18 months. The predominant species were evenly distributed (Shannon evenness index 0.82±0.06 of the T-RFLP fragments). An increase in richness during the maturation of the community in the pilot-plant filter was accompanied by an increase in relative abundance of predominant fragments, confirming the importance of these bacteria in the microbial activity.

The classification of the predominant bacteria and the metabolic properties of their cultured relatives, indicated that most bacteria on GAC are chemo-organotrophic aerobes that grow slowly at low concentrations of carboxylic and aromatic acids or chemolithrophic nitrifiers and can utilize either ammonia or nitrite as a source of energy. The nutritional versatility and growth kinetics of two *Polaromonas* strains isolated from two GACFs, representing the predominating bacteria in the investigated GACFs, were studied in batch culture with mixed and individual substrates at concentrations typical for drinking water (**Chapter 5**). The favoured substrates for growth are carboxylic acids and aromatic acids, while only a few amino acids and carbohydrates were utilized a sole source of energy and carbon. Enhanced growth on the mixture of amino acids, when compared to growth on individual amino acids, indicated that most amino acids may be utilized as source of carbon. The maximum growth rate (0.09-0.11 h^{-1}) was attained at low substrate concentrations, and low half-saturation constants were observed for the growth on acetate (0.015μM) and benzoate (0.039 μM), respectively. Hence, in GACFs low-molecular-weight organic compounds can be utilized at the microgram-per-litre level.

To elucidate the relationship between adsorption and biodegradation of NOM, bacterial growth and the removal of dissolved organic carbon (DOC) were analyzed in a pilot plant GACF that was operated at the surface-water treatment pilot plant of Weesperkarspel (Waternet, The Netherlands) for 602 days as described in **Chapter 6**. NOM removal was approximately four times faster in the initial phase I (days 0-100) than in the second phase II (days 100-602). The bacterial accumulation rate (BAR) on GAC was higher after 7 minutes of empty bed contact time (1.6 ng ATP g^{-1} GAC day^{-1}) than after 23 minutes (1.1 ng ATP g^{-1} GAC day^{-1}) reaching a maximal concentration of 315 – 530 ng ATP g^{-1} GAC after approximately 250 days. From these data, it was derived that the net specific growth rate of the bacteria on GAC was very low (< 0.016 d^{-1}), i.e. a mean doubling time > 40 days. At a temperature above 15 °C the bacterial ATP concentration decreased rapidly, but oxygen consumption increased, indicating biomass turnover by protozoan and invertebrate activity in the filter bed. The ratio of oxygen consumption and DOC removal exceeded the theoretical value (2.8 mg O_2 mg C^{-1}) for complete NOM oxidation at temperatures >10°C (3.9-11.2 mg O_2 mg $^{-1}$ C), thus confirming biomass turnover at elevated temperature. The apparent specific substrate utilization rate (0.2 – 1.8 μg DOC ng $^{-1}$ ATP day^{-1}) in phase II approached the maintenance coefficient of Polaromonas for growth on acetate at lower temperatures and the maximal specific utilization rate at high temperatures. Adsorption and biodegradation occurred simultaneously in both phases, but the removal of NOM by adsorption predominated in phase I, whereas biodegradation was more pronounced in phase II.

The surface characteristics of GAC affect the microbial activity in GACFs in different ways. The hydrophobic and charged GAC surface strengthens bacterial attachment, and the high porosity of the outer surface prevents the washout of bacteria and protect them from protozoan predation. Excess oxygen utilization as compared to the removal of dissolved organic carbon (DOC) was associated with a decrease of the concentration of bacterial biomass on GAC at temperatures > 15°C, indicating the presence of accumulated dead and living biomass, originating from the influent and/or from microbial growth. The high ratio of the cumulative oxygen removal and the cumulative DOC removal may also be attributed the binding of oxygen by GAC surface, and/or to the utilization of adsorbed compounds. Direct utilization of adsorbed compounds is unlikely to occur, but the reassembling of supramolecular aggregates of humic substances adsorbed onto GAC is a potential mechanism for the release of biodegradable compounds from the adsorbed humic aggregates. Calculations however, indicate that the contribution of these released compounds to the microbial activity is most likely negligible when compared to the activity induced by the uptake of biodegradable compounds from the water.

In conclusion, the investigations described in this thesis revealed that the bacterial activity in GACFs is affected by the GAC-surface properties promoting bacterial attachment. The microbial activity in GAC filters is usually regarded as beneficial due to the additional removal of NOM from the influent by biodegradation processes that result in an extension of periods between regenerations. However, the strong attachment of bacteria to GAC and poor backwashing efficiency enhance accumulation of dead and living biomass in GACFs, resulting in biomass-turnover processes in summer. Release of compounds and (micro)organisms, e.g. invertebrates, produced by these processes will affect the effluent water quality. Apparently, benefits and disadvantages of microbial activity in GAC filters need to be balanced.

