Chapter 10
Disinfection Technologies for Household Greywater

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Abstract The treatment technologies for greywater are followed by the disinfection processes in order to achieve safe disposal into the environment. The disinfection technologies aim at reducing or minimising the concentrations of the pathogenic microorganism of greywater which have a high potential risk for humans and plants, and, thus, provide safe and aesthetically acceptable greywater that is appropriate for the purpose of irrigation. The disinfection processes include chemical (chlorination and ozonation), physical or mechanical (filtration process) and radiation disinfection (UV irradiation, solar disinfection (SODIS)). The degree of the disinfection process proposed must take into account the type of reuse and the risk of exposure to the population. In this chapter, the disinfection techniques of greywater are reviewed and discussed based on their efficiency to eliminate the pathogenic bacteria and other toxic by-products. The objective of this chapter was to discuss the advantages and disadvantages of disinfection processes. Among the several disinfectant technologies for greywater, SODIS appears to be the most potent technology which is widely applicable in most of the developing countries experiencing arid and semi-arid

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atmospheric conditions due to the high density of sunlight which is more effective for inactivating pathogenic microorganisms.

Keywords SODIS · AOPs · Pathogenic bacteria · PGP · Non-culture methods

10.1 Introduction

The disinfection of greywater is the last stage of the greywater treatment process. It should be conducted after the suspended solids and organic matter are removed to enhance the inactivation of pathogenic organisms. In the disinfection process, the greywater is subjected to the chemical and physical processes in which the concentrations of the infectious agents are reduced to less than the detection limits. However, this process exhibits different efficiency in the inactivation percentages which depends on the mechanism of the inactivation process as well as the characteristics of the greywater. The concept of the disinfection processes is quite different for the sterilisation processes which aimed to irreversible inactivation of the pathogens. The disinfection technologies are divided into chemical (chlorination and ozonation) physical or mechanical (filtration process, heat and pasteurisation) and radiation disinfection (UV irradiation, SODIS) or combination of these techniques. In the physical disinfection process, the pathogens are inactivated as a result of the destruction of the cells, where the target is to damage the cell morphology and then their functions. In the filtration techniques, the pathogens are adsorbed on the surface of the filter as live cells. In contrast, the chemical disinfection acts by inactivation of the metabolic and anabolic pathways by the inactivation of the main enzymes used in these processes. The radiation disinfection acts by the damage of the DNA of the cells which may lead to the inhibition of the protein synthesis and the cell growth. The degree of disinfection process proposed must consider the type of reuse and the risk the exposure will pose to the population. It has to be mentioned that the microbial loads in the greywater are fewer than that in the blackwater. Therefore, the disinfection processes provide the alternative resource for the water which might be used for toilet flushing or car washing (WHO 2006). Moreover, the effectiveness of the disinfection process depends on the occurrence of regrowth in the disinfected greywater. This chapter focuses on reviewing various disinfection techniques of greywater based on the efficiency of the treatment system to eliminate the pathogenic bacteria and other toxic by-products. The advantages and disadvantages of the techniques used for the inactivation of pathogens in the greywater as well as the detection of the potential of pathogenic growth in the disinfected greywater are discussed.

10.2 Disinfection Technologies of Greywater

The disinfection of the greywater is a treatment process that has been widely reported by several authors in the literature. Many of the technologies have been suggested for the reduction of infectious agents such as pathogenic bacteria, parasites and viruses. However, the reduction of bacteria in the treated greywater is the major challenge confronting researchers due to their ability to regrow in the disinfected greywater. This is because they have no intermediate hosts compared to the parasites and viruses. Therefore, the disinfection processes should have the potential to reduce the bacterial cells to be lower than the detection limits. There are some concepts which indicate that the pathogens should be reduced to less than their infective dose, but there is only limited dose–response information available for pathogenic bacteria and viruses, while the infective dose for most of the pathogens still remains unconfirmed because it depends on the immunity of the host (Rowe and Abdel-Magid 1995). The most common technologies used in the disinfection of greywater are presented in the next session.

10.2.1 Filtration Systems

The filtration system is a simple and inexpensive technology for the removal of pathogens from the water. One of the effective filtration systems is the slow sand filtration system. The principle of the system depends on the passing of the water or greywater slowly through a chamber or multi-chambers consisting of a bed of porous sand and gravel layers. The efficiency of this system in removing the pathogenic cells depends on the pores size and flow rate since the rapid filtration results in less removal efficiency. This slow sand filtration system is more applicable in the developing countries as a non-central system. The sand filtration removes the pathogens by decrease the pore size due to trapped of the pores by the particles presented in the greywater to be less than the diameter of the pathogens cell size. However, the increase in the level of suspended solids might lead to clogging of the pores and reduce the removal efficiency. The biofilter system has also been used as the alternative technology for the removal of pathogens and biodegradable organic matters simultaneously (Haarhoff and Cleasby 1991). In this system, the degradation of organic matter takes place in the natural microorganisms present in the sand, which might act as decomposers for the organic matter from the greywater.

