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Role of the Biofilms in Wastewater Treatment

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Abstract

Biological wastewater treatment systems play an important role in improving water quality and human health. This chapter thus briefly discusses different biological methods, specially biofilm technologies, the development of biofilms on different filter media, factors affecting their development as well as their structure and function. It also tackles various conventional and modern molecular techniques for detailed exploration of the composition, diversity and dynamics of biofilms. These data are crucial to improve the performance, robustness and stability of biofilm-based wastewater treatment technologies.

Keywords: biofilm, wastewater treatment, biofilm technologies, molecular methods, biofilter media

1. Introduction

Water is a basic necessity, but its availability for human use is hardly about 1%. Current global water crises are due to a rapid increase in population, climatic variation, environmental pollution, urbanization, industrialization and contamination of existing water reservoirs. The quality of freshwater in rivers and streams is affected because much of the wastage is discharged without prior treatment from industries, municipal sewers and agricultural areas. The quality of groundwater is declining due to unprocessed sewage containing domestic waste along with human and animal excretion products, leading to worldwide deaths and other environmental factors, including biodiversity reduction and an increasing number of water-related infections, among others. According to WHO, approximately 30% of all diseases and 40% of deaths throughout the world are due to polluted water [1].

Wastewater is a broad term comprising effluents or discharge from household seepage, agriculture, industries and storm water [2]. The organic material present in wastewater includes detergents, pesticides, fats and oils. In addition, many types of microorganisms, including bacteria, viruses, protozoa and helminths, can be present in wastewater. Basic nutrients (nitrogen, phosphorous and ammonia, etc.) as well as metals and inorganic materials (mercury, lead, cadmium, nickel and hydrogen sulfide, etc.) are also present in wastewater. By keeping the hazardous effects of wastewater and its usage for daily lives, wastewater treatment plants have become a focal path in securing our future water supply.

2. Types of biological wastewater treatment systems

There are a number of wastewater treatment processes based on the physical and chemical removal of contaminants. These processes offer varying degrees of effectiveness in addition to presenting environmental and economic disadvantages. However, biological wastewater treatment technologies have been gaining much attention in recent years. They offer low operational costs, provide easy handling and have comparatively less harmful effects on the corresponding environment. On the basis of structural configuration of biomass, biological wastewater treatment processes can be divided into two basic configurations: dispersed growth system and attached growth system.

2.1. Dispersed growth system

In dispersed/suspended growth systems, biomass grows in suspended or dispersed form in liquid medium without any attachment to the surface (**Figure 1**).
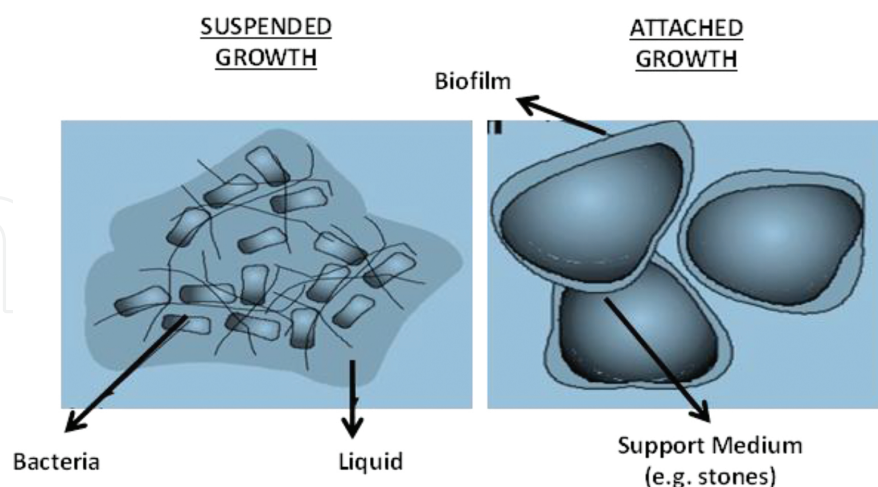

Figure 1. Typical examples of biomass growth [3].

Microorganisms in biomass absorb organic matter and nutrients in their vicinity, which allows them to grow and reproduce to form microcolonies. These microcolonies settle as sludge, which is then either removed or treated in a sludge treatment process or reused in the process

by being resuspended. In dispersed growth systems, the density of dispersed biomass is close to the sewage and moves in the same direction and velocity thereof. Thus, biomass is exposed to the same fraction of liquid for a larger interval with less substrate concentration in the neighboring cell, leading to low bacterial activity and substrate removal rate. The hydraulic retention time (average time water molecules stay in the system) has to be greater than the doubling time of microorganisms (time required to generate new cells) to increase bacterial activity and population size. Bacteria can easily be "washed out" of the system if the hydraulic retention time is shorter than the bacterial doubling time [4]. This is the main hurdle in sizing biological reactors, as reactor volume and retention time are directly related to each other. Some of the commonly used dispersed growth systems are described in the following subsections.

2.1.1. Activated sludge technology

Activated sludge systems comprise a multichamber reactor unit in which aerobic microorganisms are used to degrade organic components of wastewater to produce a high-quality effluent. Constant supply of oxygen is required to maintain aerobic conditions in an aeration tank. Besides aerobic bacteria, anaerobic and/or nitrifying bacteria along with higher organisms can be present. These microorganisms oxidize the organic carbon present in wastewater to produce carbon dioxide, water and new cells that form small clusters or flocs during the aeration and mixing process. After aeration, the mixture is transferred to a secondary clarifier for settling of floc particles and the effluent moves on for further treatment or discharge. The sludge is then recycled back to the aeration tank, where the process is repeated. A schematic of the entire process is shown in **Figure 2**. Activated sludge technology is most commonly used
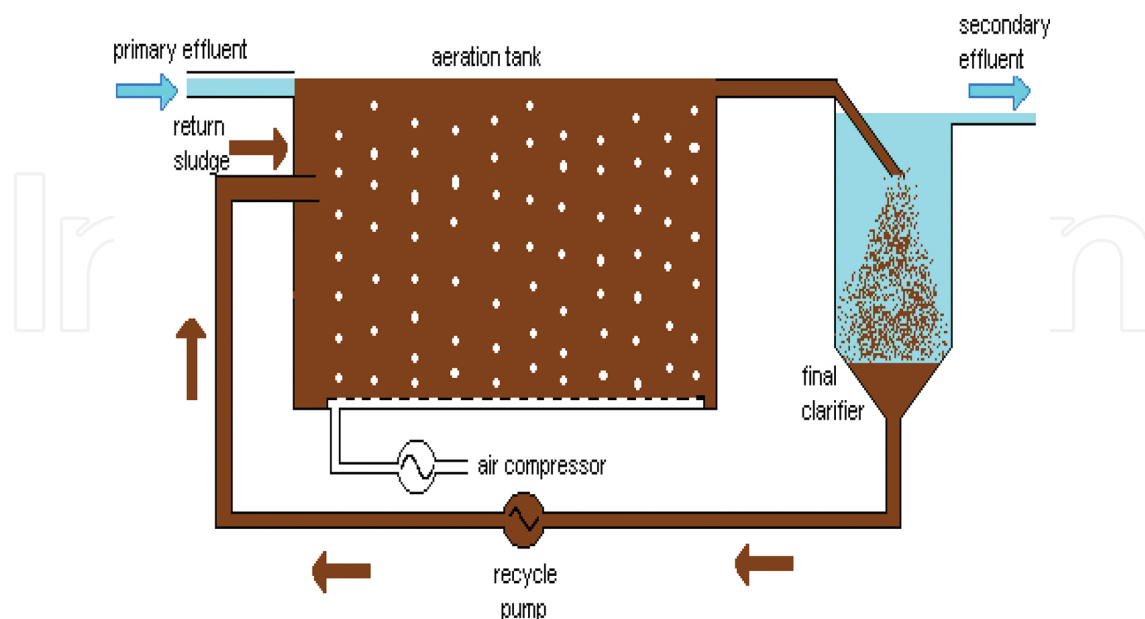