GAC materials with more macropores accessible for bacterial attachment and growth are suitable if GAC filtration is used to remove biodegradable compounds from the effluent. However, the effects of GAC filtration on biological stability of the effluent under these conditions need to be reevaluated regarding the emission of living and dead biomass (aggregates) from the GACF. If removal of micropollutants is the main goal of GAC filtration, microporous GAC materials with less mesopores and macropores could provide a better performance. To limit accumulation and release of biomass (aggregates) in the effluent an adequate pretreatment (rapid sand filtration or dual-media filtration) or posttreatment (slow sand filtration or ultrafiltration) may further improve the benefits of biological processes in GAC filters. Optimization of the backwashing procedure might also be considered.

The concentration and properties of available growth substrates in GACFs require further studies, as well as the impact of particulate organic carbon on biological activity in GACFs. Efforts to isolate the predominant and yet uncultivated microorganisms and study their preferred growth substrates and other characteristic will add to the information presented here about the importance of the release of bioavailable components from supramolecular structures of NOM induced by adsorption to the GAC surface. This knowledge may lead to further optimization of biodegradation processes in GAC filters.

SAMENVATTING

Het onderzoek beschreven in dit proefschrift richtte zich op de bijdrage van de bacteriële gemeenschap aan de verwijdering van natuurlijk organisch materiaal (NOM) in granulaire actieve koolfilters (GACFs) bij de drinkwaterbereiding. Metingen in praktijkinstallaties, proefinstallaties en laboratoriumexperimenten zijn uitgevoerd om de microbiële activiteit in GACFs te meten en interacties met adsorptieprocessen te beschrijven. Hierbij werd (i) een methode voor het meten van de concentratie van actieve bacteriën op GAC ontwikkeld en geëvalueerd; (ii) werden de dominante bacterietypen van de bacteriële gemeenschappen in GACFs geïsoleerd en geïdentificeerd met kweekafhankelijke en moleculaire technieken, (iii) werden de fysiologische eigenschappen van enkele dominante bacteriesoorten opgehelderd en (iv) werden de microbiële activiteit en adsorptie van NOM gekwantificeerd en hun relaties geanalyseerd in een GACF proefinstallatie.

Hoofdstuk 2 beschrijft de ontwikkeling van een methode voor het meten van de concentratie van de actieve bacteriële biomassa in GACFs. Deze methode is gebaseerd op het gebruik van hoog energetisch ultrasoon geluid met een vermogen van 40 W voor het losmaken van de geadsorbeerde micro-organismen en de analyse van adenosinetrifosfaat (ATP) voor de kwantificering van de microbiële activiteit. Een reeks van zes tot acht behandelingen met hoog energetisch ultrasoon geluid van twee minuten bleek nodig om meer dan 90% van de geadsorbeerde bacteriën te verwijderen van het GAC-oppervlak, hetgeen duidt op sterke hechting van de bacteriën aan GAC. De ATP-concentratie in 30 praktijk GACFs en negen snelle zandfilters (RSFs) in negen drinkwaterinstallaties in Nederland varieerde tussen 25-5000 ng ATP cm^{-3} filtermateriaal. De hoogste concentraties werden waargenomen in de filters met lange looptijden en filters die werden gevoed met water dat is behandeld met ozon. een In de RSFs en GACFs werd een vergelijkbare activiteit gevonden, maar de mediaanwaarde van het ATP-gehalte per bacteriecel was in de RSFs (3.6×10^{-7} ng ATP/cel) meer dan tien keer hoger dan van de bacteriën in de GACFs (2.1×10^{-8} ng ATP/cel). Blijkbaar zijn de meeste bacteriën in de GACFs in de stationaire groeifase, in tegenstelling tot de bacteriën in de RSFs die in de exponentiële groeifase zijn.