The mechanical filtration system is effective for the removal of organic matter but not for the inactivation of pathogenic bacteria, which are active even after the separation from the greywater (Al-Gheethi et al. 2016). Therefore, the use of membrane bioreactors such as membrane chemical reactor and membrane bioreactor (MBR) is getting research attention by authors. In this system, two or more of the processes are combined such as aerobic reactor with UV and titanium dioxide. Winward et al. (2008) indicated that this system removed TC and *Pseudomonas*

aeruginosa by 4.0 and 2.0 $\log_{10}$ CFU 100 mL^{-1}, respectively. Jong et al. (2010) revealed that this system removed *Escherichia coli* by 67.5, *Staphylococcus aureus* by 27.7 and *Salmonella typhimurium* by 20.4%. The submerged membrane bioreactor (SMBR) system which is another system of the bioreactors exhibited 99.99% of the TC and FC removal within 42 days of treatment (Bani-Melhem et al. 2015). These findings indicate that the filtration system has better efficiency for the removal of pathogens but the limitations are its need for prolonged periods to achieve high removal percentage and their less efficiency in the highly polluted wastes.

10.2.2 Chemical Disinfection

The chemical disinfection techniques include bromine chloride, chlorine, calcium hypochlorite, hydrogen peroxide and ozone. Chlorination is the most common process which is used extensively for the disinfection of water and wastewater due to the low cost and the simple usage as well as its effectiveness for the inactivation of most of the infectious agents (Ottosson 2003). The mechanism in which the chlorine deactivates the pathogenic cells is through the generation of chlorine radical which acts as the oxidation of organic components of the cells such as enzymes. The behaviour of the pathogens in the greywater for the disinfection through chlorination depends on the organic matter present in the water, since the high contents of the organic compounds might consume the chlorine by the oxidation reactions, and thus the chlorine residues are not enough to achieve high reduction on the microbial loads (Al-Gheethi et al. 2016). In order to overcome this challenge, some of the authors suggested that a preliminary treatment process for the removal of the organic content should be undertaken before chlorination (Santasmasas et al. 2013). The turbidity of the greywater might also affect negatively the disinfection efficiency, therefore a double dose of chlorination is needed (Mohamed et al. 2015).

The adverse effect of chlorination is the toxicity to aquatic life as a result of the presence of free and combined chlorine residues which are often classified as carcinogenic compounds. Some of these compounds include nitrosodimethylamine (NDMA), trihalomethane (TTHM) and haloacetic acid (HAA) (Cantor et al. 1987; Pehlivanoglu-Mantas et al. 2006). Therefore, in the USA, chlorination is not used for the disinfection of drinking water, although it is still in use in most of the developing countries due to its low cost. Other limitations for the utilisation of chlorination are the microbial resistance and the regrowth which is associated with the highly reactive characteristics that may lead to accelerating the chlorine decay process (Tal et al. 2011). This limitation occurs more in the antibiotic-resistant bacteria since the resistance to the antibiotics is correlated with that for chlorine (Shi et al. 2013). In greywater, the potential of the pathogenic bacteria to resist for chlorine might be more than the one for the bacteria in the drinking water because the chlorine is available in the greywater generated from the washing machines and kitchens. As a result, the bacteria have developed a mechanism for the resistance of chlorine residues and became more resistant during the disinfection process by the chlorine (Al-Gheethi et al. 2016).

The chlorine has less efficiency against parasites and protozoa organism and therefore, the ozonation is the alternative most effective method for inactivating protozoan cysts (Robertson et al. 1994). Ozonation is one of the most efficient methods for the disinfection of waters due to the high oxidative potential of ozone which could lead to the destruction of semipermeable membrane of the cells and, as a result, bacterial cell death (Facile et al. 2000). The extensive use of the ozonation is due to the cheap and low energy needed for the inactivation as well as the fast reactions which can reduce the pathogenic bacteria cells by 98% in less than 5 min (Tripathi et al. 2011). In the greywater, the factors which might affect negatively on the efficiency of ozonation are the chemical oxygen demand (COD) and total suspended solids (TSS). Both parameters might also induce the bacterial resistance and the regrowth in the disinfected greywater (Janex et al. 2000; Xu et al. 2002). Nevertheless, the occurrence of toxic by-products by ozonation such as aldehydes, bromate ions (BrO_3) and peroxides has also been reported (Vital et al. 2010).