Figure 2. Schematic of a typical activated sludge system [6].

in industrialized countries for the removal of biological solids by sedimentation. Poor settling of these solid pollutants can lead to increased solid treatment costs, increased effluent solid concentrations, decreased disinfection efficiencies, washout/low biomass concentration and increased risks to downstream ecosystems and public health [5].

2.1.2. Extended aeration system

The extended aeration system is one of the modifications of the activated sludge process. It is a complete mixed system that provides biological treatment for the removal of biodegradable organic waste under aerobic conditions. Air may be supplied by mechanical or diffused aeration means. The raw sewage directly flows into the aerobic digestion chamber where all the solids are digested by aerobic bacteria. This is possible because the sewage is aerated for a minimum of 24 h, giving vastly increased time for almost complete digestion of all solids. Since there is complete stabilization in the aeration tank, there is no need for a separate sludge digester. Furthermore, there is no need for a primary settling tank as organic solids are allowed to settle in the aeration tank due to their long detention time. The major advantages of extended aeration include ease of construction as well as operation, high oxygen transfer efficiency, absence of odor, less sludge yield and exceptional mixing energy from a controlled aeration chain environment. However, extended aeration plants do not achieve denitrification and phosphorus removal without additional unit processes.

2.2. Attached growth system

In attached growth systems, the biomass grows attached to a support medium to create a biofilm. Attachment to the support medium is influenced by composition of the media used, cell-cell interactions and the presence of polymer molecules on the surface [7]. The support medium can be immersed in the liquid medium or receive continuous or intermittent discharges. The support medium can be of any nature, such as solid natural (rocks, stones, gravels, sand and soil), artificial (rubber, plastic) or agglomerates of the biomass itself (granules). These biofilms grow on support media by feeding off the organic matter and nutrients in the wastewater that flows over them. In attached growth systems, there is a difference in the density gradient of the support medium together with biomass and the density of the liquid inside the reactor that allows the velocity gradient between the liquid and the external layer of biofilm. Therefore, bacterial cells being continually exposed to new substrates tend to increase their activity. Some of the commonly used attached growth systems are described in the following subsections.

2.2.1. Trickling filters

Wastewater treatment through trickling filters (TFs) is among the oldest and most well characterized treatment technologies. TFs generally comprise a vessel packed with inert media (rocks, coke, lava, slag, gravel, polyurethane foam, ceramic, sphagnum peat moss or plastic media). The distribution system is used to sprinkle wastewater over filter media, and the wastewater trickles through the filter media supporting biomass under the influence of gravitational force. A biological slime layer grows on the media, and treatment is provided by

the microbes that absorb dissolved organic matter for their growth and reproduction as the wastewater cascades randomly through the voids between the media [8]. A schematic of the entire process is shown in **Figure 3**. TFs are suitable for small- to medium-sized communities with a high filter loading rate and marked by their ease of operation, self-cleaning capacity and efficient removal of ammonia. However, additional treatment may be needed for the effluent to meet strict discharge standards as it generates large amounts of sludge and a relatively high incidence of clogging [9].
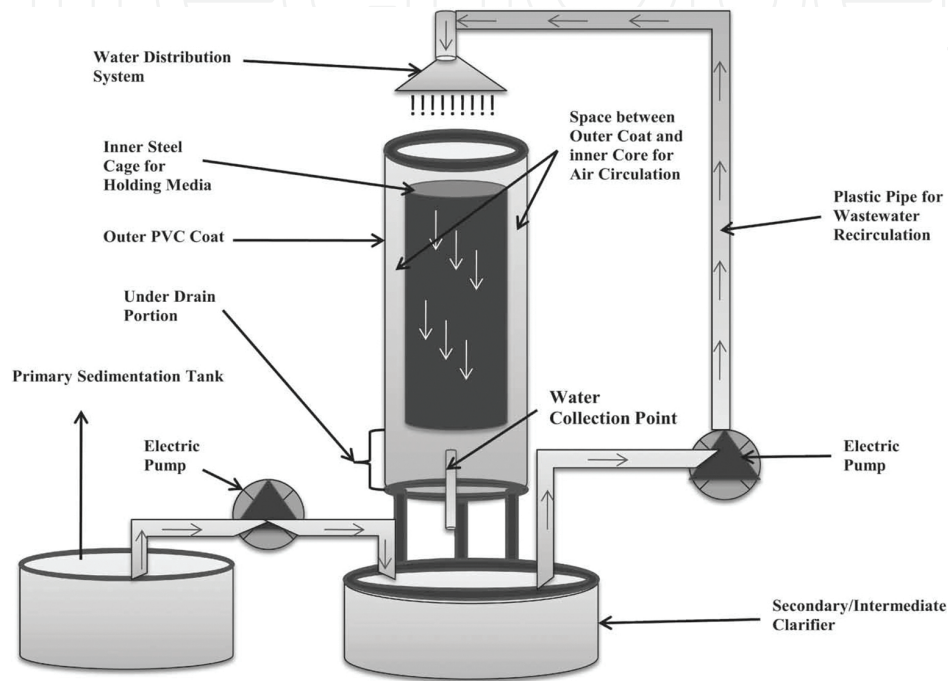

Figure 3. Schematic of a typical trickling filter system [10].