In **hoofdstuk 3** wordt de identificatie beschreven van de kweekbare bacteriën die dominant zijn in GACFs. Onderzoek van bacteriën uit 21 GACFs van negen waterbehandelingsinstallaties in Nederland met behulp van genomische fingerprints (amplified fragment length polymorphism, AFLP) en sequentie-analyse van het 16S ribosomaal RNA-gen wees uit dat de dat het merendeel van de dominante soorten in GACFs behoort tot de *Betaproteobacteria* (68%) en de *Alphaproteobacteria* (25 %). Bacteriën van het geslacht *Polaromonas* zijn bijna altijd aangetroffen in deze GACFs (86%), terwijl vertegenwoordigers van de geslachten *Hydrogenophaga, Sphingomonas* en *Afipia* werden geïsoleerd uit respectievelijk 43%, 33% en 29% van de filters. Het geslacht *Polaromonas*, dat voornamelijk wordt waargenomen in oligotrofe aquatische milieus, was vertegenwoordigd met 23 verschillende genotypen, gebaseerd op de AFLP-analyse.

Een kweek-onafhankelijke studie, gebaseerd op de sequentie-analyse en de T-RFLP fingerprint (Terminal Restriction Fragment Length Polymorphism) van bacteriële 16S rRNA genen, afkomstig uit GACFs van vijf praktijkinstallaties en twee proefinstallaties, resulteerde in de

detectie van in totaal 135 operationele taxonomische eenheden (OTU's), (**hoofdstuk 4**). Evenals bij de kweek-afhankelijke studie, behoorden de meest dominante OTU's uit de GACFs tot de *Betaproteobacteria* (43%) en *Alphaproteobacteria* (38%), met vertegenwoordigers uit de geslachten *Polaromonas* (11%), *Afipia, Sphingomonas, Variovorax* en familie *Commamonadaceae*. Bovendien, bleek uit de toepassing van de kweek-onafhankelijke werkwijze dat een aantal groepen die niet werden gekweekt een belangrijk deel uitmaken van de bacteriële gemeenschappen in de onderzochte GACFs. Hierbij vormden een nog niet gekweekte groep binnen de *Betaproteobacteria* ("TRA3-20 groep", 14.5%), de *Acidobacteria* (7%), en vertegenwoordigers van de families *Bradyrhizobiaceae* (9%) en *Hyphomicrobiaceae* (7%) van de orde *Rhizobiales* de belangrijkste groepen.

De beoordeling van bacteriële diversiteit met de t-RFLP analyse toonde een relatief hoge mate van overeenkomst (> 60%) tussen de gemeenschappen in de GACFs van de verschillende locaties. In filters uit een proefinstallatie werd een hoge temporele en ruimtelijke stabiliteit van de bacteriële gemeenschap waargenomen over een periode van 18 maanden. De dominante soorten waren gelijkmatig verdeeld (Shannon index 0.82 ± 0.06 van de t-RFLP fragmenten). Een toename van het aantal soorten tijdens de rijping van de bacteriële gemeenschap in het proefinstallatiefilter ging gepaard met een toename van het relatieve aandeel van de dominante soorten, waarmee het belang van deze bacteriën in de microbiële activiteit werd bevestigd.

De classificatie van de belangrijkste bacteriën en de fysiologische eigenschappen van de meest verwante gekweekte soorten geven aan dat de meeste bacteriën op GAC behoren tot aërobe chemo-organotrofe soorten, die kunnen groeien bij lage concentraties van carbonzuren en aromatische zuren. Ook zijn chemolithotrofe bacteriën die ammoniak of nitriet als energiebron kunnen gebruiken, aangetroffen. De substraatvoorkeur en de groeikinetiek van twee *Polaromonas* stammen, geïsoleerd uit twee GACFs, werden onderzocht in batchexperimenten met mengsels en individuele afbreekbare stoffen bij concentraties van enkele microgrammen per liter (**hoofdstuk 5**). De voorkeursubstraten voor groei van deze twee *Polaromonas* stammen waren carbonzuren en aromatische zuren, terwijl slechts enkele aminozuren en koolhydraten werden gebruikt als enige bron van koolstof en energie. De relatief sterke groei op een mengsel van aminozuren, geeft aan dat de meeste aminozuren wel gebruikt kunnen worden als koolstofbron. De maximale groeisnelheid (0.09-0.11 h^{-1}) werd bereikt bij een lage substraat concentratie, en lage Monod-constanten werden waargenomen voor de groei op acetaat (0.015 μM) en benzoaat (0.039 μM). Dit betekent dat in GACFs laag-moleculaire organische verbindingen kunnen worden omgezet bij concentraties van enkele microgrammen-per-liter.