10.2.3 UV Irradiation

The application of UV irradiation is one of the radiation disinfection processes which is used for the greywater reuse excluding the toilet flush water due to the high contents of the suspended solids which prevent the penetration of UV. This technology is more appropriate for the greywater treated with constructed wetland (Lindgren and Grette 1998). UV irradiation is an advanced disinfection method, in comparison with chlorination, and has several advantages including the applicability for small-scale treatment plants without the need for dosing apparatus or a storage process of disinfected greywater as well as the absence of toxic by-products (USEPA 2003).

The mechanism in which UV deactivate the pathogen cells lies in the localisation lesions in DNA as a result of the mutation caused by the formation of pyrimidine dimers particularly thymine (Smith and Hanawalt 1969). The selection of UV with 260 nm for the disinfection of water and inactivation of microbial cells was based on the laboratory results which revealed that the high absorption spectrum of DNA by the spectrophotometer is 260 nm (Setlow 1968). UV disinfection occurred due to the high reduction of many of the pathogenic bacteria, however, other bacterial species have also exhibited a resistance for the UV action, due to their ability to repair the damage caused in the DNA by UV. The disinfection of the greywater using UV might be more applicable in terms of the absence of residual disinfection by-products as in the case of chlorination. In the drinking water, the presence of residual chlorine in the disinfected water might be necessary in order to face any possible contamination. Conversely, these precautions might also be necessary for the disinfected greywater to prevent the regrowth of inactivated pathogens since the chlorination would not totally destroy them, and some might remain available in the dormant state and might grow back in the absence of residual chlorine as in the disinfected greywater with UV. Therefore, the UV technology is an alternative for

the chlorination in terms of the absence of toxic by-products, but the chlorination might be the best option for the microbial regrowth (Chang et al. 1985).

In fact, the authors have demonstrated regrowth of pathogens inactivated by UV or chemical disinfections. In the UV disinfection, the regrowth appears as a result of dark repair of damages caused by the UV in the DNA structure. In contrast, in the case of the chemical disinfection, the regrowth occur due to the oxidation process of organic matter which could lead to producing AOC which are nutrients inducible for the microbial growth (Al-Gheethi et al. 2015). Gilboa and Friedler (2008) studied the UV disinfection kinetics and the efficiency for the inactivation of HPC, FC, *S. aureus* and *P. aeruginosa* as well as the survival and regrowth of these pathogens in the disinfected greywater. The greywater samples were first treated with rotating biological contactor (RBC) followed by sedimentation. The inactivation rate coefficient of UV with 69 mWS cm^{-2} of the dose was 0.0687, 0.201 cm^2 $mW^{-1}S^{-1}$ for FC, 0.113 for HPC, 0.129 for *P. aeruginosa* and 0.201 cm^2 $mW^{-1}S^{-1}$ for *S. aureus*. Among these pathogens, FC exhibited the highest resistance for the UV, the microscopic examination indicated that was due to the FC self-aggregate in the greywater. FC, *S. aureus* and *P. aeruginosa* have no regrowth in the next 6 months of the disinfection process with UV doses (19–439 mWS cm^{-2}). In contrast, the regrowth of HPC in the disinfected greywater was explained because of the absence of the competition with other bacteria which were eliminated by the irradiation.

10.2.4 Solar Disinfection (SODIS)

The utilisation of SODIS appeared to be a promising disinfection technology for the greywater when these waters are used for toilet flushing or car washing. More attention and research should be conducted to evaluate this technology in the reduction of pathogens in the greywater if these waters will be used for irrigation of disposal for the natural water systems. Since some of the studies indicated that the efficiency of SODIS might be low against some pathogens due to the ability of these infectious agents to regrow after the disinfection process. SODIS is more applicable for the developing countries especially those located in the arid and semi-arid region because the solar radiation might reach more than at least 500 W/m^2 which is the minimum solar radiation required to achieve an acceptable reduction in the microbial load of the greywater. In SODIS, two or more factors are the keys for the inactivation of the pathogens which include the temperature and UV radiation. The photocatalysis by the visible light might also play an important role in the reduction of pathogens because this process could lead to the oxidation of organic matter and then the release of the inhibitory substances for pathogen growth.

The temperature is the main factor in the inactivation of pathogens cells because it leads to deactivation or destruction of cell enzymes, but it should be more than the optimal temperature required for the microbial growth. Since the ambient temperature has no significant role in the destruction of the pathogenic cell. One of the solutions used for increasing the temperature to be between 45 and 50 °C is to use transparent

polyethylene terephthalate (PET). However, the use of PET is not applicable for the large-scale treatment system and therefore the plastic bag SODIS reactors are the alternative method capable of absorbing a greater quantity of solar radiation and then more reduction of the microbes can be achieved in the water (Walker et al. 2004). The selection of plastic bag polymers depends on the potential of the polymers to transmit high quantities of UV and the stability at significant low unitary cost, therefore, the low-density polyethylene (LDPE) is the most commonly used. In order to achieve high inactivation, the LDPE-SODIS reactors are performed by washing it with H_2O_2 or TiO_2 (Sciacca et al. 2010; Ciavola 2011). In this manner, Harding and Schwab (2012) investigated the efficiency of SODIS with limes and psoralens to enhance the inactivation of *E. coli*. The study revealed that the limes and psoralens exhibited a synergistic effect with UV radiation to accelerate the reduction of microbes. The reduction of *E. coli* was >6.1 logs by SODIS coupled with lime slurry and 5.6 logs by SODIS coupled with lime juice within 30 min of solar exposure, while it was 1.5 log reduction by SODIS alone.

