MICROBIAL INTERACTIONS

IN

DRINKING WATER SYSTEMS

BY

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

W. Khan **Date**

SUMMARY

Microorganisms show a tendency to accumulate on surfaces in aqueous environments to form biofilms. Microbial biofilms represent a significant problem in public health microbiology as the development of these microbial communities, especially in water distribution systems, may lead to (i) the enhanced growth of opportunistic pathogens, (ii) the development of organoleptic problems, (iii) the reduction in the flow rate and (iv) the regrowth of microorganisms.

In this project, biofilm monitors were installed in a large water distribution system to study biofilm phenomena in drinking water systems, and to deduce the biological stability and quality of the potable water. Measurements of biofilm formation potential showed that biofilms did not reach a steady state after 100 to 150 days. The microbial cells in these biofilms were mostly non-culturable. The contribution of the heterotrophic colony count to active biomass, as determined with cell numbers based on ATP measurements were often < 1%, while the ratio of heterotrophic plate counts and direct acridine orange counts were also <1%. The ratio between cell numbers based on ATP measurements and direct acridine orange counts were often < 100%. Results also showed that under certain conditions, such as those investigated in the present study, 1 pg of ATP may not be equal to approximately 10^4 active bacteria/cells, as stipulated by previous investigations, and that the average ATP content per active bacterial cell is indeed less than 10^{-16} – 10^{-15} g. It was calculated that threshold values for assimilable, and dissolved organic carbon below ~5 µg C/l and ~0.5 mg C/l, respectively, should be target values for the control of biofilm formation in this system. It was shown that polyethylene, polyvinylchloride, teflon, plexiglass, copper, zinc-coated steel and aluminium provide favourable attachment surfaces that allowed primary colonisation and subsequent biofilm formation. Significant ($p < 0.05$) differences in surface colonisation on the materials were observed, indicating that the composition of the material has a direct influence on microbial colonisation. The two grades of stainless steel evaluated in this study were the least favourable materials for biofilm formation. It was further demonstrated that the nature of the surface of these materials, flow conditions and water type all had a direct influence on biofilm formation. While modification of the attachment surface did not result in significant differences ($p > 0.05$) in disinfection efficiency of two commonly used biocides, the concentration of the biocide, as well as the material to which the biofilm is attached, greatly influenced biocidal efficiency. The results show that biofilm monitoring needs to be implemented at the water treatment plants in addition to common biostability measurements.

OPSOMMING

Mikro-organismes neig om te akkumuleer aan oppervlaktes in akwatiese omgewings om biofilms te vorm. Mikrobiese biofilms verteenwoordig 'n betekenisvolle probleem in publieke gesondheidsmikrobiologie omdat die ontwikkeling van hierdie mikrobiese gemeenskappe in waterverspreidingsisteme mag lei tot (i) die verhoogde groei van opportunistiese patogene, (ii) ontwikkeling van organoleptiese probleme, (iii) die vermindering in die vloeitempo en (iv) die hergroei van mikro-organismes.

In hierdie projek was biofilm monitors geïnstalleer in 'n groot waterverspreidingsisteem om biofilm fenomene in drinkwatersisteme to bestudeer, en om die biologiese stabiliteit en kwaliteit van drinkwater af te lei. Bepalings van biofilmvormingspotensiaal het aangetoon dat biofilms nie 'n stabiele stadium na 100 tot 150 dae bereik nie. Die mikrobiese selle in hierdie biofilms was meestal nie-kweekbaar. Die bydrae van die heterotrofiese kolonie tellings tot aktiewe biomassa, soos bepaal deur seltellings gebaseer op ATP metings was dikwels < 1%, terwyl die verhouding van die heterotrofiese plaattellings en direkte akridien oranje tellings ook < 1% was. Die verhouding tussen seltellings, gebaseer op ATP metings en direkte akridien oranje tellings was dikwels < 100%. Resultate het ook aangetoon dat onder sekere omstandighede, soos dié wat ondersoek was in die huidige studie, 1 pg ATP nie gelyk is aan min of meer 10^4 aktiewe bakterieë/selle soos gestipuleer deur vorige ondersoeke nie, en dat die gemiddelde ATP inhoud per aktiewe bakteriële sel inderdaad minder as 10^{-16} tot 10^{-15} g is. Dit was bereken dat die drempelwaardes vir assimileerbare en opgeloste organiese koolstof onder ~5µg C/L en ~0.5 mg C/L, onderskeidelik, teikens moet wees vir die beheer van biofilmvorming in hierdie sisteem. Dit was aangetoon dat polyetileen, polyvinielchlroried, teflon, plexiglas, koper, sink-bedekte staal en aluminium gunstige aanhegtings oppervlaktes voorsien wat primêre kolonisering en daaropvolgende biofilmvorming toelaat. Betekinisvolle ($p < 0.05$) verskille in oppervlak kolinisering op die materiale was waargeneem, wat aandui dat die samestelling van die materiaal 'n direkte invloed op mikrobiese kolonisering het. Die twee tipes vlekvryestaal wat geëvalueer was in hierdie studie, was die minder gunstige materiale vir biofilmvorming. Dit was verder gedemonstreer dat die aard van die oppervlak van hierdie materiale, vloeitoestande, en water tipe almal 'n direkte invloed het op biofilmvorming. Terwyl die aanpassing van aanhegtingsoppervlak nie die ontsmettingsdoeltreffendheid resultaat van die twee algemeen-gebruikte biosiede betekinisvol ($p > 0.05$) beïnvloed het nie, het die konsentrasie van die biosiede asook die aanhegtings-materiaal, biosied doeltreffendheid grootliks beïnvloed. Die resultate het aangetoon dat biofilm monitering geïmplementeer moet word by waterbehandelingsaanlegte as 'n alternatief vir algemene biostabiliteit metings.

BIOGRAPHICAL SKETCH

Wesaal Khan was born in Cape Town, South Africa, on the 18th February 1975. She attended Accordion School Primary and matriculated at Excelsior Secondary School in 1992. She enrolled at the University of the Western Cape in 1993 and obtained a B.Sc. degree in Biochemistry and Microbiology in 1995. In 1996 she completed a B.Sc. (Hons.) degree in Microbiology at the same university. From October 1997 to February 2000, Wesaal was employed by the ARC at the ARC-Fruit, Vine and Wine Research Institute, Nietvoorbij Centre for Vine and Wine. She enrolled at the University of Stellenbosch in 1997 and obtained her M.Sc. degree in Microbiology in 1999. She is presently a full-time employee of Cape Technikon in the capacity of researcher.

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CONTENTS

LIST OF ABBREVIATIONS

Adenosine Tri Phosphate –	ATP
Assimilable organic carbon (µg C/L) –	AOC (10 µg C/L in biologically stable water)
Biofilm formation rate pg ATP/(cm^2 x day) –	BFR
Biofilm formation potential pg ATP/cm^2 –	BFP
Colony forming units –	CFU
Direct acridine orange count –	DAOC
Dissolved organic carbon (mg C/L) –	DOC (Accepted range of DOC 0.2 mg C/L to 0.5mg C/L)
Extracellular polymeric substances –	EPS
Green fluorescent protein –	GFP
Hydrogen peroxide –	H_2O_2
Polyethylene –	PE
Polyvinylchloride –	PVC
Sodium hypochlorite –	NaOCl

With reference to the map on page 66 (Chapter 2)

Site 1 –	Water Works Holthausen:	Control site for research conducted
Site 2 –	Faerberstrasse:	Situated in the city centre
Site 3 –	Reservoir Hardt:	Facility with drinking water storage tanks
Site 4 –	Reservoir Sandberg:	Facility with drinking water storage tanks
Site 5 –	Pumping station Gau:	A groundwater source situated on the outskirts of Duesseldorf

1. LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Drinking Water

Water, one of the most abundant and widely distributed substances in nature, not only forms the oceans, lakes and rivers, but as a vapour surrounds us, permeating even the solid mass of the earth. Moreover, it is an essential element in the maintenance of all forms of life, as most living organisms can survive for only short periods of time without water. The demand for potable water is thus obvious. To ensure a stable supply of potable water of a high quality is of significant international concern, as not only must the drinking water be in abundant supply but it must also comply with specific criteria and standards. The primary issues associated with the use of water by humans are thus quantity and quality. By implication the water source as well as the selection of the water treatment process are complicated tasks that ultimately must lead to the continued delivery of affordable, drinkable water.

1.1.2 Water Sources

When selecting a new drinking water source it is important to consider whether: (1) the water quality is satisfactory or can be improved by treatment to make it suitable for drinking; (2) the source will yield enough water to meet the needs of the community not only under the normal conditions of an average annual cycle, but also under unusual conditions, such as extended drought periods; (3) under normal conditions, the change in local water flow patterns will not cause any unacceptable deterioration in the quality of the water used; and (4) the water used can be protected against pollution (World Health Organisation, 1996).

The two most important water sources generally used for drinking water and agricultural purposes are groundwater and surface water. Groundwater is water that saturates the tiny underground spaces between the alluvial material (sand, gravel, silt, clay) or the crevices or fractures in rocks. It moves extremely slow through these porous geological layers and spaces, in aquifers, with the water then brought naturally to the surface through a spring or discharged into lakes and streams. Groundwater may also be extracted through a well drilled into an aquifer (The

Groundwater Foundation, 2001). Surface water on the other hand includes lakes, rivers and those waters stored as snow or ice. Groundwater and surface water are connected in various ways. Surface waters evaporate from the earth under influence of the sun's energy to form clouds. Depending on temperature and other weather conditions, the vapour condensates and returns to the earth as different types of precipitation. Some of this precipitation seeps into the ground and is stored as groundwater, with most of this fraction then feeding the various forms of surface water or extracted from the subsurface by humans using wells (Conservation Technology Information Centre, 2002; The Groundwater Foundation, 2001).

1.1.3 Pollution of Water Sources

Groundwater and surface water present the major sources of available water, which are both susceptible to pollution, sometimes by the same contaminants. Contamination of these water sources occurs when man-made products such as petroleum, oil, road salts and chemicals get into the water and cause it to become unsafe and unfit for human use and /or detrimental to the environment (**Figure 1.1**).

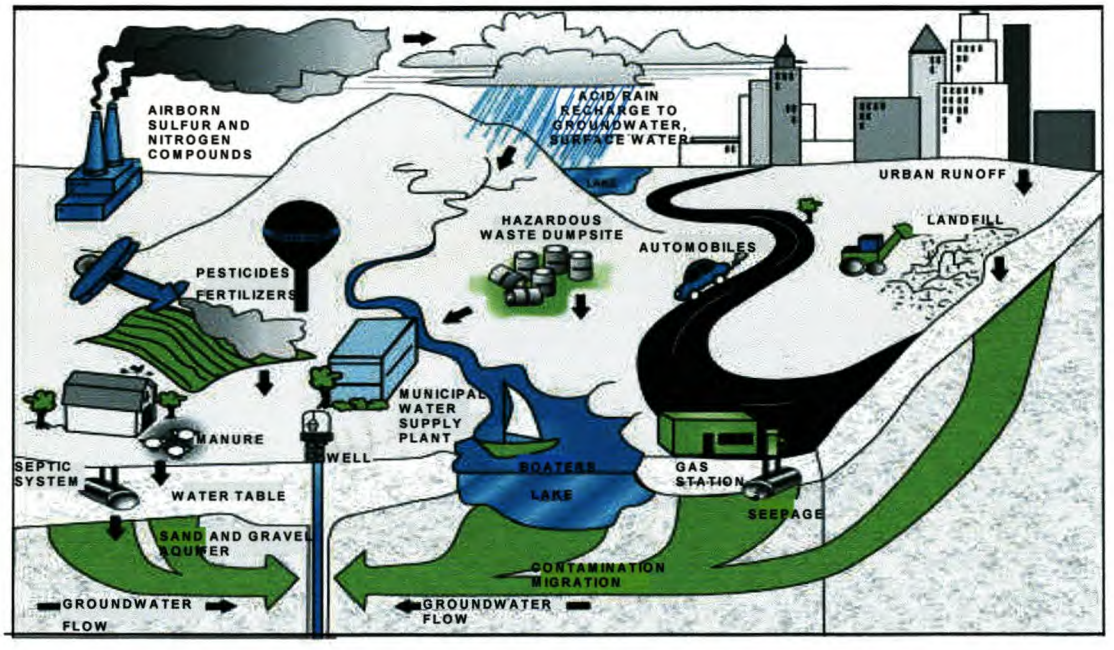


Figure 1.1. Typical Sources of Groundwater and Surface water contamination. (Adapted from The Groundwater Foundation, 2001)

Some of the major sources of these products are chemical storage tanks, septic systems, hazardous waste sites, landfills and toxic substances from mining sites, as presented in **Table 1.1**. It is important to note that water quality also

changes with the seasons and between geographic areas, even when no pollution is present.

Table 1.1. Potential sources of contamination of Groundwater and Surface water (Tchobanoglous & Schroeder, 1985; Conservation Technology Information Centre, 2002).

Source	Possible contaminant
Accidental Spills	Various inorganic and organic chemicals
Acid rain	Oxides of Sulphur and Nitrogen
Agricultural activities	Fertilizers, pesticides, herbicides, fumigants, nitrates, hydrocarbons, and pathogens
Animal Feedlots	Organic matter, nitrogen and phosphorous
Auto repair shops and salvage yards	Hydrocarbons
De-icing of roads	Chlorides, sodium and calcium
Deep-well injection of wastes	Variety of inorganic and organic compounds, radioactive materials and radionuclides
Hazardous waste disposal sites	Various inorganic compounds (especially heavy metals) and organic compounds (e.g., pesticides and priority pollutants)
Home fertilizer	Nitrates
Households	Hydrocarbons
Industrial liquid waste storage ponds and lagoons	Organic compounds, heavy metals and various cleaning solvents and degreasing compounds
Industrial floor drains	Hydrocarbons
Injection wells	Hydrocarbons
Landfills, industrial	Wide variety of inorganic and organic compounds
Landfills, municipal	Heavy metals, gases, organic compounds, and inorganic compounds (e.g., calcium, chlorides and sodium)
Land disposal of municipal wastewater and waste sludge	Organic compounds, inorganic compounds, heavy metals, microbiological contaminants.
Manure handling	Nitrates, pathogens
Mining	Minerals and acid mine drainage
Petrol Stations	Hydrocarbons
Recycling facilities	Hydrocarbons
Salting practices and storage	Inorganic salts and chlorides
Septic tank leaching fields or beds (soil absorption areas)	Organic matter, nitrogen, phosphorous, bacteria, viruses
Small quantity generators	Hazardous materials
Underground Storage tanks	Organic cleaning and degreasing compounds, petroleum products and other hazardous wastes

As there are no universally applied standards for well installation, and because wells are built from different materials, they can easily contribute to contamination if the construction was not properly done or if toxic materials are released into them. Furthermore, (toxic) material spilled or dumped near a well can leach into the aquifer and contaminate the groundwater. Toxins that have leached into water supplies and sources may also cause poisoning. Therefore wells should be constructed in such a way that they can be tested for the presence and quantities of chemicals (The Groundwater Foundation, 2001; Critto *et al.,* 2003; Reid *et al.,* 2003).

Remediation of contaminated water is usually a complex and expensive process. Numerous tools are typically employed to manage groundwater and surface water sources (**Table 1.2**). Many steps by manufacturers and consumers are also currently being undertaken to minimise pollutants reaching groundwater supplies. Examples include manufacturers that are using fewer toxic raw materials and consumers that have switched to phosphate-free detergents and other less polluting household products.

Table 1.2 Groundwater and Surface water protection tools and management practices (Conservation Technology Information Centre, 2002).

Technique	Protection Tools and Management Practices
Zoning Districts	Define water resource protected districts Prohibition of land uses e.g. petrol stations, sewage treatment plants, etc. Special permitting to regulate uses and structures that potentially degrade water and land quality Extending or developing community sewage treatment systems Set up performance standards so as not to overload a water resource Management of Underground storage tanks Artificial wetlands Identification and closure of abandoned wells
Health Regulations	Maintenance and upgrading of underground fuel storage systems, sewage treatment plants, wells and designated groundwater and surface water resources. Stipulating regulations for water quality
Other non-regulatory	Public education campaigns Frequent monitoring of drinking water supplies Monitoring of groundwater and surface water sources

Groundwater contaminants are often trapped within the soil matrix in areas that are not readily accessible to groundwater flow. Residual contamination may then gradually diffuse from the less permeable areas into more permeable areas where it can be carried downstream by the groundwater to monitoring wells or drinking water supplies. The pumping of an inexpensive carbon or nutrient source into groundwater at a specific site could encourage the growth and proliferation of microbial communities. This tactic can then be used to clean up contaminated groundwater, as the microbes create a barrier that ultimately block or slow the flow of water through those regions, cleaning up the polluted area (DeFrancesco, 2003).

The best course of action, however, is for governments to implement appropriate and realistic laws protecting all ground- and surface water supplies from being contaminated. Indeed Water Conservation Acts and campaigns providing guidelines on the protection and conservation of drinking water supplies have contributed extensively in sustaining water sources (World Summit on Sustainable Development, 2002; UNICEF, 2003).

1.1.4 Microbial Pollution of Water Sources

Serious health risks to humans and wildlife are associated with drinking contaminated water. Of the 37 major diseases in developing countries, 21 can be related to inadequate water treatment and poor sanitation processes. Throughout the developing countries, water-related diseases are also the most important causes of infant mortality and the principle cause of illness in adults (World Health Organisation, 1996).

It is thus recognised that humans may acquire various diseases such as hepatitis, dysentery and cholera from contaminated aquatic environments. However, until it was understood that microbes or pathogens such as bacteria, viruses, helminths and protozoa, associated with the water were responsible for disease, not much could be done to prevent waterborne illness.

Traditionally, when analysing the contaminated water, the primary isolates consited of the free-floating or planktonic microorganisms. However, it has been increasingly realised that most viable and productive microorganisms in contaminated or in aqueous environments in general, show a tendency to accumulate on surfaces in cell aggregates (Hallam *et al.,* 2001). These attached or sessile microorganisms grow and actively multiply in such a way, which allows them to trap organic and inorganic debris, nutrients as well as other microorganisms,

leading to the formation of a biofilm. The formation of the biofilm occurs in a number of stages namely, the formation of the conditioning film, microbial association with the surface, adhesion to the surface, growth and proliferation, recruitment of other microorganisms and detachment (Kumar & Anand, 1998). The detachment of microbial components, as single organisms or as large aggregates, into the bulk aqueous phase is a critical stage as it maintains the biofilm at an equilibrium or steady state which is dictated by the organisms, nutrient availability and environmental conditions (Stewart *et al.,* 1993). The exact time period that it takes for the biofilm to reach and remain in the steady or equilibrium state is also dependant on various external factors and conditions optimum for microbial growth and proliferation.

Microbial biofilms constitute one of the most important issues in public health microbiology, requiring attention in such diverse areas as fouling in the food and beverage industry, to biofilm accumulation on prostheses and catheters in medicine (Costerton *et al.,* 1999). A major concern also arises from the fact that biofilms provide microenvironments that can protect pathogenic bacteria, viruses and protozoa from the physical, chemical and biological stresses of conventional water and wastewater treatment processes (Percival *et al.*, 2000). Problems can arise in water distribution systems when clusters of biofilm-associated organisms become detached from pipe walls by hydrodynamic and especially shear forces. The release of attached biofilm materials through subsequent processes of erosion and sloughing compromises the microbiological quality of distribution water by providing a continual source of contamination of the bulk water phase (Water Quality News, 2000; Hallam *et al.,* 2001). An increased emphasis is thus placed on the ecological role of biofilms as reservoirs of waterborne bacteria, protozoa and viruses in distribution systems.

The standard techniques employed to evaluate biofilm form and function generally involve the combination of microscopy and a redox dye or stain. Additionally, the application of molecular techniques such as the polymerase chain reaction, denaturing gradient gel electrophoresis, DNA microarray and fluorescence in situ hybridisation are useful tools in analysing the interactions in biofilm systems.

The majority of the routine water quality monitoring methods performed by the water treatment plants generally involve plate count techniques and do not take biofilm formation into account. Adenosine tri phosphate (ATP) is an energy carrier present in all viable cells and has been successfully employed to enumerate the active biomass portion of a biofilm community in the water distribution systems (Van

der Kooij & Veenendaal, 1993). The success of this technique is directly linked to the fact that it not only enumerates viable and culturable cells, as is the case with plate count techniques, but it also enumerates the viable cells not contributing to this counts, such as the viable and non-culturable portion of the biofilm population. Additionally factors such as the rate of biomass accumulation as well as the potential for the biofilm to form once a steady state of biofilm growth has been reached can be calculated from ATP concentrations. The need then arises for more effective, biofilm-based methods to monitor water quality to be employed by the water treatment plants as the development of bacterial biofilms, in particular in water distribution systems may lead to (i) the enhanced growth of opportunistic pathogens (Goshko *et al.,* 1983), (ii) the development of organoleptic problems (Servais *et al.,* 1995), (iii) the reduction in the flow rate and (iv) the regrowth of microorganisms (Olsen *et al.*, 1991).