2.2.2. Rotating biological contactor (RBC) system

The rotating biological contactor (RBC) is an efficient attached growth system that purifies wastewater from different industries, namely food and beverage, refinery and petrochemical, pulp and paper industries. In addition, it is efficient in purifying municipal wastewater, landfill leachate and lagoon effluent. The system consists of biomass media, usually plastic (polyethylene, polyvinyl chloride [PVC] and expanded polystyrene), that are partially immersed in wastewater. As it slowly rotates, it lifts a film of wastewater into the air. The wastewater trickles down across the media and absorbs oxygen from the air provided by the rotating action. A living biomass (biofilm) attached to the discs assimilates the organic materials and nutrients in the wastewater. Any excess biomass that sloughs off the discs by shearing forces exerted with disc rotation and gravitational force is then removed from clear water through a conventional clarification process. A schematic of the entire process is shown in **Figure 4**. The RBC system has an edge over suspended growth systems in terms of reduced life cycle costs, less sludge production, less space requirement, ease of operation and high

process stability with load variations as well as high effluent quality with regard to both biological oxygen demand (BOD) and nutrients. However, RBC system optimization and adaptability under different environmental conditions and influent characteristics still pose challenges for the efficient design and use of this technology.
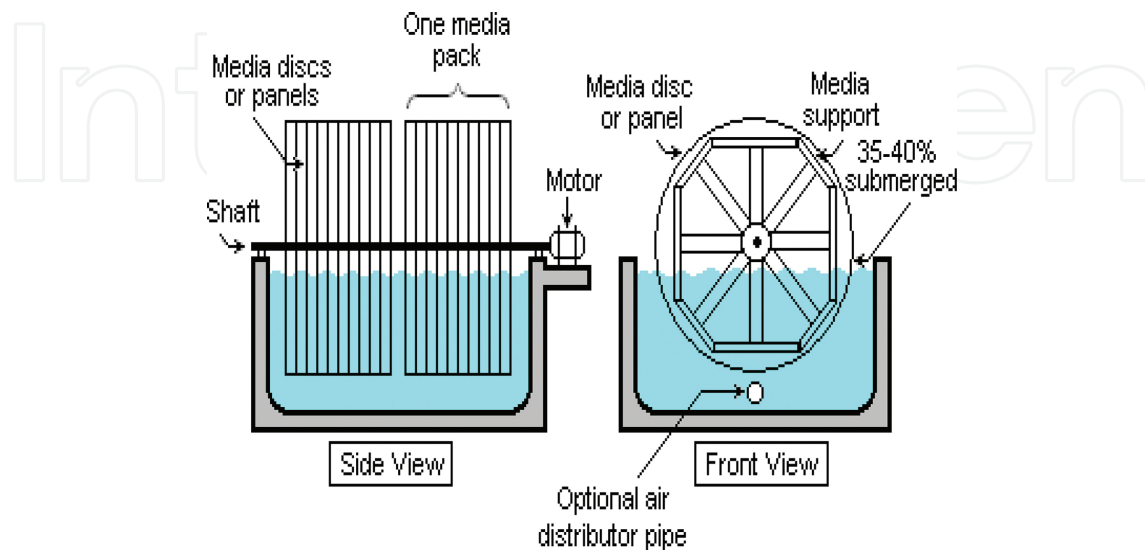

Figure 4. Schematic of a typical rotating biological contactor (RBC) [11].

2.2.3. Constructed wetland system

Constructed wetlands (CWs) are engineered attached growth or fixed film systems comprising beds loaded with inadequately sapped graded medium (sand, soil, gravel, etc.) and planted with suitable vegetation and their microbial inhabitants to treat contaminants in surface water, groundwater or waste streams. CWs generally may be categorized into two major groups: surface flow and subsurface flow. In the case of surface flow, the water runs over the surface, while for subsurface flow, it runs beneath the surface to overcome the issues of odor. In surface flow, the bacteria and substrate contact angle with water is lower than that in subsurface flow, resulting in the much enhanced treatment efficiency of subsurface flow systems [12]. Further subsurface flow systems are categorized into horizontal and vertical subsurface flow wetlands depending on the flow path. All these systems are efficient in removing contaminants and pathogens from wastewater; however, the evaporation rate of CWs in general is much higher than that of ponds or lagoons, thus posing a low potential for irrigation. The configuration of hybrid CWs (combination of vertical and horizontal flows) is considered to be an appropriate choice that has minimum water loss to overcome this flaw (**Figure 5**). Hence, the discharge of nitrified and partly denitrified effluents is possible with lower total N contents [13].

Generally, water purification in constructed wetlands involves a series of physical, chemical and biological processes, such as adsorption, filtration, sedimentation, chemical precipitation, microbial activities and macrophyte uptake. Various factors contribute to the removal

efficiency of CWs, including hydraulic retention time, temperature, macrophytes, composition of substrate or fill media and microorganisms [14]. In CWs, the role of macrophytes is very important for the removal of nutrients from wastewater, and they also speed up the purification process by increasing the chemical and biological reactions in the rhizosphere. CWs require low operational and maintenance costs, less energy consumption and a reduced amount of sludge, and they are environmentally friendly [15].
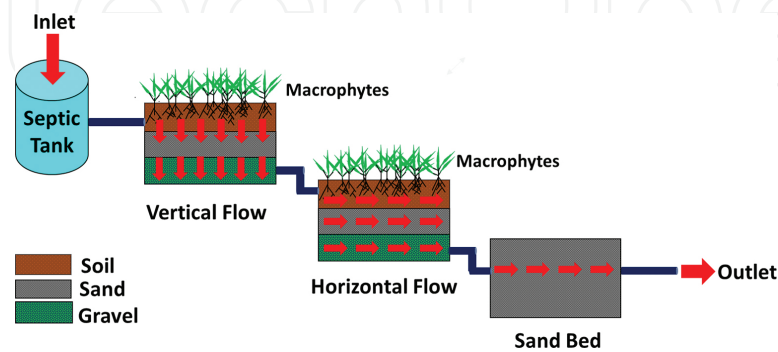

Figure 5. Schematic of a constructed wetland system for wastewater treatment [14].