The UV of the sunlight leads to the destruction of the pathogen genome by the destruction of DNA bases. It has been reported that half of the lethal effect for the SODIS is attributed to UV wavelengths which are below 370 nm and the UV wavelengths between 370 and 400 nm as well as the blue–green visible spectrum between 400 and 500 nm (Gameson and Gould 1985). Moreover, it has been noticed by the authors that the SODIS is more effective against the actively metabolising cells, which means that the presence of bacterial cells in the dormant state might make it more resistant for the SODIS.

The effectiveness of SODIS in the inactivation of pathogenic cells belongs to the formation of the reactive oxygen, which leads to the destruction of cell membrane permeability, metabolic and anabolic pathways due to the irreversible destruction of the specific enzymes required in these pathways. Although some reports indicated that the harmful wavelength of the sunlight against the cells is lower than 280 nm, others have claimed that the sunlight has a photodynamic action (Al-Gheethi et al. 2015). This process acts through the induction of the oxidation reactions for the hydroxyl groups on the cell-wall and cell-membrane to generate hydroxyl radicals which may lead to damages of the functional group of the bacterial cells wall such as an absorption of nutrients and transport system through the cell membrane into the cytoplasm. Moreover, the bacterial cells usually grew as colonies and in the water, they grew as biofilm which means they are attached together by the slim layers, and the presence of sunlight might lead to oxidation of the polymers of these slim layers. The bacterial cells grown as a biofilm are more resistant to many of the stress environmental conditions while they are very sensitive as individual cells. Furthermore, unlike the damages caused by UV, the destruction of the DNA structure by the visible light cannot be repaired by the bacterial cells (Eisenstark 1971).

The efficiency of SODIS for the reduction of the indicator and pathogenic bacteria in different water and wastewater has been reported. Al-Gheethi et al. (2013) investigated the reduction of FC, *Enterococcus faecalis*, *Salmonella* spp. and *S. aureus* in the lake water and secondary effluents using SODIS. The samples were placed in the PET bottles for a period of 8 h. The study found that the FC, *Salmonella* spp. and

S. aureus were reduced by 4 $\log_{10}$ CFU/100 mL within 6 h of the SODIS process. However, this period was not enough to eliminate completely these pathogens, since the regrowth assay for these bacteria in the laboratory revealed the ability of the inactivated pathogens to grow in the culture media after the incubation of disinfected samples for 24 h at 37 °C. In contrast, for 8 h the SODIS reduced the level of pathogens to below the detection limits without regrowing after the assay on the culture media. Among the investigated bacteria, *E. faecalis* was not totally eliminated by SODIS even after 8 h. Moreover, the bacteria was more sensitive for the storage system where it has reduced to less than the detection limits (1 CFU/100 mL) after 16 days of the storage at room temperature. These findings indicate that the efficiency of the SODIS depends on the bacterial species. In some cases, the SODIS should be followed by the storage system in order to achieve the high reduction in the bacterial loads.

Bosshard et al. (2009) examined the effect of SODIS on *S. typhimurium* and *Shigella flexneri*, and the inactivation level of SODIS on the pathogens was evaluated based on efflux pump activity, cellular ATP levels, polarisation and integrity of the cytoplasmic membrane, as well as glucose uptake ability. The study revealed that the respiratory chain was the main target of sunlight and UVA irradiation. In order to study the behaviour of the inactivated bacteria after the SODIS, the pathogens were stored in the dark and the results indicated that the physiological state of the cells continued to deteriorate even in the absence of irradiation. These findings concluded that the investigated pathogens are very sensitive for SODIS and a small light dose (700 W m^2) might be enough to irreversibly damage the cells without the need for the chemical additives.

10.2.5 Advanced Oxidation Processes (AOPs)

Advanced oxidation processes (AOPs) are a combination of chemical and irradiation disinfection techniques and more specifically by photocatalytic processes. This technology has been used mainly for the degradation of non-degradable compounds in the wastewater such as XOCs, as discussed in Chap. 9. However, the AOPs are also efficient in the disinfection processes. Examples for the AOPs include the combination of titanium dioxide (TiO_2) and UV (TiO_2-UV). TiO_2 alone is effective against bacterial cells due to the formation of highly reactive hydroxyl radicals (Joo et al. 2005). Recently, TiO_2 is frequently used in the nanotechnology treatment of the water, due to the large surface area which can adsorb many of the pollutants from the water (Khalaphallah et al. 2012). However, one of the limitations of the application of TiO_2 in the large-scale water treatment process is the particle size and morphology of TiO2 practices (Yu et al. 2002).