1.1.5 Aims of study

This study formed an integral part of a larger research programme conducted at the Division of Microbiology at Stadtwerke Duesseldorf AG and the Department of Microbiology at the University of Stellenbosch. The primary aim of this study was to determine the extent to which biofilms may serve as a source of contamination in drinking water systems. To achieve this aim, a hypothesis stating that the implementation of advanced treatment programmes by drinking water suppliers, and the subsequent production of good quality water, prevent biofilms from contributing notably to the microbiological contamination of water distribution systems, was tested. The alternative to this hypothesis would be that despite the numerous strategies typically employed in drinking water distribution systems, effective control of microbial growth may not be achieved. This was tested by the evaluation of operational parameters and water treatment methods which, if adjusted would influence or control the formation of biofilms in drinking water systems. The specific aims and approaches of this study were:

1.1.5.1 To investigate whether the oligotrophic conditions present throughout water distribution systems support notable biofilm formation. This was achieved by determining the biofilm formation potential and biological stability of the drinking water in the Duesseldorf area by installing Kiwa™ Biofilm Monitors at a water treatment plant and different locations throughout the distribution

system. Samples were analysed microbiologically to determine a direct acridine orange cell count, heterotrophic plate counts, biofilm formation rate and potential, and population dynamics over time.

1.1.5.2 To evaluate the ability of the two disinfectants, hydrogen peroxide and sodium hypochlorite, to effectively kill and remove biofilms, which may form on various materials used in the drinking water distribution industry. The concentration of disinfectant most effective against heavy biofilm contamination was also evaluated.

1.1.5.3 To investigate whether the type of materials relevant to the drinking water distribution industry supports microbial growth. Additionally, biofilm formation on materials used within the laboratory, and biofilm research in general (e.g. flowcells) was investigated. The influence of surface roughness, flow conditions and water type on biofilm formation and structure was also examined. A pilot plant was set-up at the Water Works Holthausen in Duesseldorf, Germany to examine biofilm formation potential under all these parameters. Characterisation of the biofilms was done by confocal scanning laser microscopy (CSLM) and BacLight™ stain to determine biofilm thickness, area coverage, live/dead ratio and microbiological analysis as outlined in 1.1.5.1 above.

1.1.5.4 The extent to which biofilms offer protection to naturally isolated waterborne organisms within water distribution systems was examined by tagging frequently found waterborne pathogens with green fluorescent protein (GFP) and adding these organisms to native drinking water biofilms *in vitro*. (See Addendum 1).

1.2 WATER TREATMENT PROCESSES

Water treatment is a complex activity, with numerous options available. The choice of process(es) will obviously depend on the water to be treated, and the desired final product. Many of the treatment options are applicable for treatment of waters ranging from potable water to sewage water. Furthermore, although most potable water is derived from ground and surface waters, treated wastewaters find increasing use for household (potable) purposes. Therefore, a discussion of treatment of not only drinking, but also wastewater is in order and thus included in this section.

Historically, clear water meant clean water. Public drinking water treatment only began on a large scale during the 19th century, with the city of Paisley in Scotland known as the first city that employed settling followed by filtration to treat the water supply. This practise spread through Europe and by the end of the century most major municipal supplies had slow sand filters (Peavey & Rowe, 1985). Even though today an increased scientific knowledge has led to the development of advanced technical equipment and an increase in water treatment plant efficiency, the fundamental purpose of water treatment is still to produce water that is biologically and chemically safe for human consumption.

In addition to providing good quality water, treatment processes are also aimed at removing those impurities, which may damage pipes or other items with which the water comes into contact with, rendering operation in the water treatment plant more difficult or costly. Contaminants that may have to be removed from the main water sources, groundwater and surface water, to meet specific water quality objectives are identified in **Table 1.3** (Tchobanoglous & Schroeder, 1985; American Water Works Association, 1999).

Table 1.3 Typical contaminants found in various water sources that may need to be removed to meet specific water quality objectives* (Tchobanoglous & Schroeder, 1985; American Water Works Association, 1999)

Class	Typical contaminants found in:	
	Groundwater	Surface water
Floating and suspended materials	None	Branches, leaves, algal mats, soil particles
Colloidal materials	Microorganisms, trace organic and inorganic constituents	Clay, silt, organic materials, pathogenic organisms, algae, other microorganisms
Dissolved material	Iron and manganese, hardness ions, inorganic salts, trace organic compounds	Organic compounds, tannic acids, hardness ions, inorganic salts
Dissolved gases	Carbon dioxide, hydrogen sulfide	¥
Immiscible liquids	Unusual in natural groundwater aquifers	Oils and greases

* Specific water quality objectives may be related to drinking water standards, industrial use requirements, effluent discharge requirements or agricultural reuse.
¥ Gas supersaturation may have to be reduced if surface water is to be used in fish hatcheries.

The treatment required for water depends primarily on the quality of the water source. For example, the treatment of surface water may involve screening, coagulation, sedimentation, filtration and disinfection. Groundwater from deep wells may require no treatment at all. Treatment of a good quality drinking water can thus be achieved by introducing numerous successive barriers, with the arrangement and variations on each differing from treatment facility to facility and around the world (World Health Organisation, 1996).

The principal unit operations and processes used to remove contaminants are summarized in **Table 1.4** (Tchobanoglous & Schroeder, 1985). Commonly used drinking water treatment methods are either physical operations or chemical processes. Biological processes are not used because appreciable amounts of organic matter are not present in most natural waters and biological processes are generally not suitable in situations where contaminant concentrations are low. In general, effluents from biological treatment processes do not meet the standards for domestic water supplies.

Table 1.4 Treatment processes and strategies used to remove the major contaminants found in water (Tchobanoglous & Schroeder, 1985).

Contaminant	Process or Treatment System	Classification *
Pathogenic organisms	Chlorination	C
	Ozonation	C
Turbidity and Suspended matter	Screening	P
	Sedimentation	P
	Filtration	P
	Coagulation	C
Colour	Adsorption	P/C
	Ion exchange	C
	Coagulation/flocculation/Sedimentation/filtration	C/P/P/P
Tastes and Odours	Oxidation (aeration)	P/C
	Adsorption	P
	Chemical oxidation	C
Organic matter	Adsorption	P/C
	Ion exchange	C
	Ozonation	C
	Coagulation/flocculation/Sedimentation/filtration	C/P/P/P
Hardness ions, Ca^{+2} + Mg^{+2}	Chemical precipitation	C
	Ion exchange	C
Dissolved gases	Aeration	P
	Vacuum deaeration	P
	Chlorination	C
	Ion exchange	C
Heavy metals	Chemical precipitation	C
	Ion exchange	C
Iron and manganese	Ion exchange	C
	Oxidation/precipitation/filtration	C/P/P
Dissolved solids	Reverse osmosis	P
	Distillation	P

*C = chemical, P = physical.

Discharging insufficiently purified municipal waters and other materials into a river or stream represents an obvious source of pollution. The purification of wastewater thus provides a critical link in maintaining public health and safety and includes not only the separation of wastes from wastewater but also the prevention of pollution of the receiving waters (Sly *et al.*, 1988; Pipes & Zmuda, 1997).

Two treatment systems, involving a series of processes, are common for all wastewater treatment plants. One system involves the treatment of the wastewater itself and the other system involves the removal of waste from water resulting in solid material typically called sludge. Conventional sewage treatment involves the

controlled increase of self-purification processes and may include primary, secondary and tertiary treatments (Cowan *et al.*, 1995; American Water Works Association, 1999). Primary treatment involves the removal of insoluble materials present in particulate form by screening, the precipitation of small particulates and finally settling in basins or tanks. Biological or secondary treatment involves the removal of dissolved organic matter using various approaches such as trickling filters, activated sludge, lagoons, extended aeration systems and anaerobic digesters. Finally, tertiary treatment of wastewater involves the biological and chemical removal of inorganic nutrients, but this treatment is costly and is usually only employed when necessary. The ultimate aim of these treatment systems is to dispose of the waste materials with as little expense and operational effort as possible (Prescott *et al.,* 2002).

1.2.1 Preliminary Water Treatment Processes

1.2.1.1 Screening and straining

The first stage in preliminary treatment usually involves a simple screening or straining operation to remove large solids. In the case of water treatment some form of protective boom or coarse screen with openings of about 75 mm is used to prevent large objects reaching the intake. The main screens are usually provided in the form of a mesh with openings of 5-20 mm and arranged as a continuous belt, a disc or a drum through which the flow must pass. The screening mesh is usually slowly rotated so that the material collected can be removed before an excessive head loss is reached. On small works, intermittent hand cleaning of screens is possible but on larger installations automatic mechanical cleaning is provided either on the basis of elapsed time or initiated by the build-up of head loss across the screen.

In sewage water the content of paper and rags is often high and the nature of the materials is such that a mesh screen could be difficult to keep clean. It is therefore customary to use a bar screen arrangement with a spacing between bars of 20-60 mm. Sewage screenings are usually disposed of by burial or incineration. Alternatively, they may be passed through a macerator, which shreds them to small sizes so that they can be returned to the flow for removal with the rest of the settleable solids during the main treatment process. In some situations the use of a

comminutor, which shreds the solids *in situ*, may be preferred (Tebbutt, 1992; American Water Works Association, 1999).

The microstrainer is a development of the drum screen, which uses a fine woven stainless-steel mesh with aperture sizes of 20-60 µm to provide removal of relatively small solids. In water treatment it can be used to remove algae and similar sized particles from waters of otherwise good quality. Microstraining is also employed as a final tertiary stage to produce a high quality sewage effluent. Clogging of the small mesh apertures occurs rapidly. The drum is however, rotated at a peripheral speed of about 0.5 m/s, with the mesh continually washed clean by high-pressure sprays. The design of the microstrainer installations is based on the laboratory determination of an empirical characteristic of the suspension known as the filterability index. This parameter measures the behaviour of the suspension with reference to its clogging properties and can be used to determine the allowable straining rate to prevent excessive clogging and possible physical damage to the mesh (Boucher, 1961; American Water Works Association, 1999).

1.2.1.2 Grit removal

In most sewage systems and particularly those with combined sewers, considerable amounts of sand and stone particles are carried along in the flow and these materials, if not removed could cause damage to mechanical parts of the treatment plant. As the grit particles are relatively large, with a high density compared with the organic particles in sewage, they are often removed using the principle of differential settling. Grit particles with a diameter of 0.2 mm have a settling velocity of about 1.2 m/min, whereas most of the suspended solids in sewage have considerably lower settling velocities. By using a parabolic section channel it is possible to provide a constant horizontal velocity of around 0.3 m/s at all flow rates. Under these conditions, a channel of sufficient length to provide a retention time of 30-60 seconds, will allow the grit particles to settle to the bottom whilst the remaining suspended solids are still transported by the flow. The grit is removed at intervals, washed and then disposed of for re-use. Other types of grit removal devices may involve an aerated spiral-flow chamber to achieve the desired separation or the use of a short retention-settling tank. Any organic solids removed with the grit are washed back into the flow before the grit is discharged (Tebbutt, 1992).

1.2.2 Water Clarification Processes

1.2.2.1 Pre-chlorination and Peroxone

Chlorine could be added at the beginning of the water treatment process when the water source is surface water or when the source contains high numbers of planktonic cells. Maximum time of contact is then provided between the chemical and the organism, ensuring efficient bactericidal action. Chlorination at this point effectively reduces the counts of faecal bacteria and pathogens, destroys animal life and algae (Lamb, 1985; Haas, 1999) and oxidizes ammoniacal nitrogen, iron and manganese, thereby assisting in their removal. The combined and free chlorine, which remains, effectively inhibits microbial activities, such as protozoan predation and nitrification, as well as microbial growth during subsequent filtration (World Health Organisation, 1996). Investigations have however shown that pre-chlorination of organically enriched surface waters produce a substantial increase in the content of trihalomethanes (THM's). For this reason, it has been considered postponing this step to a later point in the treatment process, when most of the humic materials, which are precursors of the formation of THM's, have been removed (Singer *et al.*, 1981; Singer, 1994).

The Peroxone process is an advanced oxidation process, which involves the addition of hydrogen peroxide and ozone. Some water utilities have considered using ozone as an alternative to chlorine for a number of reasons: to reduce halogenated organic by-products; to remove objectionable tastes and odours; to oxidize reduced metals and specific synthetic organics; for potential benefits during coagulation and filtration; and to increase the biodegradability of organic matter for subsequent treatment. Investigations have however, revealed that preoxidation with ozone or peroxone does not allow for a reduction in coagulant doses (combined alum and cationic polymer) for good treatment, and may cause increased filter run lengths resulting from head loss or delayed turbidity breakthrough. Peroxone is moderately more effective than ozone alone in eliminating odours in filtered waters (Tobiason *et al.*, 1992; Haas, 1999).

1.2.2.2 Aeration

The principle applications of aeration in water quality management are related to the addition and removal of gases from water. Aeration is used in water supply systems to reduce the concentration of volatile taste- and odour- causing substances, such as hydrogen sulphide, for the removal of carbon dioxide from groundwaters and for oxidation of ions such as iron and manganese. Sprays, cascades, multiple trays, bubble diffusers, and surface turbines are the most commonly used aeration devices. In wastewater management systems, aeration is used in sewers and treatment plant head works to control hydrogen sulphide formation, to strip volatile organic and inorganic compounds from wastewater, to supply oxygen for aerobic biological wastewater treatment, to raise treated effluent dissolved oxygen concentrations to levels acceptable for discharge, and in some cases, to increase the oxygen concentration of receiving waters. Energy requirements for aeration in wastewater management are a function of the wastewater characteristics and consequently vary considerably from location to location. Aeration is usually the single largest energy sink in a wastewater treatment system (Tchobanoglous & Schroeder, 1985; Hand *et al.,* 1999).

1.2.2.3 Coagulation

To remove particulate matter, a water treatment plant will generally include equipment for coagulation and flocculation, followed by sedimentation and filtration. Coagulation involves the rapid dispersion of a coagulant by the addition of a metal salt (e.g. aluminium sulphate, iron sulphate, ferric sulphate or ferric chloride) or a synthetic organic polymer into the flow stream to neutralize the charges on particles and facilitate their agglomeration during the slow mixing provided in the flocculation step. Flocs thus formed co-precipitate, adsorb and entrap natural colour and mineral particles, and can bring about major reductions in counts of protozoa, faecal bacteria, pathogens and viruses.

The primary consideration in a flocculation design is the provision of optimum velocity gradients. Too high velocity gradients will shear floc particles. Too low velocity gradients will fail to provide sufficient agitation for all flocculation to complete. Chemical agents such as activated silica, or polyelectrolytes are sometimes used to increase floc strength, especially during cold water conditions. Coagulation and

flocculation require a high level of operational skill. Chemical dosages and pH control must be correct, and the plant must be designed to ensure proper floc formation (Tebbutt, 1992). Before it is decided to use coagulation as part of a treatment process, careful consideration must be given to the likelihood of process stability, the availability of regular supplies of chemicals and the need for qualified personnel (Coad, 1990; World Health Organisation, 1996).

1.2.2.4 Sedimentation and flotation

Many of the impurities in water and wastewater occur as suspended matter which remains in suspension in flowing liquids, but which will move vertically under the influence of gravity in inactive or semi-quiescent conditions. Usually the particles are denser than the surrounding liquid so that sedimentation takes place but with very small, low density particles flotation may offer a more satisfactory clarification process. The purpose of sedimentation is to permit settleable floc to be deposited and thus reduce the concentration of suspended solids that must be removed by filters. The factors which influence sedimentation include: size, shape and weight of the floc; viscosity and temperature of the water; the retention time; the number, depth and areas of the basins; the surface overflow rate; the velocity of the flow; and the inlet and outlet design (Tebbutt, 1992; Matilainen *et al.,* 2002; Vinneras & Jonsson, 2002).

Flotation is an alternative to sedimentation, and has advantages when the amount of floc is small. This process separates low density solids or liquid particles from a liquid phase. Separation is brought about by introduction of gas (usually air) bubbles into the liquid phase. Suspended solids or liquid particles, e.g., oil, are floated by these minute bubbles, causing them to rise to the surface of the tank (Tebbutt, 1992; Matilainen *et al.,* 2002; Vinneras & Jonsson, 2002).

1.2.2.5 Filtration

Filtration of suspensions through porous media, usually sand, is an important stage of the treatment of potable waters to achieve final clarification. Although about 90% of the turbidity and colour are removed in coagulation and sedimentation, a fraction of floc is usually carried over from settling tanks and requires removal.

Typically rapid sand filters are 0.4-1.2 m deep, usually with an effective sand particle size of 0.5-1.0 mm, supported by gravel and underdrains. In recent years, single-medium filters have been replaced by dual-medium or multimedia systems. During filtration, residual particles of floc not removed by sedimentation are trapped in the interstices of the bed, and may induce further flocculation of particles. Both sand and mixed media filters are normally cleaned by reversal of the flow through the bed (backwashing). Backwash water is either discharged to the sewer or drying beds, or recycled after removal of sludge (Le Dantec *et al.*, 2002; Vega *et al.,* 2003).

Slow sand filters consist of 0.5-1.5 m of silica sand with an effective particle size of 0.3-0.6 mm. The upper layer of fine sand is supported on gravel and a system of underdrains. This system is easier to operate than rapid filtration, as frequent backwashing is not required. It effectively removes biodegradable organic carbon and oxidises ammonia. When the filter is first brought into use a biofilm community develops at the surface of the bed. This consists of bacteria, free-living ciliated protozoa and amoebae, crustacae, and invertebrate larvae acting in food chains, resulting in the oxidation of organic substances in the water and of ammoniacal nitrogen to nitrate. Pathogenic bacteria, viruses and resting stages of parasites are removed, principally by adsorption on to the mucous biofilm and by subsequent predation (Tebbutt, 1992; Bahgat *et al.,* 1999; Van Loon *et al.,* 2002).

Activated carbon filters are also successfully being used in water treatment plants. To achieve a sufficiently long lifespan the carbon must be strong enough to withstand crushing and friction, otherwise breaking down would occur in the passage of water, resulting in loss of head and wastage of material. The carbon should be heavier than water and fairly deep beds are essential. Possibly one of the best types of filter is one similar to the ordinary sand filter using pressure. Fine-grained carbons have greater purifying capacity than coarse-grained carbon, but their use is limited by the high resistance to pumping they offer, resulting in increased energy consumption when pumping. A thick bed of coarse-grained carbon is more slowly exhausted than a thin bed of fine carbon, thus requiring longer intervals between regeneration. The granules of high-grade carbons are specially shaped to improve water flow (James, 1971; Bouwer & McCarty, 1983; Matilainen *et al.*, 2002).

1.2.2.6 Other procedures

The addition of lime after filtration reduces the acidity that results from adding alum, as well as the corrosiveness of finished water to the distribution system and plumbing in homes. Also, Martin *et al.* (1982) showed that an increase in pH by lime addition effectively eliminates coliform growth in water distribution systems.

The process of reverse osmosis (RO) removes the particles contained in water from the aqueous component and is primarily used for the desalination of seawater for the production of consumable water. As heat is employed to separate the aqueous phase of water from the particles present, distillation is the surest way of providing pathogen free water. However, large amounts of energy are required to operate this process, and the production of drinkable water is slow, making this process much less attractive than RO (Margolin, 1997).

SOLAIR is an alternative disinfection method utilising natural sunlight (specifically UV-A and UV-B radiation) and oxygen (from atmospheric air) to damage, inactivate and/or kill the coliform bacteria found in contaminated water. It is a natural process with no need to add any potentially hazardous chemicals or to use sophisticated and expensive equipment. In South Africa where more than 8 million people are still using water obtained directly from alternative sources such as rivers, streams, boreholes, wells, community taps and dams, SOLAIR could prove to be an efficient and an economically feasible method to be used for disinfection of hand-drawn water to an acceptable potable standard (Meyer & Reed, 2001).

1.2.3 Disinfection

As indicated earlier, drinking water comes primarily from two types of sources: surface water and groundwater. How the water is treated depends on its origin. There may be no apparent need for treatment of many groundwaters, as in most cases it already meets the bacteriological requirements and has a low potential for re-growth. However, the presence of bacteria and viruses is always possible (Tebbutt, 1992). Surface waters on the other hand are exposed to environmental elements such as urban and agricultural waste, faecal contaminants, etc., and in many cases require extensive treatment.