2.2.4. Membrane bioreactors (MBRs)

Membrane bioreactors (MBRs), which are used for municipal/industrial wastewater treatment, are a combination of a suspended growth treatment method with membrane filtration equipped with low-pressure microfiltration (MF) or ultrafiltration (UF) membranes. A membrane is simply a two-dimensional material used to separate components of fluids usually on the basis of their relative size or electrical charge. MBRs are generally categorized into the following: (i) vacuum or gravity-driven systems, immersed and normally employing hollow fiber or flat sheet membranes installed in bioreactors or a subsequent membrane tank and (ii) pressure-driven systems or pipe cartridge systems located external to the bioreactor. A schematic of MBRs is depicted in **Figure 6**.

An MBR system is often composed of 10 or 11 subsystems and includes fine screening, the membrane zone and, in most cases, some type of post-disinfection process. The initial step in a biological process occurs in membrane zones where microbes are used to degrade pollutants that are then filtered by a series of submerged membranes. The individual membranes are housed in units known as modules, cassettes or racks, and a combined series of these modules is referred to as a working membrane unit. Air is introduced through integral diffusers to continually scour membrane surfaces during filtration, facilitate mixing and, in some cases, contribute oxygen to the biological process. The major advantage of MBRs is that they allow high concentrations of mixed liquor suspended solids (MLSSs) with low sludge production, increased removal efficiencies of BOD and COD, water reclamation, reduced footprints and no further polishing requirement for disinfection/clarification. However, membrane surface fouling is a major obstacle to the wide application of MBRs. Additionally,

membrane channel clogging and process complexity are the main cause of increased capital as well as running costs of the entire system [16].
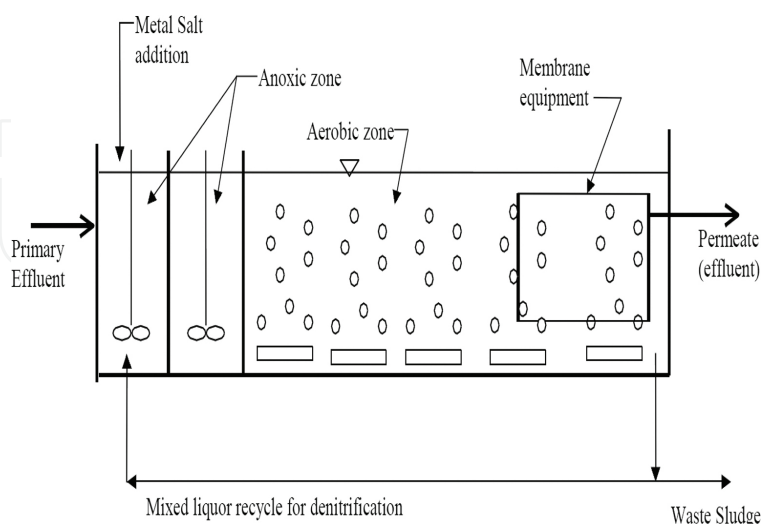

Figure 6. Typical schematic for a membrane bioreactor [17].

3. Biofilm development: structure and function

An assemblage of microbial cells enclosed in a matrix of bacterial self-generated extracellular polymeric substances (EPSs) irreversibly associated with a surface is termed a *biofilm*. Generally, the development of biofilms is composed of five main stages (**Figure 7**): (1) initial attachment of planktonic microorganisms with the exposure of a surface to an aqueous
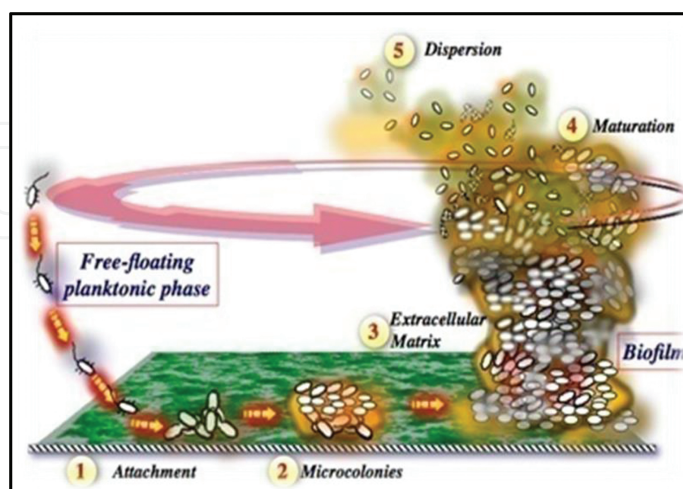

Figure 7. Stages of biofilm development [20]: (1) initial attachment; (2) irreversible attachment; (3) replication; (4) maturation and (5) dispersion.

medium; (2) irreversible adhesion upon the production of microorganism-mediated EPSs as polyhydroxyl groups in EPSs colonize bacteria to the surface via hydrogen bonding [18]; (3) formation of monolayer microcolonies on the fixed surface due to replication of early colonizers; (4) maturation of biofilm into a three-dimensional arrangement by attaching debris from the adjacent environment and by employing new planktonic bacteria and (5) dispersion or expansion by active and passive processes in which sessile, matrix-encased biofilm cells convert to freely swimming planktonic bacteria through quorum sensing (QS) or a cell-to-cell signaling mechanism [19].

3.1. Factors effecting biofilm formation

The following subsections discuss the factors that help in promoting the process of biofilm formation.

3.1.1. Effects of nutrients, pH and temperature

Biofilm formation varies under diverse nutrient conditions ranging from high to almost non-detectable. However, they are more abundant and dense in a nutrient-rich environment as it promotes the transition of bacterial cells from planktonic to biofilm state, while depletion of these nutrients causes detachment of biofilm cells from surfaces. There are different means by which bacterial biofilms obtain nutrients: (i) concentrating trace organics on surfaces through extracellular polymer, (ii) using the waste products from secondary colonizers and (iii) pooling the biochemical resources with the help of different enzymes to break down food supplies.

Any change in pH greatly affects the growth and development of bacterial and biofilm formation as it can overwhelm different mechanisms and have negative or killing effects on the microorganisms. In response to internal or external changes in pH, bacteria quickly adjust the activity and synthesis of proteins that are associated with different cellular processes. However, some of the cellular processes, including excretion of exopolymeric substances or polysaccharides, do not adapt to pH variations so easily. The optimum pH for polysaccharide production varies among different species, but for the majority of bacteria, it is around 7 [21]. Microbial activities are very sensitive to change in temperature. Optimum temperature results in healthy growth of bacterial populations, whereas a slight variation may reduce bacterial growth efficiency. The reason for this is a reduction in bacterial enzyme reaction rates. For many bacteria found in cooling water systems, the optimum temperature for maximum growth is about 40°C [22].