Om de relatie tussen adsorptie en biologische afbraak van NOM nader te bestuderen werd de groei van bacteriën en de verwijdering van opgeloste organische koolstof (DOC) gemeten in een GACF-proefinstallatie die gedurende 602 dagen in bedrijf was op een locatie waar drinkwater wordt bereid uit oppervlaktewater (Weesperkarspel, Waternet, Nederland, **hoofdstuk 6**). De verwijdering van NOM was in fase I (dagen 0-100) ongeveer vier keer sneller dan in fase II (100-602 dagen) van de looptijd. De snelheid van bacteriële accumulatie (BAR) op GAC was hoger na 7 minuten contacttijd (1.6 ng ATP g^{-1} GAC dag^{-1}) dan na 23 minuten (1,1 ng ATP g^{-1} GAC dag^{-1}). Een maximale concentratie van 315-530 ng ATP g^{-1} GAC werd bereikt na een looptijd van ongeveer 250 dagen. Uit de gegevens werd afgeleid dat de netto specifieke

groeisnelheid van de bacteriën op GAC erg laag was (<0,016 d^{-1}), d.w.z. een gemiddelde verdubbelingstijd van meer dan 40 dagen. Bij een temperatuur boven 15 °C daalde de bacteriële ATP-concentratie snel, terwijl het zuurstofverbruik sterk toenam hetgeen duidt op de consumptie van geaccumuleerde bacteriële biomassa door protozoa en (activiteit van) andere ongewervelde dieren in het filterbed. Bij temperaturen boven 10 °C is de verhouding tussen zuurstofverbruik en DOC-verwijdering (3.9-11.2 mg O_2 mg^{-1} C) hoger dan de hoeveelheid (2.8 mg O_2 mg^{-1} C) die nodig is voor complete NOM-oxidatie en bevestigt de omzetting van biomassa bij verhoogde temperatuur. De schijnbare specifieke substraatomzettingssnelheid (0,2-1,8 μg DOC ng $^{-1}$ ATP dag^{-1}) in fase II benaderde de handhavingscoëfficiënt van *Polaromonas* voor groei op acetaat bij lagere temperaturen en de maximale specifieke omzettingssnelheid bij hoge temperaturen. Adsorptie en biodegradatie traden naast elkaar op in beide fasen, maar de verwijdering van NOM door adsorptie overheerste in fase I, terwijl de biologische afbraak relatief sterk was in fase II.

De eigenschappen van het GAC-oppervlak beïnvloeden de microbiële activiteit in GACFs op verschillende manieren. Het hydrofobe en geladen GAC-oppervlak versterkt de aanhechting van bacteriën, en de hoge porositeit van het buitenoppervlak voorkomt de uitspoeling van bacteriën en biedt bescherming tegen predatie door protozoa. De overmaat van zuurstofverbruik, in verhouding met de verwijdering van opgeloste organische koolstof (DOC), en de gelijktijdige afname van de concentratie van bacteriële biomassa op GAC bij temperaturen > 15 °C, wijst op de aanwezigheid van geaccumuleerde dode en levende biomassa, afkomstig van het influent en van microbiële groei. Het overmatige zuurstofverbruik kan ook worden toegeschreven aan de consumptie van zuurstof door GAC oppervlak of de omzetting van geadsorbeerde verbindingen. Directe benutting van de geadsorbeerde organische verbindingen is onwaarschijnlijk, maar de verandering van structuur van de supramoleculaire aggregaten van de op GAC-geadsorbeerde humuscomponenten is een potentieel mechanisme voor het vrijkomen van biologisch afbreekbare verbindingen. Uit de berekeningen blijkt echter dat microbiologische activiteit door omzetting van verbindingen die eventueel vrijkomen uit geadsorbeerde humuscomponenten waarschijnlijk verwaarloosbaar is ten opzichte van de activiteit als gevolg van de opname van biologisch afbreekbare verbindingen uit het water.

Op basis van het in dit proefschrift beschreven onderzoek kan worden geconcludeerd dat de bacteriële activiteit in GACFs wordt beïnvloed door de eigenschappen van het GAC-oppervlak die de aanhechting en retentie van bacteriën sterk bevorderen. De microbiële activiteit in GAC-filters wordt meestal beschouwd als gunstig vanwege de bijkomende verwijdering van NOM uit het influent door biologische processen, die kan leiden tot een verlenging van de periode tussen de regeneraties. Echter, de sterke hechting van bacteriën aan het GAC-oppervlak en de geringe efficiëntie van het terugspoelen van GACFs leiden tot ophoping van dode en levende biomassa in GACFs met versterkte omzetting van biomassa in de zomer als gevolg. Het vrijkomen van biomassabestanddelen en (micro) organismen, waaronder ongewervelde dieren, door deze processen beïnvloedt de effluentkwaliteit. De voor- en nadelen van de microbiële activiteit in GAC-filters dienen dus goed te worden afgewogen.