The microbiological requirements on drinking water need to be guaranteed by the protection of the raw water sources, the treatment of the water and by disinfection, which is regarded as the last safety step. The small size of microorganisms means that their complete removal from water by processes such as coagulation and filtration cannot be guaranteed.

Terminal disinfection of piped drinking water supplies is of paramount importance and is almost universal, since it is the final barrier to the transmission of waterborne bacterial and viral diseases. It usually consists of the use of an oxidative process, which although it removes microorganisms, may also enhance the formation of easily biodegradable organic substances. Microorganisms can easily utilize these substances as an energy source and promote biofilm formation in distribution systems (Glaze, 1987; Gilbert, 1988). Although chlorine and hypochlorite are most often used, water may also be disinfected with chloramines, chlorine dioxide, ozone, ultraviolet irradiation and the addition of hydrogen peroxide. The presence of the disinfectant residual in the distribution system also helps to maintain water quality by preventing the growth of nuisance microorganisms (Schwartz *et al.,* 1998; DiGiano *et al.,* 2000).

The disinfection procedure, however, not only differs from country to country but in many cases differ on a regional basis, and even from treatment facility to facility in a region. Regulations passed by the individual governments not only demand proper disinfection but also careful control of disinfection by-products. These regulations then lead to many countries, especially the highly developed first world countries, investigating the development of alternative disinfection methods with the same function as conventional treatment. However, until these alternative procedures have been developed and tested, many water treatment plants still employ the conventional methods.

1.2.3.1 Chlorination

Chlorination is applied to most domestic waters supplied from surface water, most groundwaters used, recycled cooling and process waters and many treated wastewaters. Chlorination is the addition of chlorine directly to the water. It is usually added in the form of Cl_2 gas, chlorine dioxide (ClO_2), sodium hypochlorite (NaOCl), and calcium hypochlorite [Ca $(OCl)_2$]. Although the use of sodium or calcium hypochlorite is more expensive than the use of chlorine gas, many treatment facilities

have switched to the use of these chemicals because of the greater safety involved in their handling, compared with chlorine gas (Tchobanoglous & Schroeder, 1985; American Water Works Association, 1999).

Liquid chlorine was first produced in 1909 and was first used for water disinfection in Philadelphia in the USA in 1913 (Peavy & Rowe, 1985; Haas, 1999). Factors contributing to the widespread use of chlorine include its relatively low cost, ease of application, proven reliability, detectability and familiarity with its use (White, 1972; Haas, 1999). Decades of experience has shown that prescribed levels of residual chlorine remaining in the water after a prescribed time of contact assures the consumer that the water is safe to drink by providing long-term protection of the water distribution network against contamination and microbial re-growth.

Chlorine's disinfectant effect is influenced by pH, and is lowest in the alkaline range where the hypochlorite ion is the dominant component of free chlorine. The effect of chlorine is further weakened by ammonia, and particularly organic nitrogen compounds, which bind to free chlorine to form inorganic and organic chloramines. As would be expected, the chlorine dose is related to the quality of the water to be disinfected, in other words considerably more chlorine is required for disinfection of wastewaters than for domestic water supplies. In many countries however, the range of chlorine, which can be added to drinking water falls between 0.1-6 mg/l, with 0.05-0.4 mg/l allowed for chlorine dioxide (Wricke, 1998). The chlorine demand of water is therefore the amount of chlorine required to react with organic substances as well as other substances, which may occur in the water. Turbid water and water containing algae, for example, will have a greater chlorine demand than clear water (Wium & Coetzee, 1985). Important factors that affect the disinfection process in water and wastewater, particularly with chlorine, include initial contact, contact time, form and concentration of disinfectant, type and number of organisms, and environmental variables, including pH and temperature (Tchobanoglous & Schroeder, 1985; American Water Works Association, 1999).

Hypochlorous acid (HOCl) has a small molecule size and has a neutral charge, meaning it would easily be able to penetrate the cell wall. It is generally considered to be a destructive, non-selective oxidant, which reacts with all biological molecules. Research has shown that hypochlorous acid terminates adenosine triphosphate (ATP) production in *Escherichia coli*, by inhibiting transport of the fermentative substrate, glucose and respiratory succinate, while simultaneously inactivating the membrane-localised proton-translocating ATP-synthetase, which is of

utmost importance during respiration (Barrette *et al.*, 1989; Haas, 1999). Early damage of bacterial DNA has also been observed when adding chlorine (Haas & Engelbrecht, 1980; Haas, 1999), although the high resistance of bacterial spores and acid-fast bacteria to chlorine has been revealed. This may be ascribed to the failure of hypoclorous acid to penetrate the bacterial cell wall and thus reach the cell membrane (Grabow *et al.*, 1980; American Water Works Association, 1999).

Chlorine can react with organic matter to form organohalogented compounds. Chloroform and certain other trihalomethanes are formed principally during chlorination. The magnitude of their production depends on the concentration of precursor in the influent water, contact time, as well as water quality factors such as pH and temperature (Allonier *et al.,* 1999). It has also been reported that the addition of chlorine at a sufficiently high concentration will kill most bacteria but the chlorine residual soon disappears in a distribution system with a high chlorine demand, allowing for bacterial re-growth. The initial chlorine dose is thus determined by its influence on the organoleptic properties of the water as well as the formation of trihalomethanes (Gibbs *et al.*, 1990).

1.2.3.2 Chloramination

Ammonia may be added to waters lacking in ammonia to allow for the formation of chloramines, which tend to cause less trouble with tastes and odours than do free residuals. Chloramination is the action of chlorine on ammonia or the ammonia-chlorine process (combination of free chlorine and ammonia) (Tebbutt, 1992). The reaction is dependant on the pH, temperature, contact time and initial ratio of chlorine to ammonia. In most cases, the predominant species are monochloramine (NH_2Cl) and dichloramine ($NHCl_2$) (Tchobanoglous & Schroeder, 1985; American Water Works Association, 1999).

Chloramination is most commonly used as a secondary disinfectant, especially in high chlorine demand waters where they can provide bactericidal protection, control of algae, and bacterial after-growth in lieu of free chlorine which would be exhausted in the extremities of the distribution system. Chloramines have also been used in place of free chlorine to minimise taste (White, 1972; Haas, 1999). On the other hand it has been demonstrated that free chlorine (as HOCl or OCl^{-1}) is a better disinfectant over a shorter time than chloramines (Kreft *et al.*, 1985; Haas, 1999). Chloramine residuals, however, have been reported to be more effective over

a long period of time than free chlorine because of its persistence (Kruse *et al.*, 1981). It has also been useful for balancing the conflicting goals of disinfection and the control of disinfection by-products (DBP). Although chloramine treatment may not be effective in all systems, many utilities are rediscovering the use of chloramine as a primary or secondary disinfectant in their quest to reduce THM's. The disadvantages of using chloramines include their limited disinfection capabilities and longer contact times required when compared to chlorine. Chloramines are also less effective bactericides and viricides than either hypochlorous acid or hypochlorite ion. It is also limited to a pH range of 6 to 9 for disinfection (Tchobanoglous & Schroeder, 1985; American Water Works Association, 1999).

1.2.3.3 Ozonation

Ozone (O_3) is an allotropic form of oxygen produced by passing dry oxygen or air through an electrical discharge (5000 – 20 000 V). It is an unstable, highly toxic blue gas, which has a characteristic penetrating odour, readily detectable at concentrations as low as 0.01 to 0.05 ppm. The maximum concentration of ozone, which can be added to treat drinking water, is 10 mg/l (Wricke, 1998). The most widely used method for commercial ozone generation in water treatment and disinfection is a process which uses a strong alternating current electric field to produce corona discharge in the feed gas, which is usually air that has been filtered and dried (Langlais *et al.,* 1991; Lehtola *et al.,* 2001)

A powerful oxidising agent, ozone is an efficient disinfectant and useful in bleaching colour and removing tastes and odours (Glaze, 1987). Like oxygen it is only slightly soluble in water and because of its unstable form it leaves no residual. Applications of ozonation include oxidation of organic and inorganic materials, flocculation and micro-flocculation for removal of turbidity or suspended solids, as well as the promotion of aerobic biological processes conducted in filter and adsorption media (Gracia *et al.,* 2000). Unless cheap energy is available ozone treatment is however, much more expensive than chlorination but it does have the advantage of good colour removal. Because of the absence of ozone residuals in the distribution system, biological growth with attendant colour, taste and odour problems may result. Adding a small dose of chlorine after ozonation can usually prevent such growth in the distribution system (Tebbutt, 1992).

Ozonation has long been a proven technology for water disinfection and is well established at over 1300 plants around the world (e.g. Budapest, Duesseldorf, Los Angeles, Montreal, Moscow, Paris and Zuerich) as disinfectant and oxidant (Schalekamp, 1988). In South Africa, ozone is used in full scale by the Western Gauteng Regional Water Company (Pietersens *et al.*, 1993). When comparing ozone to chlorine, ozone has been shown to be superior to chlorine as a coagulant (Prendiville, 1986). Its application as an alternative to chlorine has two major drawbacks. Its instability in water, in some cases results in a short half-life, with the result that a residual disinfectant capacity will not remain at the far reaches of a large distribution system. Ozone also reacts with natural organic substances to produce low molecular weight oxygenated by-products that are generally more biodegradable than their precursors. These substances will promote biological growth in a distribution system, further limiting the disinfection efficacy of ozone. For this reason, ozone should be used in combination with other disinfectants that maintain an active residual for longer periods and it should be combined with some method of filtration for removing biodegradable material (Glaze, 1987).

1.2.3.4 Ultraviolet Irradiation

Various forms of radiation can be effective disinfecting agents, and ultraviolet radiation has been used as a non-chemical form of disinfectant for the treatment of small water supplies for many years. The water to be disinfected passes through a chamber where it is exposed to ultraviolet light, at a wavelength of around 254 nm, which affects sensitive and biologically essential components of living cells such as RNA or DNA molecules (Margolin, 1997). In practise the water to be treated flows between mercury arc discharge tubes and polished metal reflector tubes which gives efficient disinfection with a retention time of a few seconds although at a rather high power requirement of 10-20 W/m^3h. The necessary dosage for the killing of microorgansims depends on the morphology and factors such as the physiological state of the specific microorganism. In general, bacteria with thick cell enclosures are harder to destroy than bacteria with thin cells enclosures. Algae are more difficult to kill than bacteria, and require approximately 100 times more energy for destruction (Van der Westhuizen, 1995).

The efficiency of UV irradiation is directly dependant on the quality of the water, especially in relation to the turbidity of the water, agglomeration of

microorganims and organic and inorganic dissolved substances. The advantages of UV disinfection include no formation of tastes and odours, minimum maintenance, and easy automatic control with no danger from overdosing. Disadvantages of UV irradiation are that no long-term residual germicidal effect occurs, the high costs involved and the need for high clarity in the water (Tebbutt, 1992).

1.2.3.5 Hydrogen Peroxide

Hydrogen peroxide is a disinfectant with bactericidal and sporocidal properties (Weiner *et al.,* 2000). It is effective against a broad range of bacteria, including many which have become resistant to chlorine-based chemicals, such as strains of *Pseudomonas aeruginosa* (Reimann, 1992). The antimicrobial action of hydrogen peroxide may involve the impingement of surface membranes through hydroxy radical formation (OH), oxidation of protein sulphydryl groups and double bonds. Bacterial surfaces exposed to hydrogen peroxide have also exhibited erosion of protective polysaccharide coatings and cleavage of cell chains (Baldry & Fraser, 1988). As it also has a sporocidal effect, hydrogen peroxide causes lysis of spores, which includes damage to the spore coat, oxidative cortex hydrolysis or germination-like changes due to activation of cortex lytic enzymes (Foster & Johnstone, 1987; Weiner *et al.,* 2000).

The application of hydrogen peroxide as a disinfectant is not difficult, but the quantity required often presents a problem. As this disinfectant requires a higher residual concentration for effective sanitation than chlorine, it is uneconomical to consider it as a direct replacement in large-scale drinking water treatment. However, it can be used on a large scale in conjunction with other disinfectants such as UV irradiation and ozone. Hydrogen peroxide also provides a long-lasting residual in water, which is stable towards sunlight and temperature changes (Tobiason *et al.*, 1992; Weiner *et al.,* 2000).

1.2.4 Wetland Treatment Systems

Constructed wetland treatment systems are gaining increasing interest as an alternative to the more traditional biological treatment methods as it is more economical and requires less maintenance. The wetland systems are aquatic treatment systems consisting of a natural or prepared wetland to which wastewater is

applied for the purpose of treatment (Van Loon *et al.*, 2002). Wetlands are comparatively shallow (typically less than 0.6 m) bodies of slow moving water in which dense stands of plants such as cattails, bulrushes or water hyacinths are grown. Types of engineered wetlands used as aquatic treatment systems include artificial marshes, ponds and trenches. Bacteria attached to the submersed roots and stems of aquatic plants growing in the wetlands are of particular importance in the removal of soluble and colloidal biological oxygen demand (BOD) from a wastewater. Wetlands wastewater treatment systems can be used as secondary or advanced secondary wastewater treatment processes.

Removal of biodegradable organic compounds - Wetland systems appear to be able to remove BOD at high rates during the warmer seasons and when the bacterial support structure provided by aquatic plants in the form of roots and stems is greatest. During the cooler seasons the BOD removal performance of wetlands systems decreases because of the slowing of the metabolic activity of both bacteria and the plants that support and in part sustain the bacteria.

Removal of nitrogen – the principle nitrogen removal mechanisms operational in wetlands systems is bacterial nitrification/denitrification (not plant uptake). This concept is directly influenced by climatic conditions, as is the case for BOD removal. However, nitrogen removal is even more sensitive to climatic factors, as nitrifying bacteria in wastewater environments are ineffective below approximately 10°C (Tchobanoglous & Schroeder, 1985).

Wetlands systems have also been shown to greatly reduce pathogen levels by as much as 99.9%. However, microbial ecology within wetland treatment systems has not been extensively researched, and the mechanisms involved in pathogen removal are not well understood. It is clear though, that similar to other treatment systems, a range of physical, chemical and biological removal mechanisms are in effect within wetlands (Barcina *et al.,* 1997; Khatiwada & Polpraset, 1999). Van Loon *et al.* (2002) found that tracer methods in combination with bench-scale wetland mesocosms under controlled conditions, was an effective method of assessing pathogen removal kinetics. An interesting observation was that biological activity such as protozoan grazing and biofilm filtration, seemed to be the primary pathogen removal mechanism within wetlands.

In a constructed wetland system microbial communites can also be used as clean-up mechanisms by removing excess nutrients from a polluted area. For example, in mine remediation sulphate reducing bacteria (SRBs) are used to

scavenge metals that accumulate in the tailings and open streams around the mines, which may threaten surface- and groundwater in the area (DeFrancesco, 2003).

1.3 POTABLE WATER DISTRIBUTION NETWORKS

A potable water distribution system is a network of pipes and appurtenances that transports water from the place of treatment to the consumer. The aim is always to ensure that the consumer receives a sufficient and uninterrupted supply and that contamination is not introduced in transit. It is therefore essential that the water quality remains stable from the treatment plant, throughout this intricate distribution system, to the consumer.

Water distribution systems differ widely in their size and distances, and the design of the distribution system will be governed both by the topography and the location and size of the community. A series of transmission lines and pumps are used to move the water flow from the source to the treatment plant and subsequently from there to the distribution system in the community. The reliability of the system, including the maintenance of constant internal pressure, requires careful attention to the interconnection of pipes to provide close loops. This allows the flow to approach any point in the network from more than one direction and helps ensure continued water supply and maintenance of pressure everywhere in the event of pipeline failures. The most prevalent problems associated with water quality control in distribution systems are taste and odour. A further problem is the deterioration of bacteriological quality, which is usually attributed to three phenomena; 1) biofouling, due to the proliferation of microorganisms, which causes tastes and odours, with the loss of carrying capacity of pipes and sometimes severe corrosion, 2) chemical and electrolytic corrosion, resulting in undesirable end products, such as metallic and brackish tastes as well as failure of hot water heaters and residence water piping, and 3) appearance of coliform organisms in the distribution system, indicating recontamination of an otherwise safe water (Chemuliti *et al.,* 2002; Egorov *et al.,* 2002; Hem, 2002).

A slow flow rate along pipe walls facilitates a build-up of biofilms and although chlorine will attack biofilms, it cannot prevent bacterial growth on pipe walls [LeChevallier *et al.*, 1988 (a)]. Many mechanisms can introduce these bacteria into the drinking water distribution system. These include open reservoirs, enclosed reservoirs to which no chlorine is added, breakages due to new constructions that

may disturb the existing distribution system, mains breaks as the distribution system ages, back pressure, and dead-ends in mains with stagnant water (Rossie, 1975).

Microbial contamination can also occur as a result of the use of unsuitable materials for items coming into contact with water, such as materials used for washers, joining and packing materials, pipe and tank lining compounds, and polymeric materials used in pipes, tanks and faucets, all of which can deteriorate to form substances that support the growth of microorganisms. Such materials should be subject to approval by the authority responsible for the water-supply system.

1.3.1 Flow measurement and distribution

In order to operate a treatment plant efficiently it is necessary to be able to measure flow rates into the plant and through the various units. In open channel systems this is usually achieved by using a hydraulic control structure, such as a venturi flume, which has a head discharge relationship. For closed-pipe systems a venturimetre, orifice plate or electromagnetic flow meter is used as appropriate. Flow division is of great importance when treatment capacity is split between several units in parallel. The most effective form of flow splitting is that provided by free-fall weirs arranged in a chamber with a separate length of weir discharging to each unit to be fed. The choice of flow measuring and splitting devices in a treatment plant can be restricted by their head loss characteristics, since gravity flow through the whole plant is desirable and thus the head available for hydraulic control structures is often limited (White, 1982; American Water Works Association, 1999). A reduction of the water flow pressure in the system may also result in back siphonage (flow against the normal direction of the water network) (Geldreich, 1989; American Water Works Association, 1999).

1.3.2 Drinking Water Quality Standards

Setting definite rules or principals for drinking water quality is clearly a difficult task as the definition of a good water quality is closely related to the intended use of the water, and setting this standard based on a given use potential is inappropriate.

Two general methods are in use for setting standards: governmental stipulation; and a policy of minimum degradation. The United States has gravitated toward a modified minimal degradation policy that incorporates a best-available-

treatment (BAT) criterion. Stream water quality standards are set, and wastewater discharges are not allowed to violate these standards. Defining BAT is not always a simple procedure, and it must be recognised that BAT changes with time. The Federal Republic of Germany (FRG) has established a carrot and stick approach based on a minimum degradation philosophy. Compliance with their drinking water standards is established through a series of announced and unannounced samplings (Tchobanoglous & Schroeder, 1985; Wricke, 1998). The European Union has also adopted a new Drinking Water Directive (Council Directive, 1998), which will for the first time set uniform standards on the quality of water intended for human consumption in Europe (Breach, 1999). Guidelines by the South African Bureau of Standards (SABS) (2001) has been adopted by the majority of the water treatment facilities in South Africa and the quality of drinking water is defined in terms of physical, biological, organoleptic and chemical properties that are suitable for delivery as potable water (**Tables 1.5 & 1.6**).

Table 1.5 Microbiological requirements of drinking water as stipulated according to the SABS, 2001

Determinants	**Units**	**Allowable compliance contribution[a]**		
		95% of samples, min.	**4% of samples, max.**	**1% of samples, max.**
Heterotrophic plate count	Count/ml	100	1000	10000
Total coliform bacteria	Count/100 ml	Not detected	10	100
Faecal coliform bacteria[b]	Count/100 ml	Not detected	1	10
Escherichia coli[b]	Count/100 ml	Not detected	Not detected	1
Somatic coliphages	Count/10 ml	Not detected	1	10
Enteric viruses	Count/100 l	Not detected	1	10
Protozoan parasites (*Giardia/Cryptosporidium*)	Count/10 l	Not detected	Not detected	1

a- the objective of disinfection should be to attain 100% compliance to the limits indicated in column 3

b- in most cases it will not be necessary to conduct both these tests, one or the other will normally suffice as the required indicator.