3.1.2. Surface topography

Surface topography greatly influences the ability of bacteria to adhere to a surface. During the initial steps of colonization, surface roughness at nanoscale and microscale levels enhances the adhesion of bacteria to substrates by providing more surface area for cell attachment. Surface roughness reduces the shear force on bacterial cells and communities present in flowing liquids at high flow rates, such as water pipes in industrial plants. A material surface exposed in an aqueous medium will inevitably become conditioned or coated by polymers from the medium,

and the resulting chemical modification will affect the rate and extent of microbial attachment. Moreover, other factors such as charge, hydrophobicity and elasticity are also influential in microbial attachment [23].

3.1.3. Velocity, turbulence and hydrodynamics

The area from the surface where no turbulent flow is experienced is known as the boundary layer. Within this area, the flow velocity has been shown to be insufficient to remove biofilms. The area outside this layer is characterized by high levels of turbulent flow and has an influence on the attachment of cells to the surface. The size of the boundary layer is dependent on the flow velocity of water. At high velocities, the boundary layer decreases in size and the cells are exposed to a high turbulence level. Hydrodynamic conditions can influence the formation, structure, EPS production, thickness, mass and metabolic activities of biofilms [24].

3.1.4. Gene regulation and quorum sensing (QS)

Studies have shown that up-regulation and down-regulation of a number of genes are involved in the initial attachment of cells with the substratum. Approximately 22% of genes were up-regulated and 16% were down-regulated in the biofilm formation of *Pseudomonas aeruginosa* [25]. In addition, *algD*, *algU*, *rpoS* and genes controlling polyphosphokinase synthesis were also up-regulated in the biofilm formation of *P. aeruginosa* [23]. Biofilms of *Staphylococcus aureus* were up-regulated for genes encoding enzymes involved in glycolysis or fermentation, such as phosphoglycerate mutase, triphosphate and alcohol dehydrogenase [26]. Cell-to-cell signaling, also termed *QS*, has recently been proven to play a significant role in cell attachment and detachment from biofilms. Growth and development of biofilms on different surfaces are mediated by a density-dependent chemical signal released by bacterial cells densely packed with an EPS matrix. QS makes use of a transcriptional activator protein that acts in concert with small autoinducers (AIs) signaling molecules to stimulate expression of target genes, resulting in changes in chemical behavior. After accumulation of sufficient AIs, this form of intercellular communication serves to coordinate gene expression, morphological differentiation and the development responses of bacterial cells [27].

3.1.5. Production of extracellular polymeric substances (EPSs)

Extracellular polymeric substances (EPSs) are a complex mixture of high-molecular-weight polymer ($M_w = 10,000$) excreted by microorganisms, products from lysis and hydrolysis as well as adsorbed organic matters from wastewater. Generally, EPSs have been shown to be a rich matrix of polymers, including polysaccharides, proteins, glycoproteins, DNA oligomers, phospholipids and humic acids [28]. EPSs are also highly hydrated because they can incorporate large amounts of water into their structure by hydrogen bonding. EPSs are typically reported to aid in the formation of a gel-like network that keeps bacteria together in biofilms due to bridging with multivalent cations and hydrophobic interactions. In addition, EPSs also cause the adherence of biofilms to surfaces, flocculation and granulation, protect bacteria against noxious environmental conditions and enable bacteria to capture nutrients from the

surroundings [29]. Different biofilms produce different amounts of EPSs, and the amount of EPSs increases with the age of biofilms [30].

3.1.6. Extracellular DNA (eDNA)

Extracellular DNA (eDNA) has been reported to be a major constituent of various single and multispecies biofilms. eDNA or naked DNA is a central part of bacterial self-produced extracellular polymeric substances (EPSs) and has similarity to chromosomal DNA in its primary sequence [31]. Its role is very important in various stages of biofilm formation, such as initial bacterial adhesion, aggregation and microcolony formation that favors wastewater treatment. eDNA also helps strengthen biofilms, provides protection to biofilms from physical stress, antibiotics and detergents as well as serves as an excellent source of nutrients for biofilm growth [32]. In addition, eDNA can be utilized in engineering of biofilms for beneficial purposes, such as remediation of environmental pollutants and electricity or fuel production in bioelectrochemical systems or bioreactors.

3.1.7. Divalent cations

Divalent cations such as Ca^{2+} are abundant in terrestrial and aquatic environments; therefore, calcium may be one of the factors that bacteria sense during biofilm-associated growth. Recent studies showed that eDNA chelates divalent cations that help in the modification of bacterial cell surface properties and thus favor resistance of biofilms to detergents and antimicrobial agents [33]. Divalent cations, such as those of calcium, play a critical role in the initial attachment of microbial aggregates of activated sludge flocs, anaerobic sludge granules and biofilms by bridging negatively charged sites on extracellular polymers [34]. Recent studies have shown that the thickness of a biofilm can be enhanced by introducing more divalent cations, as a result of which the biofilm becomes denser and mechanically more stable [35]. Calcium has been found to not only act as a cofactor for certain proteins but also act in cell signaling, biofilm virulence, cellular and extracellular product formation and alginate regulation [36].

4. Biofilm in wastewater treatment

Biofilm system is a well-developed technology in which solid media are added to suspended growth reactors to provide attachment surfaces for biofilms, so as to increase the microbial concentration as well as rates of contaminant degradation biofilms to take advantage of a number of removal mechanisms, including biodegradation, bioaccumulation, biosorption and biomineralization [8]. The microbial communities in the biofilm break down different nutrients, such as phosphorous and nitrogen-containing compounds, carbonaceous materials as well as trapped pathogens from the wastewater. Once pollutants are removed, treated water of a biofilter is either released to the environment or used for agriculture and other recreational purposes. Removal of the pollutants from wastewater by biofilm on the filter media is schematically represented in **Figure 8**.