The examination of different AOPs in the disinfection of water has been reported in the literature. Teodoro et al. (2014) investigated the efficiency of the photo-Fenton system (Fe^{2+}/H_2O_2 coupled with UV) as an advanced oxidation process for disinfection of greywater and inactivation of *P. aeruginosa*. The greywater was treated by a treatment system consisting of an evapotranspiration tank and constructed wetland

with the horizontal flow and *Heliconia psittacorum* L.f. In one experiment, the authors examined the efficiency of H_2O_2 150 mg L^{-1} coupled with UV (4.3 mW cm^{-2}) (H_2O_2/UV), while in another experiment Fe^{2+} was used with 10 mg L^{-1} and adjusted to pH at 3 and then used in coupled with UV (Fe/UV). The study found that both systems were most efficient in the total inactivation of *P. aeruginosa*. The authors concluded that the system of H_2O_2/ UV was the main factor for the inactivation of bacteria and the exertion of greater influence compared to the system with Fe/UV at low pH.

The mechanism in which photo-Fenton system deactivates pathogenic growth is explained based on the ability of UV to regenerate of Fe^{2+} ions by the photoreduction of $Fe(OH)^{2+}$, this reaction resulting to the hydroxyl radical (OH^-).

10.3 Detection of Inactivated Pathogens in Disinfected Greywater

The concentration of pathogenic bacteria in the disinfected greywater needs a critical and accurate method because these pathogens are variable with a lower concentration than the one that can be detected by the culture-dependent method, or that is available in a dormant state which failed to grow in the culture media. Therefore, the use of the enrichment methods might be the alternative option to detect the presence or absence of these pathogens. However, the overtime required for the detection and identification of pathogens might be extended for several days. Many of the authors have suggested the use of the molecular technique such as the quantitative polymerase chain reaction (qPCR). This technique is fast, but the limitations lie in the principle of the molecular analysis, which depends on the detection of the pathogens based on the nucleic acids. The molecular analysis might be affected by the free DNA fragments of the non-viable cells, resulting in over- or underestimation of bacterial densities (Bae and Wuertz 2009; Orlofsky et al. 2015). The combination of molecular and culture-dependent methods such as non-specific enrichment and the most probable number (MPN) is a useful tool to increase the likelihood of pathogen detection with low concentrations as in the case of disinfected greywater (Krämer et al. 2011; Russo et al. 2014). In this section, the techniques used for the detection of pathogenic bacteria have been reviewed.

The isolation of pathogenic bacteria from the environment is quite difficult and different from that of clinical samples. Many factors should be considered during the isolation procedure such as the selection of the best dilution, and enrichment media. However, in many cases, conventional techniques fail to isolate these pathogens. It has been reported that 99.9% of bacteria in the environment are still uncultured (Zubair et al. 2010). Moreover, the failure to isolate these bacteria from the environment on the culture medium might be due to the failure in replicating essential aspects of their environment such as the factors necessary for their growth (Stewart 2012). However, Straškrabová (1983) claimed that the bacterial cells died due to high nutrient shock.

Culture-based techniques are more effective in determining available pathogenic microorganisms in water and wastewater samples. Utilisation of direct plating, MPN or membrane filtration depends on the density of the pathogens in the samples. In contrast, molecular-based methods (PCR, antibody-based and metabolic-based) are quite useful in the identification of pathogenic species. However, they are not efficient in distinguishing between viable and non-viable pathogens. Moreover, culture-based methods can be used for the determination of health risks associated with the exposure to pathogens due to the presence of human virulence genes while molecular methods might be able to discern cell viability. Although the presence of virulence genes can also be determined by molecular methods, the deficiency lies in the absence of entire microorganisms which makes further characterisation of pathogens limited (Center and Warrenton 2007).

In a comparison study between cultures based method and the DNA-based methods, Benami et al. (2015) used both techniques to assess the presence of pathogenic bacteria in the disinfected greywater with chlorine and UV. The culture-dependent method indicated that the concentrations of *E. coli*, FC, *Enterococcus* sp. *S. aureus*, *Salmonella enterica* and *P. aeruginosa* have differed significantly in the disinfected greywater compared with the disinfected samples. Conversely, the culture-independent DNA-based method recorded no significant differences in the concentrations of these pathogens before and after the disinfection process. The study concluded that the inactivation efficiency of the disinfection processes angst of pathogens could not be estimated by DNA-based qPCR.