Table 1.6 Physical, organoleptic and chemical requirements of drinking water as stipulated according to the SABS, (2001)

Determinants	Units	Upper limit and ranges			Class II water consumption period[a], Max.
		Class 0 (Ideal)	Class I (Acceptable)	Class II (Max. Allow.)	
Physical and Organoleptic requirements					
Colour	mg/l Pt	<15	15-20	>20-50	No limit[b]
Conductivity at 25°C	mS/m	<70	70-150	>150-370	7 years
Dissolved solids	mg/l	<450	450-1000	>1000-2400	7 years
Odour	TON	<1	1-5	>5-10	No limit[b]
pH value at 25°C	pH	6,0 – 9,0	5,0-9,5	4,0-10,0	No health effect[c]
Taste	units	<1	1-5	>5-10	No limit
Turbidity	FTN	<0,1	0,1-1	>1-10	No limit[d]
Chemical requirements – macro-determinants					
Ammonia as N	mg/l	<0,2	0,2-1,0	>1,0-2,0	No limit[d]
Calcium as Ca	mg/l	<80	80-150	>150-300	7 years
Chloride as Cl^-	mg/l	<100	100-200	>200-600	7 years
Flouride as F^-	mg/l	<0,7	0,7-1,0	>1,0-1,5	1 year
Magnesium as Mg	mg/l	<30	30-70	>70-100	7 years
Nitrate & Nitrite as N	mg/l	<6,0	6,0-10,0	>10,0-20,0	7 years
Potassium as K	mg/l	<25	25-50	>50-100	7 years
Sodium as Na	mg/l	<100	100-200	>200-400	7 years
Sulphate as $SO_4^=$	mg/l	<200	200-400	>400-600	7 years
Zinc as Zn	mg/l	<3,0	3,0-5,0	>5,0-10,0	1 year
Chemical requirements – micro-determinants					
Aluminium as Al	µg/l	<150	150-300	>300-500	1 year
Antimony as Sb	µg/l	<5	5-10	>10-50	1 year
Arsenic as As	µg/l	<10	10-50	>50-200	3 months
Cadmium as Cd	µg/l	<3	3-5	>5-10	6 months
Chromium as Cr	µg/l	<50	50-100	>100-500	3 months
Cobalt as Co	µg/l	<250	250-500	>500-1000	1 year
Copper as Cu	µg/l	<500	500-1000	>1000-2000	1 year
Cyanide (free) as CN^-	µg/l	<30	30-50	>50-70	1 week
Cyanide (recoverable) as CN^-	µg/l	<70	70-200	>200-300	1 week
Iron as Fe	µg/l	<10	10-200	>200-2000	7 years[b]
Lead as Pb	µg/l	<10	10-50	>50-100	3 months
Manganese as Mn	µg/l	<50	50-100	>100-1000	7 years
Mercury as Hg	µg/l	<1	1-2	>2-5	3 months
Nickel as Ni	µg/l	<50	50-150	>150-350	1 year
Selenium as Se	µg/l	<10	10-20	>20-50	1 year
Vanadium as V	µg/l	<100	100-200	>200-500	1 year
Chemical requirements – organic determinants					
Dissolved organic carbon as C	mg/l	<5	5-10	>10-20	3 months[e]
Total trihalomethanes	µg/l	<100	100-200	>200-300	10 years[f]
Phenols	µg/l	<5	5-10	>10-70	No limit[b]

[a]- the limits for the consumption of class II water are based on the consumption of 2 l of water per day by a person of mass 70 kg over a period of 70 years

[b]- the limits given are based on aesthetic aspects

[c]- no primary health effect- low pH values can result in structural problems in the distribution system

[d]- these values can indicate process efficiency and risks associated with pathogens

[e]- when dissolved organic carbon is deemed of natural origin, the consumption period can be extended

[f]- this is a suggested value because trihalomethanes have not been proven to have any effect on human health

1.4 MICROORGANISMS ASSOCIATED WITH DRINKING WATER

It is recognised that water plays an essential role in supporting human life, however, it also has, if contaminated, great potential for transmitting a wide variety of diseases. In the developed world water-related diseases are rare, due essentially to the utilisation of efficient water treatment distribution- and wastewater disposal- systems. The major concern in these countries involves the long-term health hazards, which may arise from the presence of trace concentrations of impurities in drinking water, with particular attention being paid to potentially carcinogenic compounds. In the developing countries, however, as many as 1.1 billion people are without safe water supplies and over 2.4 billion lack access to adequate sanitation (World Summit on Sustainable Development, 2002). As a result the toll of water-related disease in these areas is frightening in its extent (Tebbutt, 1992; World Health Organisation, 1996).

The microbial populations inhabiting the surface water (made up from rivers, lakes, ponds, etc.) and the groundwater (obtained principally from wells) can be divided into distinct categories, namely the non-pathogens, opportunistic pathogens and pathogens (Prescott *et al.,* 2002). Some of the human pathogens potentially transmitted in drinking water are listed in **Table 1.7**. These include pathogenic agents, such as bacteria, viruses, and protozoa, which may cause diseases varying in severity from mild gastroenteritis to severe and sometimes fatal diarrhoea, dysentery, hepatitis or typhoid fever. Faecal contamination of drinking water is only one of several faeco-oral mechanisms by which they can be transmitted from one person to another or, in some cases, from animals to people. The transfer of pathogen from one case to the next may appear simple, but numerous factors, including the ability to survive in the environment, play an important role. Socio-economic factors, such as the practise of food hygiene, the availability of pure water, and the adequacy of excreta disposal, are further complicating factors (Tebbutt, 1992; World Health Organisation, 1996). Not all potentially waterborne human pathogens are of equal public health significance. The agents of high health significance as indicated in **Table 1.7**, present a serious risk of disease whenever they are present in drinking water and their elimination from it should be given high priority.

The likelihood of ingesting very large numbers of a pathogen on a single occasion from drinking water is relatively small, both because enteric pathogens cannot normally multiply in water and because the water tends to disperse them. If

polluted water is permitted to contaminate food, however, bacterial pathogens, initially present in small numbers, can multiply to produce very large doses (World Health Organisation, 1996).

Table 1.7 Waterborne pathogens and their significance in water supplies (World Health Organisation, 1996)

Pathogen	Health Significance	Main Route of Exposure[a]	Persistence in Water Supplies[b]	Relative Infective dose[c]
Bacteria				
Campylobacter jejuni, C. coli	High	O	Moderate	Moderate
Pathogenic *Escherichia coli*	High	O	Moderate	High
Salmonella typhi	High	O	Moderate	High
Other Salmonellae	High	O	Long	High
Shigella spp.	High	O	Short	Moderate
Vibrio Cholera	High	O	Short	High
Yersinia enterocolitica	High	O	Long	High (?)
Legionella	Moderate	I	May multiply	High
Pseudomonas aeruginosa	Moderate	C, IN	May multiply	High (?)
Aeromonas spp.	Moderate	O, C	May multiply	High (?)
Mycobacterium, atypical	Moderate	I, C	May multiply	?
Viruses				
Adenoviruses	High	O, I, C	?	Low
Enteroviruses	High	O	Long	Low
Hepatitis A	High	O	Long	Low
Hepatitis E	High	O	?	Low
Norwalk virus	High	O	?	Low
Rotavirus	High	O	?	Moderate
Small round viruses	Moderate	O	?	Low (?)
Protozoa				
Entamoeba histolytica	High	O	Moderate	Low
Giardia intestinalis	High	O	Moderate	Low
Cryptosporidium parvum	High	O	Long	Low
Acanthamoeba spp.	Moderate	C, I	May multiply	?
Naelgleria fowlerei	Moderate	C	May multiply	Low
Balantidium coli	Moderate	O	?	Low
Helminths				
Dracunculus medinensis	High	O	Moderate	Low
Schistosoma spp.	Moderate	C	Short	Low

?- Not known or uncertain

a- O = oral (ingestion); I = inhalation in aerosol; C = contact with skin; IN = ingestion in immunosupressed patients

b- Detection period for infective stage in water at 20°C: short = up to 1 week; moderate = 1 week to 1 month; long = over 1 month

c- Dose required to cause infection in 50 % of healthy adult volunteers

1.4.1 Bacteria

1.4.1.1 Salmonella

The virulence of *Salmonella* spp. depends on serotype and strain specificity in host range, infective dose and on host status. *S.typhi* is a specific human pathogen. In particular, *S.typhi, S.paratyphi* A, and *S.paratyphi* B are able to invade tissues and cause septicaemia with high temperature rather than diarrhoea. This is known as enteric fever. In humans, the majority of the other serotypes give rise to a transient intestinal infection manifesting itself as acute gastroenteritis with diarrhoea. Certain serotypes are highly pathogenic for humans, while others are devoid of any pathogenic action. Many *Salmonella* infections are asymptomatic (Lloyd, 1983; Cohn *et al.,* 1999).

In the case of *S.typhi* and *S.paratyphi* A, human carriers are the source of infection, whereas milk-borne transmission can also occur with *S.paratyphi* B. The incidence of enteric fever decreases as a country becomes better developed in terms of controlled sewerage systems and drinking-water supplies, and the supply of pasteurised milk and dairy products increases. Most salmonellae are primarily pathogens of animals, which constitute important reservoirs for those infections (Lloyd, 1983). Salmonellae may be present in all kinds of food grown in faecally polluted environments, and are commonly isolated from poultry and livestock and foods prepared from them. Furthermore, animal feedstuffs and fertilizers prepared from animal products may be highly contaminated with salmonellae. The contamination of food and animal feedstuffs by water contaminated with salmonellae is considered to be an additional risk factor (Mersch-Sundermann & Wundt, 1987; Kirk *et al.,* 2002).

Waterborne outbreaks have been primarily associated with *S. typhi* and much less frequently with *S.paratyphi* or other *Salmonella* serotypes (Lloyd, 1983; Cohn *et al.,* 1999). Epidemiological studies of outbreaks suggest that the ingestion of relatively few cells of *S.typhi* may cause typhoid fever, whereas studies in volunteers (Blaser & Newman, 1982) indicate that, for other *Salmonella* serotypes, millions of cells are usually required to cause gastroenteritis. Faecal contamination of groundwater or surface waters, as well as insufficiently treated and inadequately disinfected drinking water, are the main causes of epidemic waterborne outbreaks caused by *Salmonella* spp. Salmonellae can be found in open wells as a result of

the drainage or flooding of contaminated surface water into unprotected well shafts. It is uncommon for salmonellae to be isolated from piped water supplies, whether treated or untreated, and their presence suggests a serious fault in the design or management of the system (Lloyd, 1983; Cohn *et al.,* 1999).

1.4.1.2 Yersinia

Research has shown that certain strains of *Yersinia enterocolitica* may cause acute gastroenteritis with diarrhoea. Biochemical and serological typing of enteric *Y.enterocolitica* strains show that serotypes O:3 and O:9 are commonly found in Africa, Asia, Canada and Europe, whereas serotype O:8 is exclusively isolated in the United States of America (Swaminathan *et al.*, 1982; Lloyd, 1983; Cohn et al., 1999).

The transmission of *Y.enterocolitica* from natural reservoirs to humans has been the subject of much debate. Many domestic and wild animals are considered to be possible reservoirs of *Y.enterocolitica* because of the high isolation rates of the organisms from such sources. Available evidence indicates that foods, especially meat and meat products, milk and dairy products, are the major vehicles for the transmission of *Y.enterocolitica*. Many other transmission routes have been suggested for this bacterium, but the ingestion of contaminated food and water is probably the most likely one (Nissen *et al.,* 2000, 2001; Ramalho *et al.,* 2001).

There is some evidence that pathogenic strains of *Y.enterocolitica* enter drinking water via contaminated surface water or as a result of pollution with sewage (Ziegert & Diesterweg, 1990). In general, pathogenic types of *Y.enterocolitica* are not isolated from untreated or treated drinking water unless faecal pollution has occurred.

1.4.1.3 Campylobacter

Worldwide, campylobacters are much more important than salmonellae as causes of acute gastroenteritis. Several major outbreaks of *Campylobacter enteritis* were linked to the ingestion of contaminated food, milk or water (Smibert, 1981; Cohn *et al.,* 1999). In the case of water hygiene, the campylobacters are of greatest significance.

Research has shown that raw sewage frequently contains $10\text{-}10^5$ campylobacters per 100 ml (Holler, 1988). The occurrence of campylobacters in

surface waters has proved to be strongly dependant on rainfall, water temperature and the presence of waterfowl. They are able to survive for several weeks in cold groundwater or unchlorinated tap water (Gondrosen, 1986).

1.4.1.4 Escherichia coli

Escherichia coli is a normal inhabitant of the intestine, and most strains are non-pathogenic. Some subtypes of this bacterium, however, are able to cause gastrointestinal disease by a variety of mechanisms (Nissen *et al.,* 2000, 2001; Ramalho *et al.,* 2001). Four classes of pathogenic *E.coli* responsible for diarrhoea are recognised: enteropathogenic, enteroinvasive, enterotoxigenic, and verocytotoxin-producing (Feachem, 1982). Enteroinvasive strains of *E.coli* produce dysentery by a mechanism similar to that found in *Shigella* spp. These organisms invade the colonic mucosa and cause bloody diarrhoea. Although enteropathogenic and enteroinvasive strains may cause serious illness, most epidemiological evidence suggests that enterotoxigenic strains are responsible for most episodes of *E.coli* diarrhoea, particularly in developing countries. The fourth class, verocytotoxic *E.coli*, was first recognised by their production of a cytotoxin active against vero cells in culture. Most of the *E. coli* that are found in the human intestine are harmless but vero cytotoxin-producing *E. coli* (VTEC) produce potent toxins and can cause severe disease in man. The toxins, termed Vero cytotoxins (VTs), were originally recognised by their ability to kill vero cells, a tissue culture line of monkey kidney cells. VTEC are responsible for a range of illnesses, which may be severe and sometimes fatal, particularly in infants, young children and the elderly. Although VTEC strains are in a wide range of O serogroups, the most important one associated with human disease is O157. Such strains are termed O157 VTEC. The organisms commonly belong to the serogroup O157 and cause disease ranging from mild diarrhoea to haemorrhagic colitis characterised by blood stained diarrhoea, usually without fever, but accompanied by abdominal pain. It is also a cause of the haemolytic ureamic syndrome, most common in infants and young children, and characterised by acute renal failure and haemolytic anaemia (World Health Organisation, 1996). Isolation of *E.coli* from a water supply indicates faecal contamination. See **Section 1.4.5** for a detailed discussion of *E.coli* as one of the microbial indicators of contamination in water supply systems.

1.4.1.5 Vibrio cholera

Cholera has historically been one of the major pandemic diseases. Cholera is usually a waterborne disease and numerous waterborne outbreaks have been documented. However, foodborne and nosocomial outbreaks are also important, and person-to-person transmission may occur under conditions of extreme crowding and poor hygiene.

The isolation of *V.cholera* O1 from water used for drinking is of major public health concern and is evidence of faecal contamination. However, evidence has accumulated to suggest that other serotypes of *V.cholera* may be part of the normal flora of some natural waters (Miller *et al.*, 1985; Attridge & Rowley, 1990). Infection with *Vibrio cholera* O1 biotype El Tor affects the small and large intestine.

1.4.1.6 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen. It is commonly found in faeces, soil, water and sewage but cannot be used as an indicator of faecal contamination, as it may multiply in an enriched aquatic environment and on the surface of organic materials in contact with water. However, its presence may be one of the factors taken into account in assessing the general cleanliness of water distribution systems and the quality of bottled waters.

Most of the illnesses in humans for which *P.aeruginosa* is responsible are caused, not by drinking water, but by contact with it. Water containing these bacteria may also contaminate food, drinks and pharmaceutical products, causing them to deteriorate and to act as secondary vehicles for transmission. Fixtures in contact with water, such as sinks and sink drains, tap fittings and showerheads, can also be contaminated with *P.aeruginosa* and can serve as reservoirs of infection in hospitals (Berkelman *et al.,* 1981; Bodey *et al.,* 1983; Rusin *et al.,* 1997).

Waterborne infections are usually associated with warm, moist environments, where bacterial counts are high and disinfection is deficient (LeChevallier *et al.*, 1988b). The presence of this organism in water supplied to hospitals and for the manufacture of pharmaceutical preparations and dressings is a matter of concern because *P.aeruginosa* is a common pathogen in infections of wounds and burns and has caused serious eye infections after the use of contaminated eye drops (Bodey *et al.*, 1983; Rusin *et al.,* 1997). The presence of this organism in potable water also

indicates a serious deterioration in bacteriological quality, and is often associated with complaints about taste and odour, turbidity linked to low flow rates in the distribution system, and a rise in water temperature.

1.4.1.7 Legionella

Legionellae are widespread in natural sources of water and may also be found in soils. They occur commonly in man-made water systems, particularly in hot water and cooling water systems. Infection is the result of the inhalation of aerosols that are small enough to penetrate the lungs and be retained by the alveoli. It has been shown that legionellae can be ingested by the trophozoites of certain amoebae and even grow intracellularly and become incorporated in their cysts (Lee & West, 1991). This may explain the difficulty in eradicating legionellae from water systems and may be a factor in the aetiology of the non-pneumonic disease (Rowbotham, 1980; Rusin *et al.*, 1997).

It is generally advisable to design and maintain systems in such a way that colonisation by *Legionella* is prevented as far as possible. Detailed instructions for achieving this have been given in several publications (Gezondheidsraad, 1986; Department of Health and Social Security, 1987) and focus on the following aspects:

- preventing the accumulation of sludge, scale, rust, algae and slime, and removing such deposits regularly;
- maintaining hot water temperatures permanently above 60°C or increasing them periodically to above 70°C, and keeping cold-water supplies below 20°C;
- selecting materials for contact with water that do not release nutrients that support the growth of *Legionella*.

1.4.2 Viruses

The viruses of greatest significance in the waterborne transmission of infectious disease are essentially those that multiply in the intestine of humans and are excreted in large numbers in the faeces of the infected individuals. Although viruses cannot multiply outside the tissues of the infected hosts, some enteric viruses appear to have considerable ability to survive in the environment and remain infective. Discharges of sewage and human excreta constitute the main source of human enteric viruses in the aquatic environment. Sewage treatment may reduce the

numbers of viruses 10-1000 fold, depending on the nature and extent of the treatment. However, such treatment typically does not entirely eliminate viruses, and the sludge produced during sewage treatment will often contain large numbers of viruses (World Health Organisation, 1996).

Waterborne outbreaks due to viruses have been recorded from developed and developing countries around the globe (Murphy *et al.*, 1983; Ticehurst, 1991). Enteric viruses are capable of producing a wide variety of syndromes, including rashes, fever, gastroenteritis, mycocarditis, meningitis, respiratory disease and hepatitis. Many different strains of viruses have been isolated from raw and treated drinking water. Isolation from water does not prove beyond all possible doubt that water is a vehicle for the transmission of disease, although it does indicate that a hazard exists. Proper treatment and disinfection should result in drinking water that is essentially virus free. In some areas, water sources may be heavily polluted, and the water treatment process used may not be reliable. For this reason, and because of the large number of persons at risk, drinking water must be regarded as having a very significant potential as a vehicle for the environmental transmission of enteric viruses. Waterborne transmission has been unequivocally demonstrated for hepatitis A and hepatitis E viruses, rotavirus and Norwalk virus (World Health Organisation, 1996; American Water Works Association, 1999).

1.4.3 Protozoa

Drinking water plays a major role in the spread of three of the intestinal protozoa pathogenic for humans, namely *Giardia intestinalis, Crytosporidium parvum*, and *Entamoeba histolytica*. *Balantidium coli* infection is uncommon although the parasite has a worldwide distribution. These pathogenic intestinal protozoa can be transmitted to humans by any mechanisms whereby material contaminated with faeces containing viable organisms from infected individuals can reach the mouth.

Epidemic giardiases associated with contaminated drinking water has been reported in Canada, England, Scotland, and Sweden. Drinking water has also been implicated as the vehicle of transmission in outbreaks occurring among travellers in the former Soviet Union (World Health Organisation, 1996). Endemic giardiases has also been associated with the consumption of contaminated drinking water in such diverse locations as the United States of America and South Africa (Esrey, 1988; Craun, 1990). In addition to endemic and epidemic giardiasis from drinking water

supplies, there have been reported outbreaks in the USA and Canada, affecting children and adults, caused by the ingestion of swimming pool water. The source of contamination in these outbreaks was apparently related to defaecation in the water by infected children (Greensmith, 1988; American Water Works Association, 1999).

Humans and other mammals are reservoirs for infection, and the contamination of water supplies with either human or animal sewage can lead to the transmission of *Cryptosporidium* through drinking water. Outbreaks have been traced to the contamination of drinking water by both human and animal wastewaters (Casemore,. 1990). Apart from *Giardia, Cryptosporidium* probably has the greatest potential for transmission through drinking water of all the waterborne parasitic protozoa since;

- oocysts from humans are infective agents for a wide variety of domestic and wild animals, and are widely distributed in the environment;
- some waterborne outbreaks have been attributed to the contamination of drinking water by oocysts of nonhuman origin;
- among the protozoa under consideration, *Cryptosporidium* spp. have the smallest and most chlorine resistant oocysts.