Wastewater treatment with biofilm systems has several advantages, including operational flexibility, low space requirements, reduced hydraulic retention time, resilience to changes in the environment, increased biomass residence time, high active biomass concentration, enhanced ability to degrade recalcitrant compounds as well as a slower microbial growth rate, resulting in lower sludge production.
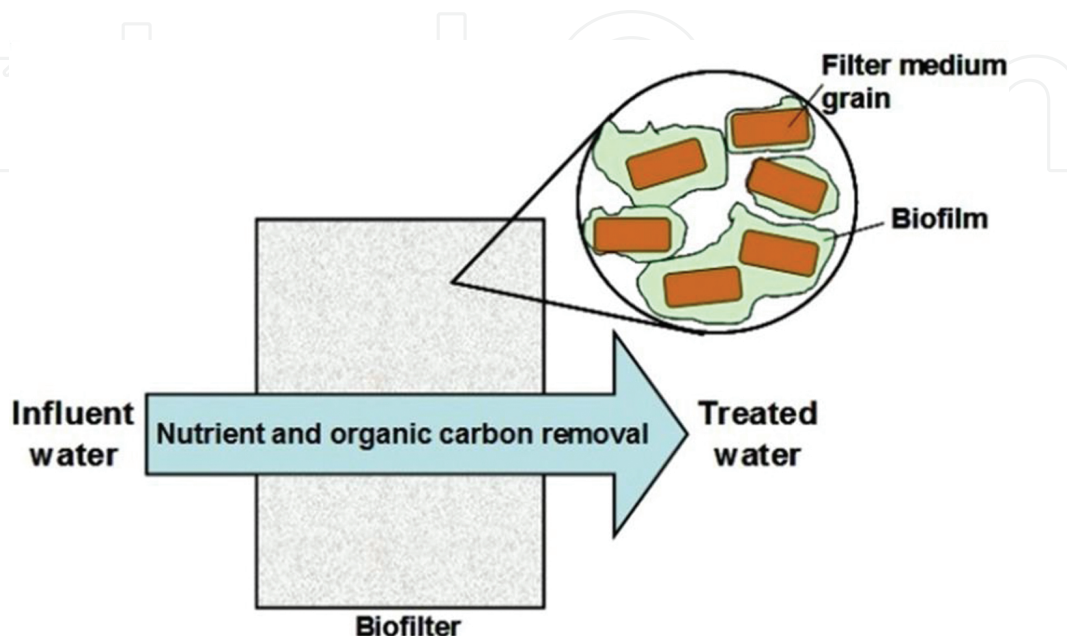

Figure 8. Removal of the pollutants from wastewater by biofilm on the filter media [37].

5. Biofilm development on different filter media

Packing or filter medium is the basic unit of attached growth wastewater treating technologies. It provides a surface for the growth of the biofilm. The filter medium needs to be durable, insoluble and resistant to chemicals. Its selection is based on size, porosity, density as well as resistance to erosion and chemicals [38]. The ideal medium provides a high specific surface area, low cost and porosity high enough to avoid clogging and promote ventilation. The surface area and geometry of the support materials affect the hydrodynamic conditions in the reactor and thus affect biofilm formation, which in turn affects wastewater treatment [39, 40]. Presently, different synthetic and natural materials have been employed. Various researchers have used polystyrene [41], polypropylene [42], tire-derived rubber [43] and pebbles [44, 45] as bio-filter media in fixed biofilm reactors for wastewater treatment. The chemical composition of the filter media is very critical, with respect to its compatibility with the developing biofilms; its elemental composition should be evaluated. For the detection and quantification of the elements in a filter medium, different spectroscopic techniques can be applied, such as X-ray photoelectron spectroscopy (XPS) and energy-dispersive X-ray spectroscopy (EDS or EDX or XEDS). XPS is a surface chemical analysis technique used to

analyze the surface chemistry of a material. It measures the elemental composition at the parts per thousand range, empirical formulas, chemical state and electronic state of the elements that exist within a material [46]. On the other hand, EDS is a useful technique applied for the elemental analysis/chemical characterization of filter media [47].

6. Biofilm community characterization approaches

The following subsections discuss various biofilm community characterization approaches.

6.1. Traditional methods

6.1.1. Determination of biofilm weight (wet weight and dry weight)

Biofilm weight can be determined in terms of dry weight and wet weight by using a digital weighing balance. The wet weight of the biofilm is measured after soft rinsing with distilled water. However, the dry weight of the biofilm is estimated by allowing it to dry under aseptic conditions in laminar flow until the attainment of the constant weight of polypropylene and polystyrene filter media [41, 42]. On the other hand, natural filter media, such as rock, granite or stone media, should be dried in the oven at 60°C to constant weight [44]. The weight of the biofilm is then calculated from the difference between the weight of medium with biofilm and that of medium without biofilm.

6.1.2. Determination of the biofilm optical density (OD)

The biofilm is also measured by the OD method. The filter media supporting biofilms are first rinsed with sterilized water to ensure the removal of any material on their surface. The biofilm is then removed from the filter media in 0.9% saline by sonication for 15 min. Finally, the spectrophotometric absorbance of dissolved biofilms is recorded at 550 nm wavelength (OD_{550}) using saline as blank [41, 42, 44].

6.1.3. Determination of heterotrophic plate count (HPC)

The HPC concentration (HPC/mL) of biofilms on filter media is determined by the conventional serial dilution method. The biofilm dissolved in 0.9% saline is serially diluted (up to 10^{-5}) and then spread on the selective growth media plates and incubated at 37°C for a specific period (24–48 h). The microbial growth appearing on specific media is enumerated in terms of HPC/mL (pathogen indicators). Pure cultures from these plates are further identified by colony morphology as well as microscopic and biochemical tests.

6.1.4. Microscopic analysis of biofilms

Non-invasive microscopic techniques provide a more accurate way of visualizing biofilms without disturbing their structure. The traditional microscopic techniques involve light microscopy (LM) and electron microscopy (SM), used for imaging analysis of biofilm sam-

ples. However, scanning electron microscopy (SEM) is a well-established fundamental technique to examine the morphology of bacteria and the topography of the material surface, and it is even capable of demonstrating the relation of biofilms to surfaces. On the other hand, other new advanced techniques have been established, including laser scanning microscopy (LSM), confocal laser scanning microscopy (CLSM), magnetic resonance imaging (MRI) and scanning transmission X-ray microscopy (STXM). These new techniques allow *in situ* analysis of the structure, composition, processes and dynamics of microbial communities. These techniques represent powerful tools for the examination of mixed microbial communities, those usually in the form of aggregates and biofilms [48].

6.1.5. Determination of biofilm activity

The metabolic activity of the microorganisms constituting biofilms can be estimated by considering the rate of the conversion of the specific substrate after inoculation with the seed of the biomass. For example, the physiological activity of *Nitrosomonas* spp. can be determined by measuring the strength of the nitrites (NO_2-N) formed in the growth medium from the known concentration of $(NH_4)_2SO_4$ after a specific period [49]. Similarly, the removal of carbonaceous (COD and BOD) and nitrogenous (NH_4—N) pollutants by biofilms can be estimated.