Bedrina et al. (2013) examined the efficiency of a combined magnetic immunocapture and enzyme immunoassay for the detection of *Legionella pneumophila* in water samples in comparison to the culture-based method. The method depends on the anti-*L. pneumophila* antibodies immobilised on magnetic microspheres. The results revealed that the method was more applicable for the fast detection of *L. pneumophila* in water samples without the need for bacterial growth on the culture medium.

PCR technique is the best and most successful technique for the identification of pathogenic bacteria since it depends on the nucleotide sequence of a DNA strand. The efficiency of this identification technique may reach up to 99.99% (Nissen and Sloots 2002). The main challenge lies in the determination of pathogenic bacteria or fungi concentrations. The determination of bacterial concentrations represents a very serious point in terms of pathogenicity which depends on the dosage of bacteria at which the bacteria might cause infections in humans.

Pathmanathan et al. (2003) investigated the potential of the PCR procedure based on the use of hilA primers for detecting *Salmonella* spp. In the study, 33 *Salmonella* strains and 15 non-*Salmonella* strains were used. The results revealed that PCR produced 784 bp DNA fragments in *Salmonella* strains but none in non-*Salmonella* strains. The detection limit of PCR was 100 pg based on the genomic DNA which is equivalent to 3×10^4 CFU mL^{-1} based on serial dilutions of bacterial culture. The use of the enrichment-PCR method has increased the sensitivity of the hilA primers' efficiency for a concentration of 3×10^2 CFU mL^{-1}. The study concluded that hilA primers are selective and specific for detecting *Salmonella* spp. in faeces through

the PCR method. However, the presence of *Salmonella* spp. with s concentration of 300 CFU mL^{-1} which was detected as a detection limit can be achieved through the direct culture method on a selective medium without the need for other enrichment methods or molecular techniques. Moreover, one of the issues in the detection of potent pathogenic bacteria such as *Salmonella* spp. and *Shigella* spp. in wastewater is when they are available in concentrations less than the detection limits of direct isolation. It is also an issue when there are high concentrations of other bacteria such as *E. coli*, FC, *Proteus* sp., *Pseudomonas* sp., *Klebsiella* sp. which might be similar to *Salmonella* spp., *Shigella* spp. colonies in terms of some morphological characteristics in their grown colonies on the selective medium. This is because even though the medium is selective for isolation specific pathogens, some other bacteria can also be grown there. In this case, the use of a more specific method is required to detect *Salmonella* spp. and *Shigella* spp.

Silbert et al. (2006) proposed a new technique for detecting bacteria based on the interaction between membrane-active compounds which are secreted by the bacterial cell and nanoparticles embedded in an agar medium which leads to the formation of phospholipids and the chromatic polymer polydiacetylene (PDA). The results revealed that the PDA changed from visible blue-to-red transformations alongside an intense fluorescence emission due to the induction process caused by the molecules released from the bacterial cells. The generated colour can be detected by the naked eye. Moreover, the time required for fluorescence change is less than that required for the bacteria to form a colony on the culture medium. This method that is called chromatic technology is acceptable in terms of its simple procedure and the short time required to obtain the results. Therefore, it might be more useful for the detection of bacterial contamination of foods as well as antibiotic-resistant bacteria.

The culture-based method depends on the measurement of metabolic activities of the cells or the level of growth in the culture medium. However, these measurements need an incubation period of 24 h. The molecular methods have the potential to measure cellular activities in less than an hour (Tanchou 2014). Noble and Weisberg (2005) reviewed advanced technologies for the rapid detection of bacteria in the water. These methods include immunoassay techniques, molecule-specific probes, quantitative PCR (qPCR), nucleic acid sequence-based amplification (NASBA) and microarrays as well as the enzyme/substrate methods which depend on the utilisation of chromogenic or fluorogenic substrates. These techniques are also effective for the detection of pathogenic bacterial cell concentration in water.

The use of electrokinetic methods such as electrophoresis (EP) and AC dielectrophoresis (DEP) to determine the bacterial concentration in samples has been reported in the literature. The DEP technique depends on the polarisation of a particle in a non-uniform electric field which will be attracted to the regions with high field or low field, based on the polarisability of these particles relative to the medium. The characteristics of particles in terms of size, shape and the conductivity play an important role in detecting the living and dead cells by DEP (Camacho-Alanis and Ros 2015).

Another technique for the detection of microbial cells in the environment is the use of spectroscopy such as ultraviolet and visible UV–Vis, infrared IR and Raman

spectroscopy. These techniques have high sensitivity towards molecular differences and complex structures of bacterial cells as well as DNA and chemical compounds. Both IR and Raman spectroscopy depends on measuring the bending, vibrating and stretching modes of the molecule bonds. Therefore, they have high sensitivity towards different molecular structures (Hou et al. 2007; Davis and Mauer 2010). The use of surface-enhanced Raman scattering (SERS) for rapid detection and identification of bacteria which is estimated to be the 20 s, but the bacteria supposed to be in log phase with the concentration of bacteria reach to 100 CFU mL^{-1}. Moreover, the bacteria need to be immobilised and this represents the main challenge for the process known as the on-chip diagnostics technique (Hou et al. 2007).