Cryptosporidium infection in immunocompetent persons have been found in 26 countries, with a reported prevalence of 0.6-20% in developed, and 4-20% in developing countries. The infection is more common in children than in adults (Soave & Armstrong, 1988). Among AIDS patients, cryptosporidiosis has a prevalence of 3-4% in the USA and over 50% in some African countries and Haiti. At present, no effective drug is available for the treatment of cryptosporidiosis.

1.4.4 Helminths

Drinking water containing infected cyclops is the only known source of infection with *Dracunculus*, which is therefore the only human parasite that can be eradicated solely by the provision of safe drinking water (World Health Organisation, 1996). Guinea worm occurs in rural areas where piped water supplies are not always available. Control is based principally on the provision of boreholes and safe wells, but also includes measures aimed at preventing contamination of water sources, filtering of water by consumers, and in some situations chemical treatment of ponds and open wells. There are no effective antihelminthic drugs for the clinical treatment of the infection (Global Surveillance Summary, 1993).

Schistosoma spp. belong to the class of trematodes or flukes, whose infective larvae are able to penetrate the human skin or mucous membranes, causing schistosomiasis. Schistosome infections are acquired when infected water is used for domestic activities, bathing or washing, or while working in contact with water. While there is a real possibility of piped untreated surface water transmitting schistosomiasis, most transmission is from unpiped sources such as pools, wells, and cisterns. In regions where schistosomiasis is endemic, the construction of dams and large reservoirs often leads to an increase in the population of the aquatic snail host and thus favours the spread of the disease. Schistosome infections are a hazard of recreational and irrigational water use rather than of drinking water (World Health Organisation, 1993).

1.4.5 Microbial Indicators of Water Quality

The recognition that faecally polluted water is responsible for spreading enteric diseases led to the development of sensitive methods of verifying that drinking water is free from faecal contamination. Even though many waterborne pathogens can now be detected, the methods involved are often difficult, relatively expensive and time-consuming. Furthermore, pathogens are introduced into water only from infected people and animals, and it is not possible to examine water for every possible pathogen that might be present. It was decided to regard as unsafe all water that contains bacteria indicative of faecal pollution, because of the risk that enteric pathogens may be present. The bacteria selected as indicators of faecal pollution should be universally present in the faeces of humans and warm-blooded animals in large numbers. It is also desirable that the faecal indicators be readily detected by simple methods and that they do not grow in natural waters (World Health Organisation, 1996). Furthermore, it is essential that their persistence in water and the extent to which they are removed by water treatment are similar to those of waterborne pathogens, as the presence of indicator organisms in finished water would then indicate that one of the barriers or treatment processes was not functioning properly (Toranzos & McFeters, 1997).

The use of normal intestinal organisms as indicators of faecal pollution rather than the pathogens themselves is universally accepted for monitoring and assessing the microbial safety of water supplies. It has, however, been suggested that no direct correlation can be drawn between the numbers of any single indicator

organism and the enteric pathogens (Grabow, 1996). In order to eliminate the uncertainty the following three groups are now recognised as microbial indicator organisms; general (process) microbial indicators; faecal indicators; and index and model organisms (Fewtrall & Bartram, 2001).

The general (process) microbial indicators are defined as the group of organisms that demonstrates the efficacy of a process such as chlorine disinfection. These include total heterotrophic bacteria or total coliforms. Coliform bacteria should thus not be detected in treated water supplies and if found, suggest inadequate treatment, post-treatment contamination, or excessive nutrients. Many water treatment plants also test for the presence of coliform organisms as an indicator of drinking water quality, largely because they are easy to detect and enumerate. By definition, coliform bacteria are Gram-negative rods, capable of growth in the presence of bile salts, able to ferment lactose at 35-37°C with the production of acid, gas and aldehyde within 24-48 hours. Coliform bacteria also display ß-galactosidase activity.

Thermotolerant (faecal) coliforms are defined as a group of coliform organisms that are able to ferment lactose at 44-45°C. They comprise the genus *Escherichia* and, to a lesser extent, species of *Klebsiella*, *Enterobacter* and *Citrobacter*. Faecal streptococci, sulphite-reducing clostridia, bacteriophages, coliphages and *Pseudomonas* spp. among others, may also be used as process indicators to determine the biological safety of treated water (World Health Organisation, 1996).

Research has indicated that coliforms survive in distribution systems as part of a bacterial biofilm on pipe surfaces (Camper *et al.*, 1991; LeChevallier *et al.,* 1996; McMath *et al.*, 1999). Despite this observation the isolation of biofilms with coliform organisms from water distribution systems has rarely been reported (LeChevallier *et al.,* 1987; McMath *et al.*, 1999). There is thus a need to further explore this phenomenon as it may have far-reaching implications for the management of drinking water distribution systems.

The faecal indicators are defined as a group of organisms that indicates the presence of faecal contamination, such as *Escherichia coli* as this organism is abundant in human and animal faeces, where numbers may reach 10^9 per gram of fresh faeces. It is found in sewage, treated effluents, and all natural waters and soils subject to recent faecal contamination, whether from humans, farm or wild animals and birds. The presence of *E.coli* in treated waters always indicates potentially

dangerous contamination which requires immediate attention (Toranzos & McFeters, 1997; Fewtrell & Bartram, 2001).

The index and model organisms are defined as a group or species indicative of the presence and behaviour of a pathogen, such as *E.coli* as an index for *Salmonella*, and F-RNA coliphages as models of human enteric viruses (Fewtrell & Bartram, 2001). F-RNA coliphages as well as other phages are similar in morphology, structure and behaviour to human enteric viruses. These similarities suggest that they should be better models for faecal pollution than the indicator bacteria when human viruses are the likely aetiological agents. Even though a poor correlation in phage numbers is expected when compared to the level of human enteric viruse titre, an important factor in the case of this model organism is that laboratory experiments with individual coliphages have confirmed, that the survival of the phages in natural water environments and their resistance to commonly used disinfectants such as chlorine, resembles that of enteric viruses (Stetler, 1984; Yates *et al.,* 1985; Grabow, 1986). The value of phages as models for viruses was also demonstrated when applying them in the routine monitoring of raw and treated drinking water supplies (Grabow *et al.,* 2000).

Even though phages are useful as models/surrogates for enteric viruses, a number of deficiencies have been noted when using this system: (a) phages are excreted by a certain percentage of humans and animals all the time while viruses are excreted only by infected individuals for a short period of time (Grabow *et al.,* 1999); (b) methods used to detect a somatic coliphages also detect a wide range of phages with different properties (Gerba, 1987; Yates *et al.,* 1985); and enteric viruses have been detected in water environment in the absence of coliphages (Montgomery, 1982; Moringio *et al.,* 1992).

1.4.6. Methods Used to Monitor Indicator Microorganisms Associated with Drinking Water

In 1905 MacConkey first described his now famous MacConkey's broth, which tests for lactose-fermenting bacteria tolerant of bile salts. As a result the broth is used diagnostically by many laboratories testing for the presence of coliforms. The most probable number (MPN) and membrane filtration (MF) methods are also widely used as the standard procedures to enumerate coliforms and *E.coli* in drinking water samples. Although both tests are simple to perform the MPN procedure is

considered to be time-consuming and requires 48 hours for the presumptive results. The inability to demonstrate gas production with membranes is considered a major drawback of the membrane filtration method (Fewtrell & Bartram, 2001).

Defined substrate methods which incorporate media without harsh selective agents but specific enzyme substrates allow for significant improvements in recoveries and identification of target bacteria. Edberg *et al.* (1988, 1990 & 1991) introduced the defined substrate method for coliforms and *E.coli.* This method has evolved into the Colilert® technique which correlates well with the traditional membrane filter and MPN methods when used to test both fresh and marine water (Clark *et al.,* 1991; Palmer *et al.,* 1993; Fricker *et al.,* 1997; Eckner, 1998). A further advantage of these enzyme-based methods is that they appear to pick up traditionally non-culturable coliforms (George *et al.,* 2000). As a result of these developments the International Standards Organisation has published a miniaturised MPN-based method for coliforms/*E.coli* and enterococci based on the defined substrate approach (ISO/FDIS 1998, 1999).

The use of chromogenic compounds, which may be added to conventional or newly developed media, has been used as a fast and reliable method to test for indicator bacteria. The detection method is based on the concept that the chromogenic substances are modified either by enzymes (typical for the specific bacteria) or by specific bacterial metabolites. After modification the chromogenic substance changes its colour or fluorescence, enabling easy detection of those colonies displaying the metabolic activity (Fewtrell & Bartram, 2001).

The application of monoclonal and polyclonal antibodies is based on the antigen-antibody concept. In other words the antibodies possess highly specific binding and recognition domains that can be targeted to specific structures of a pathogen (antigen). Polyclonal antibodies are obtained directly from the serum of immunised animals. The *in vitro* fusing of plasma cells from an immunised animal (e.g. mouse or rat) with a cell line that grows continuously in culture (ensures that the fused cells produce only one kind of antibody) produces monoclonal antibodies (Goding, 1986). These monoclonal antibodies have been successfully applied for the detection of indicator bacteria in water samples (Hübner *et al.,* 1992; Obst *et al.,* 1994). In order to ensure the detection of only viable cells, the water sample is typically subjected to precultivation in a selective medium. A major advantage of immunological detection methods is the fact that they can be easily automated in order to accommodate and handle high sample numbers (Fewtrell & Bartram, 2001).

Immunomagnetic separation is a rapid identification method of culturable and non-culturable microorganisms (Safarik *et al.,* 1995). The simple application and principles of the method rely on suitable antibody specificity and involve the biotinylation of purified antigens, which are then bound to streptoavidin-coated paramagnetic particles. The raw sample is gently mixed with the immunomagnetic beads, after which a specific magnet is used to hold the target organisms against the wall of the recovery vial, with the non-bound material poured off. The beads can be removed by simple vortexing with the target organism then cultured or identified by direct procedures (Fewtrell & Bartram, 2001).

Gene sequence based methods such as the polymerase chain reaction (PCR) and *fluorescence in situ hybridisation* (*FISH*), have a substantial potential for future application in the field of drinking water hygiene as they are rapid and can be tailored to detect specific strains or groups of organisms (Havelaar, 1993). The molecular based approach also shows the greatest promise for the development of a unified system for waterborne pathogen detection (Straub *et al.,* 2002). A major advantage in the application of gene sequence methods such as the PCR technique, for the identification of microorganisms from drinking water, is that the target organisms do not need to be culturable. A problem with PCR is that the assay volume is usually some microlitres, whereas the water sample is usually in the range of 100-1000 ml. A filtration method to concentrate the sample has however, been published (Bej & McCarthy, 1991), but a further problem is the fact that natural water samples often contain inhibitory substances (such as humic acid and iron) that concentrate with the nucleic acids. A number of *FISH* methods have also been developed for the detection of coliforms and enterococci (Meier *et al.,* 1997; Fuchs *et al.,* 1998; Patel *et al.,* 1998). FISH detection based methods also efficiently detect the presence of infective pathogens and viable indicator bacteria (Fewtrell & Bartram, 2001).

Future developments for the detection of indicator and pathogen index organisms include methods based on microarrays and biosensors. Microarray technology like PCR is based on the Watson and Crick model of stranded DNA/RNA molecules that bond together in a defined order (Fewtrell & Bartram, 2001). The methods employs gene chips i.e. probes, anchored to glass or nylon slides. Each spot on a microarray is intended to bind to a specific target sequence and using automated robotics, tens of thousands of spots can be placed on a single array. Furthermore, these probes can be used for the detection of multiple target sequences and have been used to directly detect RNA without a PCR amplification

step. This technology is thus currently being developed with real-time monitoring approaches for the detection of a multitude of microbial pathogens in water (Reynolds, 2003).

1.5 BIOFILM FORMATION

Enormous ecological, medical, agricultural and commercial significance can be obtained by the activities of microorganisms associated with soil, rivers, lakes, the sea or animals and plants. For obvious reasons of ease, economy and ethics, the great majority of research in the scientific field involves laboratory- and pilot-scale test systems. Outside the laboratory bacteria usually face a highly competitive, hostile and changing environment in which the organism must either adapt its physiology to that appropriate for the environment or it will die (Brown, 1977; Gilbert *et al.*, 1987). Hostile influences in the real world usually include a shortage or competition for essential nutrients, the presence of inhibitors, which may be xenobiotic or natural, and various biological influences.

Most environments allow for the existence of sessile (attached) and planktonic (free-living) bacterial populations in such a way that neither community completely dominates (Fletcher, 1979). The attachment of single organisms to various surfaces in nature is dependent on factors such as mechanisms of attachment and favourable environmental conditions. Colonies of cells are formed when these attached cells grow and actively multiply, with the proper colonisation of these microorganisms further aided by the essential development of organic polymers (Allison & Sutherland, 1987). A microbial biofilm is eventually formed when this mass of cells expand in such a way to enable them to trap organic and inorganic debris, nutrients and other microorganisms (Kumar & Anand, 1998).

The dynamic process of biofilm formation has been shown to involve a sequence of steps (**Figure 1.2**). The first step involves the formation of a conditioning film which entails a surface becoming coated with a layer of adsorbed (attached) organic and inorganic molecules. The properties of the film depend on the nature of the surface material and on the kind of molecules present in the aqueous environment (Characklis, 1981; Hood & Zottola, 1997). Once the conditioning film is formed on the surface of the material, it facilitates the next step in the formation of biofilms, which involves the attachment of the microorganisms.

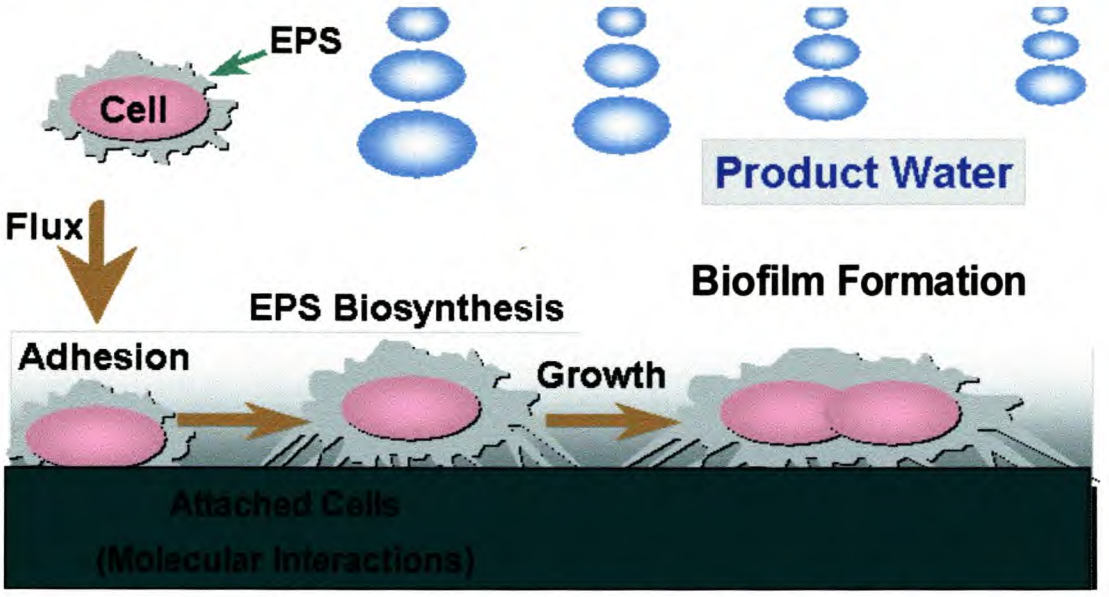


Figure 1.2 Diagrammatic representation of biofilm formation (Adapted from MicroMem Analytical, 2003)

Planktonic microorganisms as well as organic and inorganic molecules can be transported or moved towards a surface by diffusion, gravity, the turbulent flow of liquid (water flow), through microbial motility or by a combination of these processes. Various physico-chemical properties of the microorganism's cell surface, such as surface free energy, hydrophobicity and electrostatic charges, are vital in determining the adhesion of cells during this initial attachment phase (Dickson & Koohmaraie, 1989; Van Loosdrecht *et al.*, 1990). The attachment of cells generally takes place in two stages: a reversible attachment followed by an irreversible attachment. Reversible adhesion refers to the initial weak associations, which develop between the organism's cells and the substratum. During this stage, microorganisms will exhibit Brownian motion and can effortlessly be dislodged by fluid shear forces, e.g. simply by rinsing (Marshall *et al.*, 1971). The next significant step in biofilm formation is the irreversible attachment of cells. Repulsive forces are essentially involved in preventing the cells from making direct contact with the surface. However, the production of surface appendages such as flagella, fimbriae, pili and the exopolysaccharide (EPS) fibrils by the microorganism still allows for contact (Jones & Isaacson, 1983; Kumar & Anand, 1998). The polymeric fibrils form a bridge linking the bacterial cell and substratum enabling this irreversible association with the surface. In this process stronger forces such as scrubbing and scraping could possibly remove the cells (Marshall *et al.*, 1971). Nutrients present in the organic or conditioning film as well as from the surrounding fluid environment then allow these

irreversibly attached bacterial cells to grow and divide. Consequently microcolonies are formed, which expand and unite to form a layer of cells covering the surface. Additional polymer gets produced by the attached cells during this stage, which further aids in the attachment of the cells to the surface and also stabilizes the colony from the fluctuations of the environment (Wolfaardt *et al.*, 1994; Kumar & Anand, 1998). In addition, the EPS can protect the microorganism from dehydration as it can retain water several times its own mass and only slowly becomes desiccated (Roberson & Firestone, 1992; Wolfaardt *et al.,* 1999)

The stable attachment of the bacterial cells to the substratum and its ensuing growth, coupled with associated EPS production, eventually leads to the formation of a biofilm. Multilayers of microbial cells entrapped within the EPS-containing matrices are formed within the biofilm. The microorganisms associated with the biofilm then grow in the matrix-enclosed microcolonies interspersed within highly permeable water channels (Costerton *et al.*, 1994). Depending on culture conditions a biofilm can grow to a few millimetres thick in a matter of days (Kumar & Anand, 1998).

The surface adherent, biofilm mode of growth basically predominates in natural ecosystems and is quite remarkable in that to a certain extent the sessile bacteria, as a group create their own environment (Costerton *et al.*, 1987, Brown *et al.*, 1991). In addition, the biofilm mode of growth not only enables greater reproducibility and control of the micro-environment, but subsequently a greater chance of survival for the microorganism (Brown *et al.*, 1991).

Different microorganisms invariably have different nutritional requirements, implying that the composition of biofilms may be heterogenous. The initial colonising species may also possibly encourage the colonisation of species, which are physiologically compatible, while inhibiting the attachment of others.

The preferred mode of growth for most bacteria would thus be in association with a biofilm. It should however, be remembered that certain nutrients or environmental conditions may favour or limit biofilm formation. Numerous studies on the ability of various nutrients to inhibit or allow for the development of biofilms have been conducted. Mohamed *et al.* (1998) determined that phosphorous limits the growth of heterotrophic biofilm bacteria in the Fraser River, British Columbia. Their work also established that in treatments where phosphorous was not added, bacteria accumulated more EPS per bacterial cell than bacteria where phosphorous was added. They concluded that this could have been an adaptive mechanism to acquire limiting nutrients. Research by Liehr *et al.* (1989) showed that the interaction of

carbon and light limitation in algal biofilms is fairly complex and should be considered in algal biofilm modelling. The degree of adhesion of a microorganism to a contact surface could also be influenced by the pH and temperature of that surface. *Pseudomonas fragi* exhibited maximum adhesion to stainless steel surfaces, at a pH range of 7 to 8, which is optimal for its cell metabolism (Stanley, 1983). Shea *et al.* (1991) established that *Deleya mariana*, a marine bacterium, adhered maximally to a hydrophilic substratum at 25°C, the optimum temperature for growth of this organism. Studies conducted by Hallam *et al.* (2001) showed that the lower the water temperature the lower the biofilm activity, and that biofilm activity seemed to decrease by 50% at a temperature of 7°C that at 17°C. Various techniques to investigate, clarify and ultimately mimic the in-vivo behaviour and activities of biofilms in their natural habitats are constantly being developed.

1.5.1 The Influence of Surfaces on Biofilm Formation

One of the most interesting questions concerning bacterial attachment to surfaces relates to the effect of solid surfaces on bacterial activity. The general belief is that the activity of bacteria is enhanced at surfaces, particularly in low nutrient environments, but this concept is not clearly understood. It is thus considered extremely difficult to predict the effect of various surfaces on bacterial activity, but numerous studies have however, been carried out in an effort to clarify this point and consequently a number of possible explanations exist. The most commonly accepted explanation is that nutrients may be adsorbed and concentrated at surfaces and thereby made more available to attached bacteria. It is also thought that more efficient use of exoenzymes is made at the surface and finally that a wide range of other chemical and physical factors will be different at the solid/liquid interface, as compared to the bulk of the medium (Fletcher, 1979).