GAC typen met veel macroporiën die toegankelijk zijn voor aanhechting en groei van bacteriën zijn geschikt als GAC-filtratie wordt toegepast voor de verwijdering van biologisch afbreekbare

stoffen uit het water. Echter, het effect van GAC-filtratie op de biologische stabiliteit van het effluent onder deze omstandigheden moet mede worden beoordeeld met betrekking tot de levering van levende en dode biomassa (aggregaten) door de GACFs. Indien verwijdering van microverontreinigingen het belangrijkste doel is van GAC-filtratie, kan een microporeus GAC-materiaal met minder meso- en macroporiën een beter resultaat geven. Een adequate voorbehandelingstap (snelle zandfiltratie of dubbellaagsfiltratie) en/of een nabehandeling (langzame zandfiltratie of ultrafiltratie) kan de accumulatie en/of het vrijkomen van biomassa (aggregaten) beperken waardoor de voordelen van biologische processen in GAC-filters beter kunnen worden benut. Optimalisatie van de terugspoelprocedure kan eveneens worden overwogen.

De invloed van deeltjes-gebonden organische koolstof op de biologische activiteit in GACFs en op de kwaliteit van het effluent vereist verder onderzoek. Onderzoek van de dominante en nog niet gekweekte micro-organismen voor het bepalen van de favoriete groeisubstraten, de groeikinetiek en de substraatopname in de stationaire fase kan informatie geven over het vrijkomen van biologisch beschikbare componenten van supramoleculaire structuren van NOM geïnduceerd door adsorptie aan het GAC-oppervlak. Deze kennis kan leiden tot verdere optimalisatie van de biologische processen in GAC-filters.

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ABOUT THE AUTHOR

Aleksandra Knezev (1967, Novi Sad, Yugoslavia) received her degree in Bio-organic Chemistry, at the University of Novi Sad in 1990. She has lived in the Netherlands since 1991. Her carrier in the field of drinking water research started in 1996 at the Amsterdam Water Supply, Department of Water Quality. Between 2000-2005 she worked at KWR Water Cycle Research (former KIWA Water Research) and Wageningen University on her PhD. Since 2006, she has been working as Senior Consultant for Biology at Het Waterlaboratorium in Haarlem, the company providing water quality control services to the water supply companies in the west part of the Netherlands. She coordinates the research programme of the Biology Department with the main goal to maintain a high level of quality and efficiency for the routine water quality services as well as introduce novel methods and techniques into the drinking water practice. She is a member of the Thematic Group on Biological Activity of the Joint Research Programme (BTO) of the Water Supply Companies in the Netherlands and is regularly involved in various research projects. These projects involve collaboration with the Faculty of Civil Engineering and Geosciences of the TU Delft, the Netherlands and UNESCO-IHE Institute for Water Education, Delft, the Netherlands. Her main research interest is aquatic microbial ecology, in particular attached growth and control of biological stability in drinking-water systems. Aleksandra is committed to helping ensure the acceptance and implementation of novel methods into the routine monitoring of drinking water quality.

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- Research Context Activity: 'Organising workshop at Water laboratory on: Analysis of Biological Stability', Haarlem (2011)

Other PhD and Advanced MSc Courses

- Techniques for writing and presenting scientific papers (2003)
- Training in the software package ARB (2006)
- Training in analytical technique for DNA analysis: Denaturing Gradient Gel Electrophoresis - DGGE (2003)
- Training in analytical techniques for the adenosine tri-phosphate analysis (ATP), total cell count analysis (epyfluorescence microscopy), and batch growth experiments (2002)

Management and Didactic Skills Training

- Supervising BSc student with thesis entitled 'The Contribution of Bacterial Biomass to the removal of Organic Matter in Biological Activated Carbon Filtration' (2002)
- Supervising MSc student with thesis entitled 'Impact of biodegradability of natural organic matter and redox conditions on removal of pharmaceutically active compounds during riverbank filtration' (2009)
- Organising of Workshop 'Rapid methods for faecal indicators', Haarlem (2013)

Selection of Oral Presentations

- *Molecular analysis of microbial communities in water treatment: biological activated carbon filters*. 35th International Association for Danube Research (IAD) Conference, 19-23 April 2004, Novi Sad, Serbia
- *Nutritional versatility of two Polaromonas-related bacteria isolated from biological granular activated carbon filters*. 4th International Slow Sand and Alternative Biofiltration Conference, 3-5 May 2006, Mülheim an der Ruhr, Germany
- *Bacterial growth and removal of organic carbon in GAC filters in drinking water treatment: quantitative relationship*. International Water Association (IWA) Biofilm Conference: Processes in Biofilms, 27-30 October 2011, Shanghai, China
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