10.4 Pathogen Growth Potential (PGP)

The main concern in the treated wastewater lies in the ability of the inactivated pathogenic bacteria to grow in treated wastewater which is disposed into the environment or reused for irrigation. The disinfection process which includes chemical and physical disinfections has been demonstrated to reduce pathogenic bacteria in wastewater. However, the remaining bacteria even in very small populations can multiply rapidly under suitable conditions. This case is true for bacteria but not for viruses, helminths and protozoa which cannot regrow outside their specific host organism(s). Therefore, once these pathogens have been reduced through treatment, their populations cannot increase again in the environment (USEPA 2007).

The methods used for studying the potential of the inactivated pathogenic bacteria or fungi to regrow in treated wastewater include culture and non-culture techniques. Moreover, the selection of the culture media and method of isolation in the culture-based method plays an important role in detecting the presence or absence of these pathogens. Chun-ming (2007) claimed that the use of DCA or MLCB for the direct isolation of FC and *Salmonella* spp. from heat-treated cow dung is effective in detecting the presence or absence of these pathogens. The study depends on the storage of the treated samples at 30 °C for 7, 14 and 21 days, respectively, in which some of the damaged bacterial cells can repair themselves during the storage period. Besides, the study depends on optimal growth conditions for these pathogens such as the use of a selective media and an optimal temperature of 37 °C. This method might be effective if some of the pathogenic bacteria remain active after the heat treatment or if the pathogenic bacteria have not been totally damaged. However, the absence of growth on the selective media does not mean that the bacteria have been totally eliminated from the samples since in many cases, the bacteria will still be available in the samples in an inactivated state. Sugumar and Mariappan (2003) reported that *Salmonella* spp. were found to have survived for more than 3 months in water without supplemental nutrition and metabolic injury. However, they failed to grow on selective media. Besides, even after the bacteria undergo the treatment process, they have different colony characteristics compared to those of the typical colonies before the treatment. This might lead to misidentification of the grown bacteria. Markova

et al. revealed that the bacterial cells inactivated using autoclave have the ability to regrow. However, they have grown in L-form where the bacterial cells have grown without cell walls. This case also shows that bacterial cells can be inactivated by using antibiotics whose function is to prevent cell wall synthesis.

It has been demonstrated that bacterial or fungal cells under stressed conditions go into the dormant state which is called viable but non-culturable (VBNC) (Weaver et al. 2010; Al-Gheethi et al. 2016). VBNC means that the cells would not produce hydrolysis enzymes even if isolated on specific or enrichment media. The ability of microorganisms to be reactivated depends on many factors such as the surrounding incubation temperature and the availability of glucose and amino acids which represents the main source for energy and anabolism pathways (Choi et al. 1999). Therefore, the treatment process which does not lead to the reduction of nutrients in wastewater or the irreversible destruction of pathogenic cells is not considered an effective technique to produce high-quality wastewater. For instance, the use of chemical disinfectants such as chlorine or ozone has the potential to inactive the microorganism cells by inhibiting the enzymatic reactions of their energy pathway. However, some microorganism cells have alternative pathways for metabolic activities thus causing the inactivation process for these pathogens to be temporary. Using irradiation as a form of disinfection works because the cell is inactivated due to the ability of UV irradiation to form a dimer between thymine bases in the DNA nucleotide. Nevertheless, the cells have the ability to repair the damage caused by UV irradiation (Al-Gheethi et al. 2013). The possibility of pathogenic bacteria or fungi to regrow in the treated wastewater is a serious point which limits the safe handling and disposal of wastewater into the environment. However, the main challenge is to find an effective technique to determine the potential of ABNC pathogenic bacteria or fungi to regrow in the treated wastewater or the environment. Based on the physiological status of ABNC cells, the culture-based method is not efficient for recovering these pathogens on the culture medium. The absence of bacterial or fungal growth in the culture medium after inoculation with treated samples does not mean that these pathogens have been totally eliminated. Instead, it indicates that the pathogens might exist in concentrations less than the detection limits or that the cells might be in an inactivated state and need more time to regrow. Banana (2013) found that pathogenic bacteria in blood waste samples treated with supercritical carbon dioxide (SC-CO_2) were reduced to less than the detection limits. However, the sample stored at room temperature for 2 months revealed the regrowth of the pathogenic bacteria. The level of the growth was less than that in the raw samples, but the presence of the regrowth confirmed that the cells have the ability to regrow in the treated samples.