It can only be expected that microorganisms in natural environments get attracted to, and encounter a vast number of solid surfaces. In several cases these surfaces are laced with nutrients, which favour and support these organisms' ability to grow and multiply and eventually may alter their physiological and ecological behaviour (Marshall, 1997).

1.5.1.1 *Biofilm formation on potable water distribution piping materials*

Research on various aspects of bacterial biofilms, formed in association with different solid surfaces in natural environments, industrial distribution systems, wastewater treatment operations and in laboratory- and pilot-scale test systems, is being carried out in laboratories around the world. The focal points of these studies are diverse and range from mechanisms of adhesion to surfaces, general aspects of colonization and biofilm formation, methods of controlling and removing biofilms and understandably the various techniques used in studying the microbial activity in biofilms.

Piping materials used in drinking water distribution systems can generally be classified into one of three types, i.e. cement, metallic or plastic, with these materials used in varying proportions in countries throughout the world. For biofilm growth to occur, necessary elements must be available for the organisms, otherwise they cannot build new cell material. It is thus obvious that biofilms may develop on the surface of a plumbing material that is able to supply nutrients for bacterial growth. For this reason, the United Kingdom has implemented the British Standard BS6920, which examines the suitability of materials for plumbing as well as the materials influence on water quality. This ensures that the material does not contribute to poor water quality by producing unacceptable tastes or odours, releasing chemicals, or encouraging microbial growth (British Standards Institution, 1988).

The transportation of surface waters via concrete pipes has been used in the United Kingdom, Unites States of America and Germany to some extent for almost 100 years. The involvement of microorganisms in the deterioration of concrete has been considered for a number of years, but only recently have attempts been made to understand the basis of microbial mediated decay of concrete. The process is believed to be complex, involving several different groups of microorganisms and either inorganic or organic acids. Research has identified *Thiobacillus* spp. and Anaerobic Sulphate Reducing Bacteria (ASRBS) as the dominant bacteria responsible for the degradation of concrete in cooling water systems, fire water systems and sewer pipes (Kulpa & Baker, 1990; Poulton & Mixon, 1992). A study conducted by Poulton and Mixon (1992) however, showed microbial attachment of bacteria on concrete, with no deterioration effect noted even after extended periods of time.

Copper can also be used as a piping material in distribution systems as well as domestic plumbing systems. Problems have however, been encountered when copper piping is used for the plumbing of potable systems in large institutional buildings (e.g. hospitals). Copper pipe failure within the United Kingdom, Germany, Saudi Arabia and Japan have been reported to be due to abnormal forms of pitting corrosion (McEvoy & Colbourne, 1988; Fischer *et al.*, 1992). It has been demonstrated that biofilms are formed on this material, readily corroding it. Stainless steel has been proposed as an alternative to copper. Different grades of stainless steel are available, with different compositions and properties, and consequently different uses. Types 304 and 304L are the most widely used basic grades of the chromium-nickel stainless steel, with types 316 and 316L being the more corrosion resistant grades, which contain, in addition to chromium and nickel, molybdenum (Percival *et al.*, 1998). Percival *et al.* (1998) investigated biofilm formation on stainless steel grades 304 and 316 exposed to potable water for a period of 24 months. Results revealed that total cell counts were not significantly different on the two grades of stainless steel, while viable cell counts differed on the different grades. Furthermore, metal ion levels were generally low within the biofilms with no indication of molybdenum leaching. No evidence of corrosion on any of the metallic surfaces could be found, despite being exposed to water for 24 months, demonstrating the long-term stability of this metal.

Various polymeric materials, such as Polyethylene (PE), Polyvinylchloride (PVC) and Teflon, as well as various grades of stainless steel have been adapted for use as pipe and tubing material in drinking water distribution systems. Despite the many advantages of using these materials in the distribution system, research has shown that they also contribute to biofilm formation in drinking water. Zacheus *et al.* (2000) investigated biofilm formation on PVC, PE and stainless steel (grade 304) exposed to ozonated water. Their study revealed no clear difference in biofilm formation on the three different surfaces. Pedersen (1990) had similar findings when studying the formation of biofilms on PVC and stainless steel in drinking water. Rogers *et al.* (1994) however, found higher bacterial numbers on PE than on PVC, with both these numbers higher than those detected on stainless steel. Similarly, Clark *et al.* (1994) found that the biofilm accumulation was higher on the two polymeric materials, PE and PVC, than on cement. A study conducted by Hallam *et al.* (2001) demonstrated that the piping materials used within a drinking water distribution system has a marked influence on the biofilm activity, measured as mean

biofilm potential (MBP), with the highest MBP measured on PVC, then MDPE, followed by cement and finally glass. Van der Kooij *et al.* (1995a) investigated biofilm formation on teflon and glass. Their results revealed great similarities both in accumulation rate and in amount of accumulated biomass on these materials when exposed to a specific water type.

1.5.1.2 Factors that favour biofilm growth in drinking water distribution systems

It has been concluded that among the factors that primarily influence biofilm growth on materials in drinking water distribution systems are environmental factors (e.g., pH, temperature, etc.), an accumulation of organic materials and sediment in distribution pipes, the presence and effectiveness of disinfectant residuals, internal corrosion, and an increase in water temperature (Characklis & Wilderer, 1989; LeChevallier *et al.*, 1990).

Water temperature is perhaps the most important rate-controlling factor that affects and governs microbial growth (Characklis & Wilderer, 1989). Not only does temperature influence the microbial growth rate, but it also has an influence on treatment plant efficiency, disinfection efficiency, dissipation of disinfectant residuals, corrosion rates and distribution system hydraulics, as well as water velocity through customer demand. A number of investigators have observed significant microbial activity in water at temperatures of 15°C or higher (Donlan & Pipes, 1988; LeChevallier *et al.*, 1990).

The behaviour of water flowing through pipe systems in industrial applications influences both bacterial attachment and detachment and ultimately the development of the biofilm. Two extremes of flow generally exist in potable water pipe systems; laminar and turbulent (Munson *et al.*, 1990). Laminar flow is smooth, slower flow with no lateral mixing, whereas turbulent flow is often defined as irregular and chaotic. As most water flow in engineered systems is turbulent, Percival *et al.* (1999) investigated the effect of turbulent flow and surface roughness on biofilm formation in drinking water. Cell counts on slides of stainless steel grades 304 and 316 with both smooth and rough finishes showed viable and total cell counts were higher at the higher flow rates of 0.96 and 1.75 m s^{-1}, compared to a flow rate of 0.32 m s^{-1}. Extracellular polysaccharide levels were not significantly different ($P<0.05$) between each flow rate on all stainless steel surfaces studied. Results clearly showed that

surface roughness influenced the transport and attachment of microbial cells in that bacterial settlement was greater in cavities and grooves on the roughened surface. Microbial growth in drinking water requires nutrients such as organic carbon, nitrogen and phosphorus, with organic carbon, especially assimilable organic carbon (AOC) considered the main nutrient controlling microbial growth in drinking water distribution systems (Van der Kooij *et al.*, 1982; LeChevallier *et al.*, 1991). The AOC test has been used in the Netherlands for more than 10 years to help determine treatment strategies to limit bacterial growth in water (Van der Kooij, 1987, 1992; Van der Kooij *et al.*, 1995b). Recent studies have however, shown that if water contains high concentrations of organic matter, microbial growth in drinking water can be limited by phosphorus instead of organic carbon (Sathasivan *et al.*, 1997; Sathasivan & Ohgaki, 1999). Lehtola *et al.* 2001 showed that in water treatment, microbially available phosphorus (MAP) and AOC should be considered as important factors that can limit microbial growth in drinking water.

Drinking water also contains organic and inorganic matter, which is capable of accumulating at the surface of the drinking water pipelines. The formation and quality of these soft deposits mainly depend on the microbiological and chemical quality of produced drinking water and on the circumstances prevailing in the distribution system (Zacheus *et al.*, 2001).

1.5.2 Disinfection of Biofilms

The attachment of microorganisms to surfaces is ubiquitous in natural and man-made environments. A water distribution system is an example of an environment where the nutrient levels available in the flowing water are low, and where microoganisms attach to the surfaces of the pipes and equipment found in the distribution network (Ridgway & Olson, 1982; LeChevallier *et al.*, 1988b; LeChevallier *et al.*, 1988c; Hermanowicz & Filho, 1992). This biofilm formation could contribute to a range of costly problems within industrial environments. These interfacial microbial communities are directly involved in biofouling and biocorrosion, that cause significant reductions in system performance, and contribute to the accelerated deterioration of components within the drinking water distribution system (McFeters *et al.*, 1995).

Biofilm build-up on the surfaces of materials in water distribution networks could cause aesthetical problems for the consumers and economical and technical

problems for the water companies. Furthermore, a significant number of waterborne disease outbreaks have been associated with public water systems (Geldreich, 1996; World Health Organisation, 1996), which might be attributed to inadequate care during repairs of the distribution system, insufficient treatment of the water before release into the network, and possibly biofilm formation on piping materials. The magnitude and ubiquitous character of biofilm formation emphasizes the significance of controlling their growth in industry, with extensive research being conducted into the disinfection of these biofilms from the distribution systems.

Biofilms in drinking water systems have however, proved difficult to inactivate. Nagy *et al.* (1982) reported that bacterial levels in Los Angeles, California, aqueduct biofilms were high even in the presence of a residual of 1 to 2 mg of chlorine per litre. Maintenance of a residual of 3 to 5 mg of chlorine per litre was necessary to reduce bacterial biofilms by more than 99.9%. Ridgway *et al.* (1984) however, found that a residual of 15 to 20 mg of chlorine per litre was necessary to control biofouling of reverse osmosis membranes. In contrast, Momba and Binda (2002) observed the inability of bacteria to grow on stainless steel and galvanised mild steel when 0.35 mg l^{-1} residual monochloramine was maintained throughout the drinking water system.

The inactivation of biofilm bacteria and characterisation of the interaction of biocides with microbial interfaces was investigated by LeChavellier *et al.* (1988b). The research examined four disinfectants (hypochlorous acid, hypochlorite, chlorine dioxide and monochloramine), three types of surfaces (granular activated carbon, metal coupons, and glass slides), two bacterial types (heterotrophic plate count bacteria and coliforms) as well as several alternate biocides (copper, zinc, sodium chlorite and alkaline pH). Results showed that unattached bacteria were notably susceptible to the variety of disinfectants tested. Biofilm bacteria grown on the different types of surfaces were 150 to more than 3000 times more resistant to hypochlorous acid (free chlorine, pH 7) than were unattached cells. In contrast, resistance of biofilm bacteria to monochloramine disinfection ranged from 2- to 100-fold more than that of unattached cells. This study further showed that monochloramine was as effective as free chlorine for the inactivation of biofilm bacteria. Furthermore, the results indicated that the alternate biocides had some effectiveness on suspended bacteria but limited activity against biofilm microorganisms. Various authors have suggested that the limited penetration of chlorine in the biofilm matrix could be an important factor influencing the reduced

efficacy of this biocide against biofilms as compared with its action against planktonic cells (Paquin *et al.*, 1992; De Beer *et al.*, 1994).

A study conducted by Lund and Ormerod (1995) investigated the action of chlorination, UV irradiation and ozonation on biofilm formation in water distribution systems over a period of 20 months. They found that ozone allowed for a greater production of biofilm than UV irradiation. In their experiments, the residual free chlorine in the chlorinated water was sufficient to prevent biofilm formation for the duration of the experimental period. Johansen *et al.* (1997) found that the combining oxidoreductases with polysaccharide-hydrolysing enzymes resulted in bactericidal activity as well as the removal of the biofilm. Palmer and White (1997) investigated the developmental biology of biofilms, for its implications in the treatment and control of this mode of growth. It was suggested that the first opportunity for affecting biofilm development is at the substratum. Modification of the substratum to alter non-specific attachment is an attractive approach that can hinder biofilm initiation under controlled conditions. Alternatively a substance can be applied that enhances attachment of particular micoorganisms thereby reducing the efficiency of attachment of other undesirable microorganisms. These simplistic approaches do not work in complex natural or industrial situations, but they may succeed under the more controlled conditions to which catheters and other indwelling medical devices, and even dental appliances are exposed. They also suggested that as the microbial signalling of cell concentration [quorum sensing (Allison *et al.*, 1998; Davies *et al.*, 1998)], controls large banks of genes in many different organisms, it could provide an opportunity for the control of attachment and detachment of the biofilm.

Numerous applications of biofilms in bioremediation however, exists as these microbial communities are inexpensive labourers capable of cleaning up pollutants such as, metal contaminants, chlorinated solvents, crude oil, sewage water, etc. In sewage treatment plants, biofilm communities are used to remove excessive nutrients from water, as they are an inexpensive alternative to chemical cleaning. This alternative could however, have negative implications if corrosion of the piping material occurs. This can be very expensive to fix and usually involves the physical removal (scraping) of the biofilm from the pipes, which is time consuming and costly (Schachter, 2003).

1.6 TECHNIQUES FOR STUDYING THE MICROBIAL ACTIVITY IN BIOFILMS

A variety of techniques have been applied for the evaluation of biofilms in association with their natural habitats, industrial distribution systems, wastewater treatment operations and in laboratory- and pilot-scale test systems. Grooves, crevices, dead ends, corrosion patches, etc. are some of the areas where biofilms can grow and become hard to access. These limitations often arise during the sampling of biofilms (Kumar & Anand, 1998). Conventional cultivating methods for the enumeration and sampling of biofilms include swabbing, rinsing, scraping, vortexing, agar flooding and agar contact methods (Mustapha & Liewen, 1989; Frank & Koffi, 1990). More recently, advances in microscopy and fluorescent staining as well as the use of fluorescent labelling are extensively used to study the interactions of the different microorganisms in the biofilm, in contact with its habitat.

1.6.1 Cultivation Dependant Techniques

Potable water samples can be analysed for the presence of coliform bacteria using a range of techniques. The MPN and MF methods are however, the two methods specified by the Standard Methods for the examination of Water and Wastewater (1998), for the microbiological analysis of drinking water. The MPN method uses probability statistics to determine the mean concentration of bacteria as the MPN per volume of sample and is based on the random dispersion of microorganisms in a given sample (Fujioka, 1997; Toranzas & McFeters, 1997). This technique can be rather time consuming as it is generally conducted in three sequential phases, namely, presumptive, confirmatory and completed. The membrane filter method is probably the most widely used method for the cultivation of organisms because of its simplicity, speed and precision. A membrane filter is used to entrap the bacterial cells. The membrane is then placed on an appropriate agar and incubated (Geldreich, 1996; Toranzas & McFeters, 1997). Limitations of the MF method are the presence of non-target microorganisms (Clark & Pagel, 1977; Geldreich *et al.*, 1978; Geldreich, 1996; Toranzas & McFeters, 1997), and its suitability for low turbidity waters only (LeChevallier *et al.*, 1981; Geldreich *et al.*, 1978; Geldreich, 1996). The MPN method is generally recommended for high turbidity waters (Toranzas & McFeters, 1997). Although the microbiological analysis of drinking water is predominantly done using the MPN and MF methods, a method was introduced in the 1930's to simply examine for the presence or absence, i.e. the

Presence-Absence test (P-A test), for indicator organisms. Comparative studies suggest that the P-A test detected a significantly higher number of positive samples than either the MPN and MF methods (Geldreich, 1996; Toranzas & McFeters, 1997).

The total numbers of viable bacteria in a potable water sample generally provides an indication of the general water quality as well as the effectiveness of water treatment processes such as disinfection, filtration and coagulation. This can be achieved by performing the heterotrophic plant count of the sample using classical microbiological techniques such as pour or spread plating, as well as by using the MF method (Geldreich, 1996). Although the pour plate technique is relatively easy to perform, the greatest drawback of this technique is that it often leads to lower bacterial colony counts because of the selective effects of heat shock from the melted agar. It is recommended that this technique not be used for the enumeration of heat-sensitive and possibly nutritionally stressed heterotrophic bacteria in water (Klein & Wu, 1974; Geldreich, 1996). The spread plate method is considered no more difficult than the pour plate method, with spread plating providing conditions more optimal for the cultivation of heterotrophic bacteria. While it is true that spread plating gives a better indication of bacterial counts, pour plating on the other hand yields greater accuracy between replicates. The two media most frequently used to enumerate the heterotrophic plate count are standard plate count agar and R2A agar. R2A agar, however has been gaining increasing popularity as this medium yields significantly higher bacterial counts than plate count agar, which does not permit the growth of certain bacteria which may be present in the treated water sample (Reasoner & Geldreich, 1985; Geldreich, 1996).

1.6.2 Microscopy

The physiology, chemistry, characteristics, interactions and activities of bacterial biofilms have been investigated for decades. Microscopy has gained considerable attention as a highly valuable approach and has proved indispensable for the investigation of biofilms. Interference reflection microscopy, epiflourescence microscopy and atomic force microscopy are some of the techniques, that have attracted considerable interest in the study of biofilms (Ladd & Costerton, 1990; Lawrence *et al.*, 1997; Kumar & Anand, 1998). Phase-contrast microscopy is usually the most routine and convenient light microscopy technique, as it avoids the need for drying and staining and can be more readily used in continuous observation systems

(Lawrence *et al.*, 1987; Lawrence *et al.*, 1997). Environmental scanning electron microscopy (ESEM) is another technique which has been widely used in the enumeration of biofilms as it not only helps in visualising the sample without the need of conventional SEM procedures like dehydration, fixation and staining/coating, but also preserves many of the structures associated with biological samples which remain in hydrated and viable state (Kumar & Anand, 1998).

The numbers and distribution of microorganisms adhering to surfaces can be determined using scanning electron microscopy (SEM). However, SEM lacks sufficient resolution to give any convincing idea of structures involved in the process of adhesion (Marshall, 1997). More recently, confocal scanning laser microscopy (CSLM) has also been widely used as it can document biofilm morphology and physiology in four dimensions under *in situ* conditions (Kuehn *et al.,* 1998; Palmer & Sternberg, 1999). Computer enhanced microscopy employs image processing computers in conjunction with video microscopy techniques or other capturing devices to obtain quantitative data of microbial behaviour during various scenarios (Caldwell, 1985; Gualtieri *et al.*, 1985; Korber *et al.*, 1989). Korber *et al.* (1989) successfully developed computer image processing programmes to routinely and interactively analyse video tapes of microbial movement during chemotaxis studies. Furthermore image analysis provided rapid and detailed analysis of numerous behavioural parameters and delivered high-quality motility tracks regardless of debris or inactive cells present in the original image.

Two or more techniques can be used in conjunction with each other to investigate the same or parallel specimens or activities. Lawrence *et al.* (1987) used phase, darkfield and computer-enhanced microscopy to observe and analyse the microbial behaviour of *Pseudomonas fluorescens* in a flow cell set-up during bacterial colonisation. While previous authors had only referred to the reversible and irreversible phase of surface attachment for *P. fluorescens*, this study showed that the surface colonisation of *P. fluorescens* can in fact be subdivided into the following sequential colonisation phases: motile attachment, reversible attachment, irreversible attachment, growth phase and recolonisation phase. Lawrence *et al.* (1994) also studied the efficiency of flourescein and size-fractionated flour-conjugated dextrans in conjunction with CLSM to directly monitor and determine diffusion coefficients within biofilms. This study indicated that the fate of numerous fluorescent probes in the biofilm matrix can be monitored using CLSM. The *In situ* analysis of biofilms on historic window glass was successfully investigated by Mueller *et al.* (2001) using the

CLS microscope. They concluded that the information on thickness and microbial composition of the biofilm obtained using CLSM offered an essential background to optimise cleaning procedures or conservation strategies for stained glass windows. Interference reflection microscopy (IRM) and light section microscopy (LSM) together proved useful in investigating the effects of cations (Ca^{2+}, La^{3+}), a highly polar cosolvent (dimethyl sulfoxide) and a nonionic detergent (Tween 20) on the attachment and biofilm adhesives of *Pseudomonas flourescens* H_2S (Marshall *et al.*, 1989).

Fluorescent Staining- Direct counting of the total bacterial population present is one of the most significant methods for the evaluation of bacterial counts in natural waters as well as in biofilms. This can be achieved using various fluorescent dyes such as acridine orange [3,6-bis (dimethylamino) acridium chloride] and 4',6-diamidino-2-phenylindole (DAPI). Kulakov *et al.* (2002) enumerated the bacteria contaminating ultrapure water in industrial systems by both plate counts and epifluorescence microscopy using cyanotolyl tetrazolium chloride (CTC) and DAPI staining. Assessment of bacterial presence by epifluorescence microscopy and staining showed significantly higher numbers (10- to 100 times more bacteria cells were detected) than that detected by plate counts.