6.2. Advanced methods

6.2.1. Clone library technique

Cloning and sequencing of the 16S rRNA gene have been extensively and successfully employed for the study of microbial biofilms since the beginning of the 1990s, and this technique is still most widely used [50]. The cloning methodology for studying a biofilm community involves (1) extraction of the nucleic acid from the biofilm sample; (2) amplification of the 16S rRNA gene by polymerase chain reaction (PCR), usually using universal primers for bacteria or archaea, for obtaining a mixture of rDNA copies of the microorganisms; (3) cloning of the PCR products into an appropriately high number of copies of plasmid and then transformation of competent *Escherichia coli* cells with this vector; (4) selection of the transformed clones on the basis of an indicator contained in the plasmid; (5) extraction of the plasmid DNA from the colons; (6) creating a cone library by sequencing of the cloned gene and finally (7) identification and affiliation of the isolated cloned sequence with the aid of phylogenetic software and various dedicated computer programs (ARB, Seqlab, PAUP, PHYLIP).

These illustrate that the clone library method allows complete 16S rRNA sequencing and identification with very precise taxonomic studies of both cultured and non-cultured microorganisms in biofilms, design of primers for PCR and probes for fluorescence *in situ* hybridization (FISH) [51]. However, cloning is a time-consuming method, impractical for a high sample throughput and non-quantitative; in addition, extraction of a DNA pool from a microbial community can be difficult and the PCR steps are also biased. Furthermore, this technique needs specialized personnel and equipment [52]. In general, cloning and rRNA gene

library construction have been applied in combination with other advanced techniques in wastewater treatment for the exploration of biofilm communities.

6.2.2. Microbial fingerprinting methods

Microbial fingerprinting methods provide the overall profile of a biofilm community by making a distinction between microorganisms and groups of microorganisms on the basis of their distinctive characteristics of a universal component/section of a biomolecule, such as phospholipids, DNA or RNA [52, 53]. These methods include phospholipid fatty acid analysis (PLFA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP).

6.2.2.1. Phospholipid ester-linked fatty acid analysis (PLFA)

Phospholipids are a structural component of all cell membranes, but their type and proportion are distinctive to different microorganisms and break down rapidly upon cell death. Thus, the mass of PLFAs in a biofilm sample is directly proportional to viable biomass. Some groups of organisms have unique or "signature" types of PLFA [54]. PLFA analysis of the biofilm involves (1) extraction of phospholipids from the biofilm sample; (2) separation by gas chromatography with flame ionization detection; and, if required, (3) confirmation and identification by mass spectroscopy. It is not a good choice as a stand-alone method and can be combined with stable isotope probing (SIP). The SIP technique includes (1) incorporation of the stable isotope label (typically ^{13}C) into biomass, (2) incubation of microorganisms to metabolize for a specific time, (3) extraction of biomolecules from the incubated biofilm sample, (4) quantification of the extracted biomolecules by ^{13}C-PLFA using GC/IRMS and separation of unlabeled nucleic acids by density gradient ultracentrifugation and (5) identification of the genes/microorganisms by PCR or fingerprinting or sequencing.

6.2.2.2. Denaturing gradient gel electrophoresis (DGGE)

DGGE is a nucleic acid-based technique employed to generate a genetic fingerprint of a complex microbial community [51]. It encompasses the following steps: (1) extraction of the DNA or RNA from the biofilm sample; (2) amplification of the extracted nucleic acids by PCR, generating a multitude of copies of a variable region within a target gene usually with universal primers to give a mixture of DNA fragments, all of the same length and each representing a species present in the original sample; (3) separation of the DNA mixture by denaturant gradient electrophoresis on an acrylamide gel with an increasing urea/formamide gradient, with the DNA molecules migrating toward the positive pole and halting on the gel upon reaching their corresponding denaturant force (T_m), depending on the DNA sequence, with every band on the gel corresponding to a different microorganism in the sample; (4) visualization of these bands and (5) sequence identification by excision of the individual "bands" from the gel and its comparison with the 16S rDNA database for the phylogenetic affiliation of the microorganism.

DGGE is the fastest and most economical way of comparing large numbers of samples without culturing on expensive media, isolations and analysis, and it permits rapid/simple monitoring of the spatial-temporal distribution of microbial populations by only considering band. However, depending on the nature of the sample, extraction and amplification of representative genomic DNA can be difficult. The DNA copy number, proportional to the abundance of a particular microorganism, can be very different after amplification by PCR, and thus the intensity of the bands on a DGGE gel is not quantitative. Furthermore, the sequences of the bands obtained from a gel correspond to short DNA fragments (200–600 bp), and so phylogenetic relations are less reliably established and short sequences are less useful for designing new specific primers (for PCR) and probes (for FISH).

6.2.2.3. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a nucleic acid-based method and provides the profile of a microbial community, which is used to identify specific microbial populations [55]. It has four steps: (1) total community DNA or RNA extraction from a sample; (2) PCR amplification with a fluorescent PCR primer to make multiple copies of a target gene; (3) enzymatic digestion of the PCR products with restriction enzymes to cut the DNA molecule at known sequences, indicative of a specific microorganism and finally (4) fragment identification by electrophoretically separating the amplified gene sequences of different sizes.

Furthermore, it is also possible to sequence and identify the generated sequences by comparison with a sequence database. The strength of the fluorescent signal yields additional information regarding the abundance of the different species, similar to the band intensity in the patterns of a DGGE gel. T-RFLP offers more sensitivity than DGGE, and it may detect the lower number sequences in a sample and is commercially available. However, sometimes, the heterogeneous size of fragments makes phylogenetic analysis less confident [56].

6.2.3. Fluorescence in situ hybridization (FISH)

For FISH, the most commonly used target molecules are 16S rRNA, 18S rRNA, 23S rRNA and mRNA. FISH is an excellent method for the identification, localization, visualization and quantification of non-cultured microorganisms in their microcosm. The specificity of the florescent probe enables detection/identification on any desired taxonomic level, from domain down to a resolution suitable for differentiating between individual species [57].