The alternative technique to evaluate the efficiency of the disinfected or sterilised waste samples is to use non-culture methods which might be able to determine the level of destruction caused by the treatment method or to determine the possibility of inactivated cells to regrow in treated waste. Noman et al. (2016) investigated the level of destruction caused by the fungal spores inactivated by autoclave and SC-CO_2. In the study, the inactivated fungal spores were scanned using the scanning electronic microscope (SEM) technique which revealed that the spore surface was

totally damaged. The use of SEM for detecting the level of destruction in bacterial or fungal cells is a critical step but it is not enough to confirm that no regrowth will occur after the disposal of waste into the environment because the SEM technique cannot detect the validity of the cells.

One-dimensional SDS-PAGE analysis for the protein banding patterns of inactivated bacteria was used by Hossain (2013) in order to detect the validity of bacterial cells after the treatment process by SC-CO_2. The results revealed the absence of the protein banding patterns of inactivated bacteria. Kim et al. used two-dimensional electrophoresis (2-DE) and principal component analysis (PCA) to detect the protein profiling of *S. enterica* after the inactivation process. The study revealed that the cell fatty acids and proteins are alternated. The analysis of cell fatty acids by GC-MS noted that the total fatty acid quantity reduced in comparison to the control. Conducting a test on the cell membrane permeability can also be used to determine the destruction level in the inactivated cells. The test is carried out using flow cytometry. Two types of stains are used including ethidium bromide (EB) and propiumiodide (PI) to evaluate the permeability of the cell membrane and efflux pump system of bacteria. The ability of EB to stain the bacterial genomic (DNA) and the ability of PI to stain cell cytoplasm confirm that the treatment process has damaged the membrane cell and the efflux pump (Humphreys et al. 1994; Ericsson et al. 2000). In untreated cells, EB is transported into the cytoplasm, but the validity of the efflux pump leads to the stain being pumped out of the cell (Jernaes and Steen 1994). These studies have not been conducted on pathogenic bacteria inactivated in wastewater by different disinfection methods. Moreover, the analysis tools can also be applied to the inactivated bacteria or fungi regardless of the sample type.

Physical treatment such as the use of high temperature can cause denaturation of proteins and enzymes necessary for metabolic and anabolic pathways. The destruction by thermal treatment might be irreversible. In contrast, the chemical treatment might lead to change in the protein structure, but these changes are reversible. For example, the change of pH to extreme acidic or alkaline conditions may cause the destabilisation of enzymes by dissociation of the enzyme subunits or loss of correct assembly structure which may be reversible or irreversible (Kamihira et al. 1987; Poltorak et al. 1999; Fernandez-Lafuente 2009). Nevertheless, the main challenge here is to detect if these changes in the bacterial or fungal cell protein and lipids are enough for irreversible inactivation and preventing regrowth. In other words, it is important to detect if these changes can lead to the death of bacterial or fungal cells.

The detection of proteins available in disinfected samples based on the PCR technique might be unsuitable because the presence of DNA fragments in treated or disinfected samples does not mean that the bacterial or fungal cells are active. The viability of bacterial or fungal cells in the treated sample might be accessed based on metabolic activities, RNA transcripts, a positive energy status and responsiveness. Some authors have combined culture and molecular methods to detect the validity of inactivated cells or those which are present in VBNC state. Jiang et al. (2013) used MPN method and reverse transcription quantitative PCR (RT-qPCR) for the quantification of *S. typhimurium*, *E. coli* and *S. flexneri* in the VBNC state. The study

stated that this procedure provided an improved evaluation of pathogen inactivation efficiency.

In addition, the toxicity of the bacterial and fungal protein structure are important factors to be considered. The treatment process might inactivate the microorganism cells and prevent their regrowth in the environment. However, it does not mean that the health risks for these pathogens have been totally eliminated. For example, even though viruses are invalid or nonliving outside host cells, they can become more pathogenic in the cells. The microorganism protein with high molecular weight is considered toxic for other organisms even in the absence of the cells. The toxins produced by the bacterial and fungal cells consist of protein and polysaccharides which might exhibit high resistance towards chemical or physical treatment. The proteins might also pose a high risk for humans if they are not removed from the samples such as prion proteins (PrPs) which are infectious agents and consist of protein materials (Bartelt-Hunt et al. 2013).

10.5 Conclusions

The evaluation of the treatment efficiency of the inactivation of pathogenic bacteria or fungi in wastewater should include the determination of the validity of pathogens to regrow and the toxicity of proteins and other fragment structures of the cell. The best treatment method should have the ability to kill pathogens by causing irreversible destruction of the cell, energy pathways, toxicity of the proteins, polysaccharides and lipid fragments released from the cells as well as the irreversible destruction of DNA and RNA fragments to prevent transmission to other microorganisms. In contrast, the evaluation procedure should be conducted using different techniques to ensure that the cell and their components have been totally damaged by the selected treatment process.

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