The Live/Dead Baclight™ bacterial viability assay utilises mixtures of SYTO9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. These strains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When mixed in recommended proportions, SYTO9 stain produces green fluorescent staining of bacteria with intact cell membranes and propidium iodide produces red fluorescent staining of bacteria with damaged membranes (Molecular Probes, 2002). The Live/Dead Baclight™ bacterial assay used in conjunction with Epiflourescence or CLS microscopy can thus be used to determine the viability of the biofilms as well as the live/dead ratio within the biofilm community. Boulos *et al.* (1999) applied the Live/Dead BacLight™ stain to enumerate viable and total bacterial counts in drinking water. They concluded that even though Baclight viable counts were comparable to 5-cyano-2,3-ditolyl tetrazolium (CTC) counts and total counts were comparable to acridine orange counts, an increase in environmental stresses (chlorine, growth rate or temperature) induced a decrease in viability that was more pronounced for plate counts than for viable counts as obtained by these fluorescent stains.

1.6.3 Fluorescence Labelling

Green Fluorescent Protein- In 1994, the gene encoding green fluorescent protein (GFP) became available as an important visual, molecular marker of gene expression in eukaryotic organisms. It is more sensitive than other reporter genes, requires no special cofactors for detection and can be quantified with a spectrofluorometer (Chalfie, 1994; Stretton *et al.*, 1998; Errampalli *et al.*, 1999). The application of GFP as a molecular marker allows for new research that will increase the general understanding of microorganisms in the environment, particularly as it is a powerful method for non-destructive monitoring in situ (Errampalli *et al.*, 1999; Sternberg *et al.*, 1999; Timms-Wilson & Bailey, 2001). The functionality of the Tn-7 Gfp based system for single-copy insertion of cloned genes into the chromosome of *Rhodospirillum rubrum* was demonstrated by Bao *et al.* (1991). Li *et al.* (1999) tagged *Agrobacterium tumefaciens* with a mini-Tn5 transposon containing a promoterless gene encoding a GFP variant, to monitor cell morphology and gene expression associated with infection. Confocal laser scanning microscopy was then used to monitor the GFP-tagged bacterial cells *in situ* during the infection.

A panel of Tn7-based vectors for insertion of the Gfp marker gene or for delivery of cloned DNA into gram-negative bacteria at a neutral chromosomal site was developed by Koch *et al.* (2001). In one group of vectors, the mini Tn7 element, which is inserted into the chromosome, contains a multiple cloning site (MCS) and the kanamycin, streptomycin and gentamycin resistance markers. Another group of vectors intended for tagging with green fluorescent protein carries the *gfp*mut3^{+} gene controlled by the modified *lac* promoter $P_{\wedge 1/04/03}$, several transcriptional terminators and various resistance markers.

16S and 23S rRNA probes- 16S-labelled, rRNA-targeted oligonucleotide probes are increasingly used as a tool to identify bacteria within their natural habitats (Amann *et al.*, 1990; Amann, 1995). These probes can be designed to be complementary to phylogenetically more or less conserved regions of rRNA. The advantage of using this method is among others the ability to differentiate bacteria on various phylogenetic levels and, at the same time, to achieve at least qualitative information on the physiological state of the bacteria on the basis of the number of ribosomes per cell. Specific rRNA probes can also be obtained for uncultured microorganisms (Manz *et al.*, 1993). Amann *et al.* (1992) demonstrated the applicability of these probes to study biofilms. Research by Manz *et al.* (1993) demonstrated that bacteria

in drinking water and multispecies biofilms in oligotrophic environments can be studied by hybridisation with 16S and 23S rRNA-directed fluorescent oligonuleotide probes. A further observation was that the coexistance and interaction of bacteria in drinking water biofilms may be an integral part of their growth and survival strategies. Moller *et al.* (1998), successfully combined GFP expression and *in situ* hybridisation with fluorescence-labelled 16S rRNA targeting probes to identify organisms and visualise gene expression in fixed mixed culture biofilms growing in laboratory-based flow chambers.

1.6.4 Analysis of the biological stability and biofilm formation rate and potential of biofilms in drinking water systems

Assimilable Organic Carbon – Research has shown that the surest way of avoiding bacterial regrowth and deterioration of water quality in a distribution system is to sufficiently remove biodegradable organic matter from water i.e. AOC (Liu *et al.,* 2002). Van der Kooij *et al.* (1982) developed a technique to assess the concentration of easily degradable AOC, by determining the maximum colony counts of *Pseudomonas fluorescens* strain P17 and *Spirullum* sp. strain NOX in water samples which had been pasteurised to inactivate the natural microbial population. Based on their observations of AOC concentrations, a guideline of 10 µg C/l was defined for drinking water, which allowed for a limited heterotrophic growth potential (Van der Kooij, 1992).

Adenosine Tri-Phosphate Concentration – Adenosine tri phosphate (ATP) is an energy carrier present in all viable cells. The determination of the ATP concentration in a biofilm sample is representative of the total active biomass or viable population of the community (Van der Kooij & Veenendaal, 1993). ATP monitoring or detection assays can also be useful tools in determining or measuring bacterial population density ratios such as the biofilm formation rate (BFR) as well as the biofilm formation potential (BFP) of drinking water. The BFR is generally defined as the linear increase of ATP concentrations as a function of time, and is usually calculated from the period of 0 to 100 days as the biofilm is considered to be actively growing during this time (Vrouwenvelder *et al.,* 1997). The BFP is then usually calculated from the period of 100 to 150 days or in some cases until the end of the study period as the biofilm is considered to be stable at this time (Vrouwenvelder *et al.,* 1997). Comparative techniques involve CLSM microscopy used in combination with the

Baclight™ Live/Dead stain which allows for the analysis of biofilm thickness, area coverage and cell activity (Lawrence *et al.,* 1994; Mueller *et al.,* 2001). The rate and potential of biofilm growth in real-time is however difficult to determine using the CLSM and other microscopy techniques without appropriate online devices for sampling. In general, these microscopy-based techniques provide information on features such as surface coverage and percentage cell viability, which can be used to show changes in viability and growth of the microbial population present in the biofilm.

1.6.5 The MicroLog™ System

This is an easy to use yet advanced system for identifying and characterising microorganisms using a combined database, which includes over 1400 species of aerobic bacteria, anaerobic bacteria, and yeasts. This technology uses each microbe's ability to use particular carbon sources to produce a unique pattern or "fingerprint" for that microbe. As a microorganism utilises the carbon sources in the respective wells of the MicroPlate, it respires. This respiration reduces a tretrazolium dye and those wells turn purple. The end result is a pattern of purple wells on the MicroPlate that is characteristic of that microorganism. The fingerprint data is fed into MicroLog software, which searches its extensive database and makes an identification in seconds (Bochner, 1989). Different identification MicroPlates (e.g., GN MicroPlate to identify gram-negative migroorganisms) and special purpose MicroPlates (e.g., ECO MicroPlate contains three identical sets of 31 carbon sources, used to monitor and study changes in microbial communities) are routinely used (Biolog, 1999).

Since Garland and Mills (1991) first used Biolog™ (Hayward,Ca., USA) substrate utilisation tests to characterise microbial communities of terrestrial and aquatic ecosystems, this approach has repeatedly been applied in microbial ecology. Moll and Summer (1999) found the Biolog™ system useful in characterising the microbial communities in drinking water biofilters. Klinger *et al.* (1992) evaluated the Biolog™ system to identify 39 American type culture collection reference taxa and 35 gram-negative isolates from water samples. They concluded that although a total of 93% of the water isolates were identified correctly, identification of some authentic strains of *Enterobacter, Klebsiella*, and *Serratia* was unreliable.

Van der Merwe *et al.* (2003) used Biolog™ plates to analyse the functional diversity of planktonic and sessile microbial communities in a paper-mill water system. By employing this system they concluded that a high functional diversity existed in both the planktonic and sessile communities in this environment. Furthermore, the substrate utilisation profiles obtained with the Biolog™ plates were a sensitive measure to detect shifts in the microbial community within the water system.

2. MATERIALS AND METHODS

2.1 EVALUATION OF BIOFILM FORMATION CHARACTERISTICS AND BIOLOGICAL STABILITY OF DRINKING WATER

2.1.1 Background

Despite the role of water in sustaining human health, it is recognised that when contaminated, water has a potential for transmitting a wide variety of diseases. Through advances in modern technology, various processes were developed for the purpose of controlling, and when needed, eliminating various sources and forms of contamination from drinking water and effluents, in a cost- and time effective manner.

The treatment required for water depends primarily on the quality of the water source. Different water sources typically show great variation, depending on environmental factors and anthropological activity. In the Duesseldorf area, Germany, the main water source is composed primarily of riverbank filtrate from the river Rhine and to a smaller extent groundwater.

Riverbank filtration of raw water obtained from wells situated along the banks of the river Rhine was originally sufficient to produce drinking water in Germany after World War II. However, a new process for water treatment had to be developed in the 1950's when heavy pollution of the Rhine affected water quality. The Duesseldorf water treatment process (**Figure 2.1**) was introduced in 1961 and currently encompasses the application of ozonation and granular activated carbon (GAC) filtration of riverbank- and ground- water (Vrouwenvelder *et al.*, 1997). Additionally, after the water has passed through the activated carbon filters, phosphate and silicate (1 mg/l, respectively) are added to the water to prevent corrosion of the piping materials in the distribution system. Chlorine dioxide is also added as disinfectant at a concentration of 0.06 mg/l to prevent the regrowth of microorganisms on the way to the consumer (Vrouwenvelder *et al.*, 1997).

The ozone added oxidises the organic and inorganic materials in the water and thereby acts as an efficient disinfectant in addition to bleaching colour and removing tastes and odours. Similar to oxygen it is only slightly soluble in water and because of its unstable form it leaves no residual (Glaze, 1987). The GAC filter consists of a 1.5 m top layer of activated carbon, which removes nitrate and

manganese as well as other pollutants. The remaining compounds, which could influence the odour/taste of the water, are then removed by adsorption to the activated carbon in the bottom 2.5 m lower layer of the filter (**Figure 2.1**).

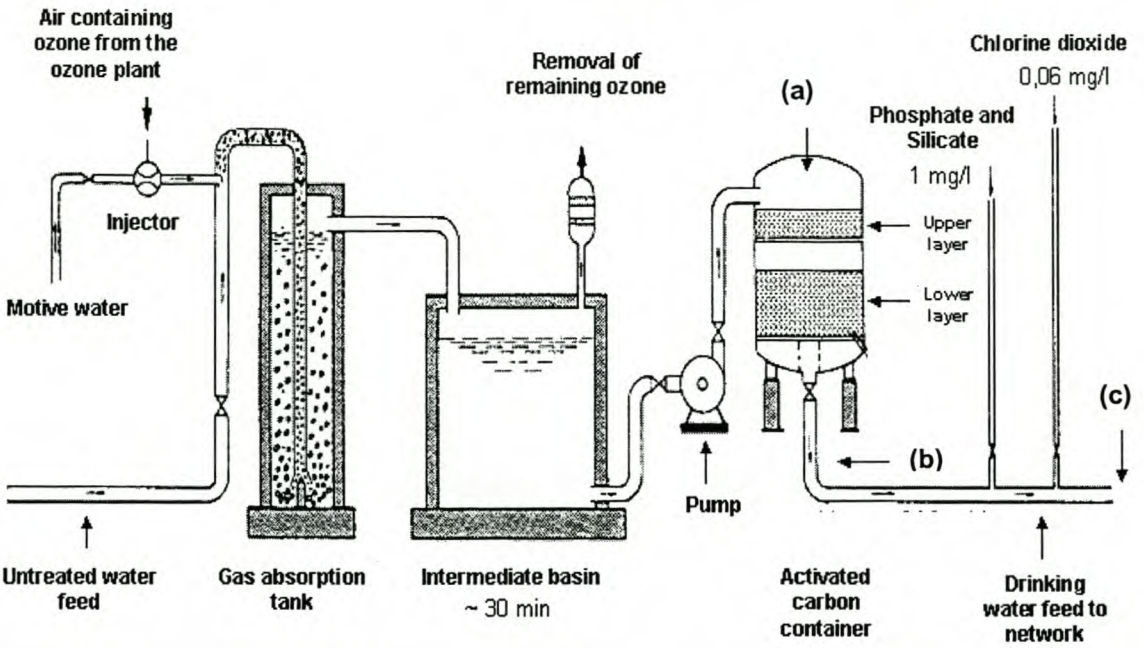


Figure 2.1 Diagrammatic representation of the Duesseldorf water treatment process. Material samples in the pilot plant (**Section 2.2**) were exposed to water from three different sections of the water treatment process, namely (**a**) ozonated raw water, (**b**) activated carbon treated water (water that had passed through the activated carbon filters) and (**c**) potable water [activated carbon treated water with chlorine dioxide (60 µg/l), phosphate (1 mg/l) and silicate (1 mg/l).

The fundamental purpose of water treatment is to produce water that is biologically and chemically safe for human consumption. The reliability of the distribution system, including the maintenance of constant internal pressure, requires careful attention to the interconnection of pipes, which allows the flow to approach any point in the network from more than one direction and helps ensure continued water supply and maintenance of pressure everywhere in the event of pipeline failures. The system, however, is not infallible with the most prevalent problems associated with water quality control in distribution systems being undesirable tastes, odours and turbidity of the potable water. A further problem is the deterioration in bacteriological quality, which is usually attributed to three phenomena: (1) biofouling, due to the proliferation of microorganisms, which causes tastes and odours, and often results in the loss of carrying capacity of pipes and sometimes severe corrosion; (2) chemical and electrolytic corrosion, resulting in undesirable end

products, such as metallic and brackish tastes as well as failure of hot water heaters and residence water piping; and (3) appearance of coliform organisms in the distribution system, indicating recontamination of an otherwise safe water (Chemuliti *et al.*, 2002; Egorov *et al.*, 2002).

It has been concluded that the factors, which primarily influence microbial regrowth and biofilm growth on materials in drinking water distribution systems, are environmental factors (e.g., pH, temperature), an accumulation of organic materials and sediment in distribution pipes, the presence and effectiveness of disinfectant residuals, internal corrosion, and water temperature (LeChevallier *et al.*, 1990). Microbial growth in drinking water requires nutrients such as organic carbon, nitrogen and phosphorus. Organic carbon, especially AOC is considered to be the main nutrient controlling microbial growth in drinking water distribution systems (Van der Kooij *et al.*, 1982; LeChevallier *et al.*, 1991). The AOC test has been used in the Netherlands for more than 10 years to assist in developing treatment strategies to limit the heterotrophic growth in water (Van der Kooij, 1987, 1992; Van der Kooij *et al.*, 1995b). Based on their observations of AOC concentrations, a guideline of 10 µg C/l was defined for drinking water, which allowed for a very limited heterotrophic growth potential (Van der Kooij, 1992). The BFR of drinking water can be determined using a Kiwa^{TM} Biofilm Monitor (Van der Kooij et al., 1995b). This system can determine the total active biomass or the concentration of the energy carrier Adenosine Tri-Phosphate (ATP) present in drinking water or in a bacterial biomass sample. ATP monitoring or detection assays can thus be useful tools in determining or measuring the bacterial population density.

Research has shown that in drinking water containing increased levels of organic matter, the total number of microorganims present can be limited by phosphorus instead of organic carbon (Sathasivan, *et al.*, 1997; Sathasivan & Ohgaki, 1999). Lehtola *et al.* (2001) also revealed that microbially available phosphorus (MAP) and AOC should be considered as important limiting factors of microbial growth in drinking water. The influence of AOC on bacterial regrowth in drinking water distribution systems in China was investigated by Liu *et al.* (2002). It was concluded that one of the surest ways of avoiding bacterial regrowth and the deterioration of water quality in a distribution system, was to remove biodegradable organic matter from water i.e. AOC.

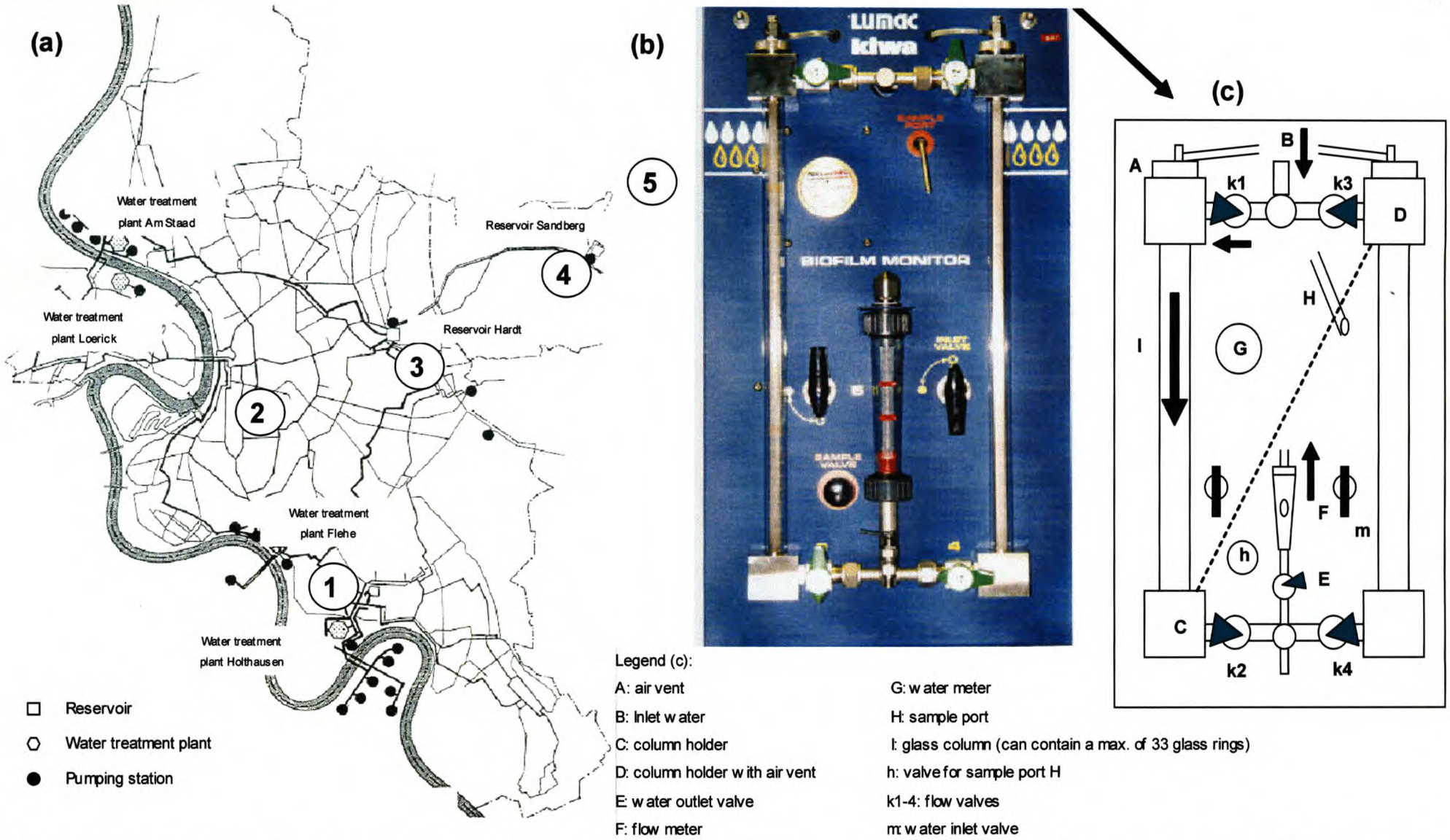
In the case of the distribution system studied in this project, it was suggested that the low nutrient load in the water throughout the distribution system would not

support notable biofilm development. To test this hypothesis, Kiwa™ biofilm monitors were installed at the water treatment plant Holthausen and at four locations to lines containing potable water in the distribution system in the Duesseldorf area. This programme monitored the biofilm formation characteristics as well as the biological stability (DOC and AOC concentrations) of the drinking water.