FISH is carried out in a few steps: (1) the specimen is fixed by precipitating agents (ethanol or methanol), cross-linking agents (aldehydes) or a mixture depending on the target organism and the type of sample; (2) the sample is prepared, with the process including specific pretreatment steps. For better attachment of specimens to glass slides, their surfaces should be treated with coating (gelatin, poly-L lysine) or silanising agents; (3) hybridization is directly carried out on the fixed sample by addition of a mixture of salts, formamide, detergents and fluorescent probe in a dark humid chamber, usually at temperatures between 37°C and 50°C. Its time varies between 30 min and several hours; (4) slides are rinsed with distilled water to remove unbound probe, dried and mounted and (5) visualization and documentation of

results are carried out with a conventional epifluorescence microscope for multicolor FISH. However, a charged coupled device (CCD) camera and appropriate image analysis software can be used for the digitalization/manipulation of images, enumeration of microorganisms and measurement of the activity of single cells in biofilms by quantification of their rRNA content.

On the other hand, CLSM is used with FISH analysis for thick samples with a high background (sludge flocs, biofilms) and for obtaining three-dimensional images. Different software packages are also available.

FISH is an easy and fast technique, and, if required, probes are available for direct visualization and quantification of microorganisms. This technique is apt for routine analyses, highly trained/specialized personnel are not necessary, and only basic knowledge of microscopy and laboratory experience is required. However, prior knowledge of the microbial habitat/ environment conditions and the target microorganisms to be detected is necessary. The rRNA sequence for a particular microorganism to be detected and quantified must also be known [51].

FISH is a widely applied technique and can be combined with other techniques to increase its sensitivity and upgrade it to overcome some of its pitfalls. FISH-based methods have revolutionized investigations into the morphology and microbial composition of biofilms and enable bacteria to be mapped [58]. These methods include FISH-MAR (FISH with micro-autoradiography, CARD-FISH (FISH with catalyzed reporter deposition), Clone-FISH (FISH preceded by generating the expression of the 16S or 18S rRNA targeted gene), CLASI (combinatorial labeling and spectral imaging with FISH), DOPE-FISH (double labeling of oligonucleotide probes with FISH), RING-FISH (recognition of individual genes with FISH), DVC-FISH (FISH with direct viable count) and RCA-FISH (FISH with rolling circle amplification). In the FISH-BrdU method, identification of the microbes is carried out by using 5-bromo-2'-deoxy-uridine (BrdU) without any need for paraformaldehyde for cell fixation or formamide for DNA denaturation. In a technique called Spike-FISH, quantification based on an internal standard (*E. coli*) is introduced by spiking the biofilm samples with known amounts of *E. coli* cells. In RAMAN-FISH, Raman microspectroscopy is combined with FISH. NanoSIMS is based on the visualization of oligonucleotide probe-conferred hybridization signals in single microbial cells and isotopic measurement using high-resolution ion microprobes [58].

6.2.4. DNA microarray technology

DNA microarray technology detects hundreds or even thousands of DNA sequences simultaneously and rapidly [59]. It involves (1) extraction of DNA from the sample, (2) amplification by PCR, (3) direct hybridization of the amplified PCR products from total DNA to known molecular probes attached on the microarrays and (4) scoring of positive signals using CLSM after hybridization of the fluorescently labeled PCR amplicons to the probes. Generally, the hybridization signal intensity on microarrays is directly proportional to the abundance of the target organism. The main pitfalls of this technique are cross-hybridization and that it is not useful in identifying and detecting novel prokaryotic taxa. Moreover, if the genus does not

have a corresponding probe on the microarray, then the biological significance of a genus could be totally missed. The application of this technique is comparatively less in the study of wastewater treating biofilms.

6.2.5. Next-generation sequencing (NGS) technology

NGS, such as pyrosequencing, is a novel DNA sequencing technology developed at the Royal Institute of Technology (KTH) based on the sequencing-by-synthesis principle [60] and on the detection of released pyrophosphate (PPi) during DNA synthesis [61]. This technology transforms microbial ecology, explores deeper layers of microbial communities and is vital in presenting an unbiased view of the composition and diversity of communities [62]. NGS platforms such as Roche/454, Illumina/Solexa, Life/APG and HeliScope/Helicos BioSciences are much faster and less expensive than the first-generation Sanger sequencing technology [63].

The steps in pyrosequencing techniques include the following:(1) extraction of the DNA from the biofilm samples; (2) quantification and detection of the purity of the extracted DNA using a NanoDrop spectrophotometer; (3) amplification of the sample 16S rRNA gene by using universal PCR primers (28F and 519R) and incorporation of different barcodes between the 454 adaptor and the forward primer, with the duplicate PCR products pooled and purified using the QIAquick Gel Extraction Kit; (4) use of the purified PCR products for pyrosequencing and then ligation of short adaptors onto both ends for the segregation of the sequences; (5) attachment of the modified products to DNA capture beads, followed by emulsion-based clonal amplification, with the beads set into the wells of a PicoTiterPlate device, with appropriate chemicals, four enzymes (DNA polymerase, ATP sulfurylase, luciferase, apyrase), adenosine 5′-phosphosulfate (APS) and luciferin, and then inserted into the Genome Sequencer according to the manufacturer's directions to record programs; (6) preprocessing of all partial 16S rRNA gene sequences using the pyrosequencing pipeline at the Ribosomal Database Project (RDP) to trim barcodes, remove primers from the partial ribotags and discard low-quality and short (<250 bp long) sequences; (7) denoising and assemblage of the sequences into clusters using the precluster command, thus generating the FASTA file data sets (*.fna and *.qual files) and (8) further analysis of these sequences through MOTHUR, with MOTHUR analysis pipeline and R-Scripts used to start sequencing the taxonomy and analyze the data

The technique of pyrosequencing has the potential advantages of accuracy, flexibility, parallel processing and easy automation. It has no need for labeled primers, labeled nucleotides and gel electrophoresis. It has been successful for both confirmatory sequencing and *de novo* sequencing [61].

7. Conclusions

Of the different wastewater treatment technologies, biofilm-based systems have potential advantages. For better designing of these biofilm wastewater treatment systems, knowledge

about the composition of filter media and developing biofilms is highly necessary. The composition and quantification of different elements in filter media can be determined using spectroscopic techniques, such as X-ray photoelectron spectroscopy (XPS) and energy-dispersive X-ray spectroscopy (EDS). The traditional methods used for the study of biofilms include analyses of their gravimetric weight spectroscopic absorbance, substrate utilization activity and viable plate count as well as microscopic techniques. Complete biofilm community profiling is carried out by advanced techniques such as microbial sequencing, clone library generation, genetic fingerprinting, DNA microarray, denaturant gradient electrophoresis (DGGE) and next-generation sequencing (NGS), on the basis of their availability, to increase the performance, stability and robustness of biofilm reactors.

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