2.1.2 Experimental

2.1.2.1 Installation of Kiwa™ Biofilm Monitors in the Duesseldorf area

The locations where the Kiwa™ Biofilm Monitors were installed, and a picture of a monitor are illustrated in **Figure 2.2 (a-c)**. The biofilm monitor is composed of two vertical glass columns containing glass rings stacked on top of each other [**Figure 2.2 (b)** and **(c)**]. A serial flow in the biofilm monitor can be obtained by a bypass from C to D [dotted line in **Figure 2.2 (c)**], with the water flowing downwards through the left column and transported through stainless steel tubing to the top of the right column [valves k2 and k3, as illustrated in **Figure 2.2 (c)**, are closed]. One biofilm monitor was installed at the water treatment plant Holthausen (Site 1; HOL) to a distribution line containing water prior to addition of chlorine dioxide, phosphate and silicate. This would give an indication of how effective the drinking water treatment is and whether the water without supplements is likely to support biofilm growth. It was however, argued that the addition of phosphate and silicate to an oligotrophic system enhances microbial growth. Consequently, a further four monitors were installed to distribution lines containing potable water (with the addition of supplements), at various points throughout the Duesseldorf area, namely; Färberstrasse (Site 2; FÄR) reservoir Hardt (Site 3; HARDT), reservoir Sandberg (Site 4; SAND) and the pumping station Gau (Site 5; GAU). After the flow rates were set at 270 l/hour, the sterile glass rings were placed in each biofilm monitor. The water supply to sites 1, 2, 3 and 4 is generally composed of approximately 80% bank filtrated water and 20% groundwater, whereas site 5 has a pure groundwater source. The exact ratio of bank filtrated water to groundwater is subject to the water levels of the Rhine and the amount of water drawn from the wells.



Legend (c):

A: air vent	G: water meter
B: Inlet water	H: sample port
C: column holder	I: glass column (can contain a max. of 33 glass rings)
D: column holder with air vent	h: valve for sample port H
E: water outlet valve	k1-4: flow valves
F: flow meter	m: water inlet valve

Figure 2.2 (a) Location of Kiwa™ biofilm monitors connected to the distribution network in Duesseldorf, Germany **(b)** Kiwa™biofilm monitor **(c)** Schematic representation of Kiwa™ biofilm monitor

2.1.2.2 *Sampling and Transport*

The biofilm monitors were sampled eight times over a period of 120 days, with the temperature of the water measured on location. The experimental work entailed collecting two glass rings and one water sample from each monitor every fortnight. The two rings were transferred directly into glass tubes (2,4 x 20 cm) containing 10 ml of chlorine free sterile water (tap water with an ATP content < 4 ng/l). The glass rings and water samples were transported while kept on ice.

2.1.2.3 *Treatment of Glass Rings Sampled from the Biofilm Monitors*

The rings were processed within 24 hours after sampling. They were sonicated for 2 minutes in 10 ml chlorine free sterile water. This sonication step was repeated three times, with fresh sterile water used after each sonication step and the suspensions pooled to result in a total of 30 ml/ring. This suspension was used for further bacteriological and chemical analysis.

2.1.2.4 *Direct Acridine Orange Count (DAOC)*

The total number of microorganisms in the biofilm was determined by a direct fluorescence microscopy cell count, with acridine orange (Sigma) as the fluorochrome. The organisms were first immobilised by drawing a range of volumes of the bacterial biomass through a black Nuclepore cellulose membrane filter (pore size 0.2 µm). The cells were stained with 1 ml acridine orange (160 mg/L) for 5 minutes. The total cell count was obtained microscopically using a Zeiss microscope Laborlux (100X magnification). A minimum of 10 different fields was enumerated for each monitor, respectively, at each sampling time.

2.1.2.5 *Heterotrophic Plate Counts*

Serial dilutions were performed on the respective bacterial biomass suspensions. This was then plated on R2A agar (100%) with the plates incubated for ten days at 25°C. After the incubation period, all colonies visible to the naked eye were counted. The use of media with a relatively low concentration of nutrients in combination with a long incubation period generally result in higher colony counts than the use of rich

yeast glucose agar media for the enumeration of samples from oligotrophic environments such as drinking water distribution systems (Vrouwenvelder *et al.*, 1997).

2.1.2.6 Adenosine Tri-Phosphate Concentrations

The Adenosine Tri-Phosphate (ATP) concentration was determined for the suspensions of biomass released from the biofilm monitor rings using the procedure as outlined in Van der Kooij *et al.* (1993) and Vrouwenvelder *et al.* (1997). An ATP calibration curve with a known standard ATP concentration (2 ng ATP/l) was used to determine the ATP concentrations of the biomass suspensions obtained from the coupon samples. The ATP assay was performed with a LUMAC reagentia (M1500 biocounter and QM_NRM kit) following the manufacturers instructions. The BFR [pg ATP/(cm^2 x day)] was calculated from the linear increase of the ATP content of the biofilm as a function of time for a period between 0 and 100 days. The BFP was defined and calculated as the average biofilm density (ATP in pg/cm^2) from the period of 100 days onwards. The classical heterotrophic plate count procedure only enumerates culturable cells, whereas the ATP method also determines bacteria not contributing to these counts (e.g. nitrifying and methane oxidising bacteria as well as viable but not culturable cells).

2.1.2.7 Population Dynamics using Biolog™Eco Microplates

Biolog™Eco (Hayward, Ca., USA) MicroPlates were used to determine whole microbial community metabolic profiles for the respective samples. Each microplate contains three identical sets of 31 carbon sources, used to monitor and study changes in microbial communities (Biolog, 1999). After a 20 µl inoculation per well of undiluted sample, the Microplates were incubated at 25°C for a period of nine days. The microbial response to (ability to utilise or not) the individual carbon sources was recorded and a fingerprint was obtained using the statistic programme Systat™.

2.1.2.8 Chemical Analyses

The dissolved organic carbon (DOC) concentration in the water sample was determined using the European standards EN1484 procedure (European Committee for Standardisation, 1997).

2.1.2.9 Assimilabe Organic Carbon (AOC) Levels

Six times during the period of 120 days, 600 ml water samples were collected in 1 L sterile glass bottles at each site. The AOC concentration of these samples were determined according to the Kiwa Standard Method LMB-004. In essence, the method is based on determining the maximum colony counts of *Pseudomonas fluorescens* strain P17 and *Spirullum* sp. strain NOX in water samples, which had been previously pasteurised to inactivate the natural microbial population. The samples were inoculated with a mixed culture of these strains and incubated at 15°C. The AOC concentration was calculated from the maximum colony counts (N_{max}, CFU/ml) of the organisms and their yield coefficients for acetate and expressed as equivalents of acetate-C/l. Details of the AOC method have been described in detail by Van der Kooij *et al.* (1982) and Van der Kooij (1992).

2.1.2.10 Statistical Analyses

Repeated Measures Anovas (RMA) were performed on all data obtained as outlined in Dunn & Clark (1987), using Statistica™. In each RMA the residuals were analysed to determine if they were normally distributed. If they were not normally distributed, Bootstrap methods were used to determine if there were significant differences among the samples (Effron & Tibshirani, 1993). In all hypothesis tests a significance level of 5% was used as standard.

2.2 BIOFILM FORMATION ON MATERIALS RELEVANT TO THE DRINKING WATER DISTRIBUTION INDUSTRY

2.2.1 Background

Most environments allow the existence of sessile (attached) and planktonic (free-living) bacterial communities in such a way that neither community completely dominates (Fletcher, 1979). The attachment of microorganisms to various surfaces in nature is dependent on factors such as mechanisms of attachment, nutrient availability and favourable environmental conditions. Colonies of cells are formed when these attached cells grow and actively multiply, with the development of a 3-D biofilm architecture aided by the production of organic polymers (Allison & Sutherland, 1987). A microbial biofilm is formed when this mass of cells and their extracellular products trap organic and inorganic debris, nutrients and other microorganisms (Kumar & Anand, 1998).

Potable water distribution systems are generally regarded as environments where microorganisms occur in low numbers. In most low nutrient habitats it has been shown that the activity of the bacteria is increased at surfaces (Fletcher, 1979). Planktonic bacteria, as well as organic and inorganic molecules are transported towards a surface by diffusion, gravity, laminar or turbulent flow of liquid, through the organism's motility, or by a combination of these processes (Marshall *et al.*, 1971; Percival *et al.*, 1999). The attachment of single organisms or microbial populations to different surfaces is dependant on various physico-chemical properties of the bacterial cell surface [surface free energy, hydrophobicity, electrostatic charges, etc.] as well as the properties of the substance itself [chemical composition, physical properties (e.g. roughness), charge, etc.] (Dickson & Koohmaraie, 1989; Van Loosdrecht *et al.*, 1990; Percival *et al.*, 1999).

In natural aqueous ecosystems the biofilm mode of growth mainly dominates, with the sessile bacteria generally creating their own microenvironment (Costerton *et al.*, 1987, Brown *et al.*, 1991). Advantages of this mode of growth is that the biofilm enables greater reproducibility and control of the microenvironment, as well as a greater chance of survival for the microorganism (Brown *et al.*, 1991).

Internationally drinking water distribution systems generally employ as piping materials varying proportions of three types of materials i.e. cement, metallic or plastic. Biofilm growth depends on the availability of necessary elements, implying that biofilms may develop on the surface of a material that is able to supply nutrients

for bacterial growth. Studies on diverse aspects of bacterial biofilms, formed in association with different solid surfaces and in varying environments, are thus being conducted in numerous laboratories around the world (Olson *et al.*, 1991; Mittelman *et al.*, 1992).

Concrete pipes have predominantly been used to transport surface waters in the United Kingdom, Unites States of America and Germany for almost a century. The possibility that microorganisms may be involved in the deterioration of concrete has been considered for a number of years, with the result that attempts have been made to understand the basis of exactly how the microbes are involved in the decay of this material. The process is believed to be a complex one involving several groups of microorganisms and either inorganic or organic acids. Yet various studies have identified *Thiobacillus* spp. and anaerobic sulphate reducing bacteria (SRB) as the main bacteria responsible for the degradation of concrete in cooling water systems, fire water systems and sewer pipes (Kulpa & Baker, 1990; Poulton & Mixon, 1992). Research conducted by Poulton and Mixon (1992) however, showed that while bacteria attached to the concrete surface, no associated deterioration effect was noted.

Water distribution as well as domestic plumbing systems also use copper as a piping material. Problems associated with corrosion have however, been encountered when using copper piping for the plumbing of potable systems in large institutional buildings (e.g. hospitals). Abnormal forms of pitting corrosion have been reported to be the main cause of copper pipe failure in the United Kingdom, Germany, Saudi Arabia and Japan (McEvoy & Colbourne, 1988; Fischer *et al.*, 1992). It is also recognised that biofilms may form on this material and can readily corrode it.

Stainless steel has been proposed as an alternative and even substitute to copper. As different grades of stainless steel, with different compositions and properties, are available, they can ultimately be used for different purposes within distribution systems. Chromium-nickel stainless steel types 304 and 304L are the most widely used basic grades, with types 316 and 316L being the more corrosion resistant grades, which contain, in addition to chromium and nickel, molybdenum (Percival *et al.*, 1998). Biofilm formation on stainless steel grades 304 and 316 exposed to potable water for a period of 24 months was investigated by Percival *et al.* (1998). Results revealed that while total cell counts were not significantly different on the two grades of stainless steel, viable cell counts were. Furthermore, the

stability of this material was demonstrated by the fact that metal ion levels were generally low within the biofilms, signifying no leaching of molybdenum, and that no presence of corrosion was present on any of the grades, despite being exposed to mains water for 24 months.

Polymeric materials, such as polyethylene (PE), polyvinylchloride (PVC) and teflon, have also been adapted and applied as pipe and tubing material in drinking water distribution systems. Even though there are many advantages associated with using these materials in distribution systems, research has shown that they also contribute to biofilm formation in drinking water. Biofilm activity on PVC, PE and stainless steel (grade 304) exposed to water disinfected with ozone was investigated by Zacheus *et al.* (2000). No clear difference in biofilm formation on the three different surfaces was demonstrated. An earlier study conducted by Pedersen (1990) regarding the formation of biofilms on PVC and stainless steel exposed to drinking water, collaborated these findings. Studies conducted, however, demonstrated that PE was more favourable for biofilm formation than PVC, with both of these materials having higher bacterial numbers than stainless steel and cement (Rogers *et al.*, 1994; Clark *et al.,* 1994). Hallam *et al.* (2001) showed that a higher mean biofilm potential was measured on PVC, then MDPE, followed by cement and finally glass. Results from a study conducted by Van der Kooij *et al.* (1995a) also revealed notable similarities both in accumulation rate and in the amount of accumulated biomass on the two materials teflon and glass, when exposed to a specific water type.

The attachment of microbes to piping material surfaces within drinking water distribution systems and the subsequent development of biofilms are often associated with technical and hygienic problems (Olsen *et al.*, 1991; Zacheus *et al.*, 2000). Research has shown that biofilms not only protect microbes against disinfection (LeChavellier *et al.*, 1988b) but also increase the possibility of corrosion of piping materials (Holden *et al.*, 1995; Hallam *et al.,* 2001).

The primary goal of this series of experiments was to evaluate the hypothesis that the type of material has a notable effect on biofilm formation. To address this, the following parameters were investigated; 1) ten different materials (relevant to the water distribution industry and used in the laboratory to evaluate biofilm formation), 2) surface roughness, 3) flow conditions and 4) water characteristics. Microbial response to these parameters was investigated in terms of (a) the Adenosine TriPhosphate (ATP) concentrations to measure active biomass; (b) total number of

organisms by direct microscopic cell counts; (c) colony counts on R2A agar; (d) whole community metabolic profiles using the Biolog™ Eco Micro Plate System; (e) biofilm thickness, area coverage and cell activity using CLSM with the BacLight™ Live/Dead stain; and (f) qualitative and quantitative analyses of elements present on the coupon surfaces using scanning electron microscopy in conjunction with energy dispersive x-ray spectroscopy (SEM/EDX) analysis.

2.2.2 Experimental

2.2.2.1 Pilot Plant Set-up

The experimental set-up of the pilot plant at the water treatment plant Holthausen in Duesseldorf, Germany is illustrated in **Figure 2.3.** The experimental work entailed sampling of material coupons and flowcells after exposure for 6, 12, 20, 28 and 36 weeks. Two systems were employed in the pilot plant set-up; a flow-cell system and a coupon system placed in a RohrEX cylinder. As illustrated in **Figure 2.3** the RohrEX cylinder is a narrow plastic container in which an additional plastic tube containing the coupons samples was placed. The coupon samples were successively replaced at each sampling time to determine the influence of an environment with a pre-adapted population on colonisation rate and intensity. These coupons were sampled after exposure for 8, 16, 24, 30 and 36 weeks.

2.2.2.2 Materials Used in Pilot Plant

The materials used in the pilot plant set-up were polyethylene (PE), polyvinylchloride (PVC), VA 1.4301 (SS 304), VA 1.4571 (SS 316), copper, aluminium (AlMgSi 0.5), zinc-coated steel, teflon (PTFE), plexiglass (PMMA) and glass. A standardised procedure to clean and prepare all material samples (size: flow-cell coupons 10 x 10 x 1 mm; RohrEx coupons 31 x 20 x 1 mm) was developed. This entailed cleaning with methanol (sonicating for 3 min) and rinsing with sterile distilled water (sonicating for 5 min). All ten materials were analysed in the coupon system of the pilot plant and polyethylene (PE), polyvinylchloride (PVC), VA 1.4301 (SS 304), VA 1.4571 (SS 316), copper, aluminium (AlMgSi 0,5), zinc-coated steel and teflon (PTFE) were analysed in the flow-cell system.

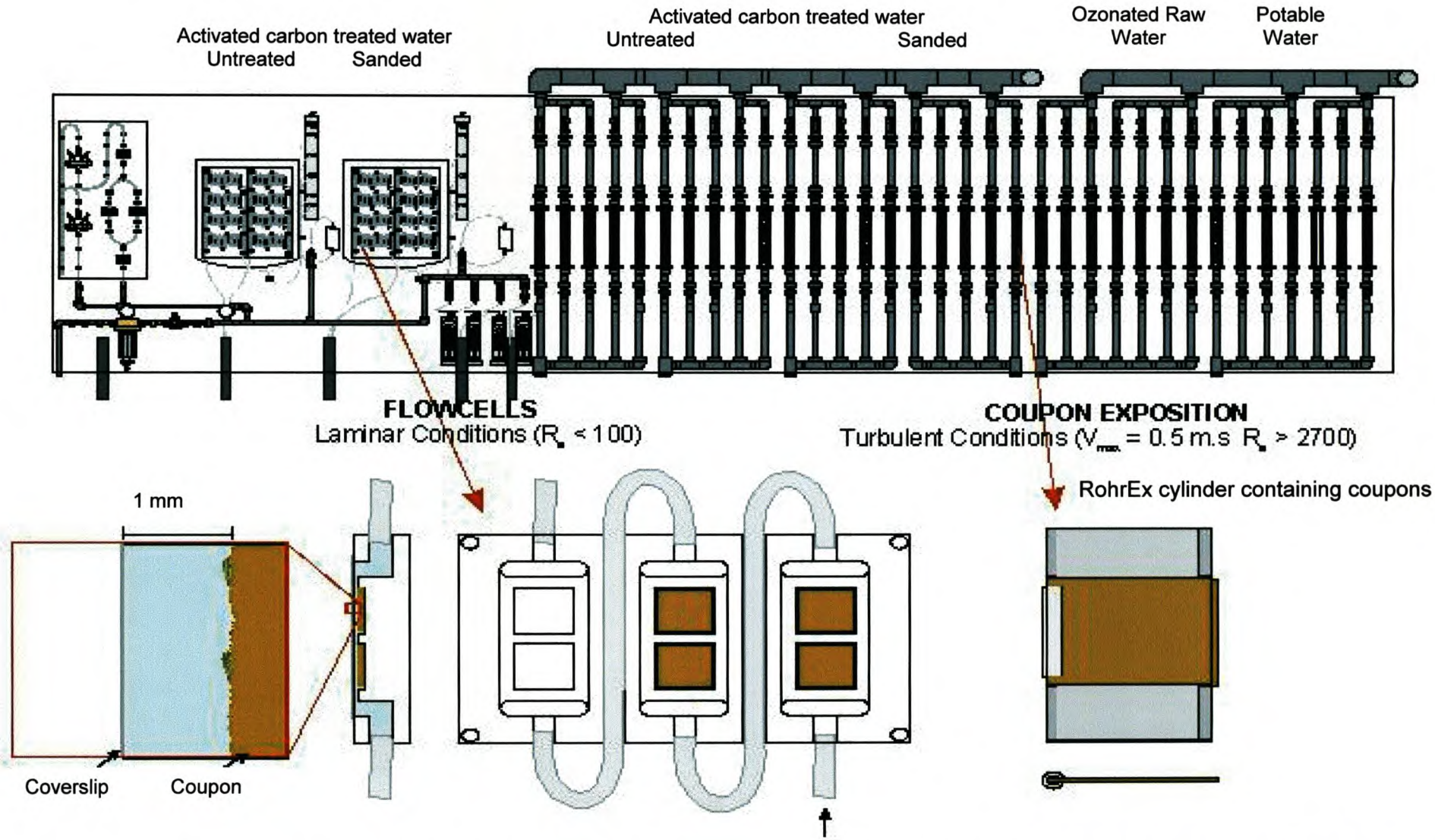


Figure 2.3 Diagrammatic representation of the pilot plant layout at the water treatment plant Holthausen in Duesseldorf, Germany

2.2.2.3 Effect of Water Characteristics, Flow conditions and Surface Conditioning

Sample coupons of the 10 materials were exposed to water from three different sections of the water purification process as illustrated in **Figure 2.1**, i.e. (**a**) ozonated raw water (PE, PVC, VA 1.4301, VA 1.4571, copper, zinc-coated steel, and glass), (**b**) activated carbon treated water [all 10 materials (water that had passed through the activated carbon filters)] and (**c**) potable water [activated carbon treated water with chlorine dioxide (60 µg/l), phosphate (1 mg/l) and silicate (1 mg/l)] (PE, PVC, VA 1.4301, VA 1.4571, copper, zinc-coated steel, and glass). The physical, organoleptic and chemical characteristics of the three water types are summarised in **Table 2.1**. Additionally these material samples were exposed to laminar (Re < 100) flow conditions in a flow-cell system and turbulent (Re > 2700) flow conditions in the coupon RohrEX system. The materials analysed in the activated carbon treated water section as well as in the flow-cell system were either used as obtained from the manufacturer, or sanded (600 grid) to determine the influence of surface roughness and damaged passivation layers on biofilm formation.

2.2.2.4 Sample Preparation

At each scheduled sampling date, two coupons were sampled per material from every section of the pilot plant, as illustrated in **Figure 2.3.** The coupons were processed within 24 hours as follows. Each coupon was sonicated for 2 minutes in 10 ml chlorine free sterile water. This sonication step was repeated three times, with fresh sterile water and the resulting suspensions pooled to result in a total of 30 ml/coupon. The respective suspensions were used for further microbiological analysis.

Table 2.1 Physcial, organoleptic and chemical composition of the three water sources (indicated on **Figure 2.1**) used in the pilot plant.

Determinants	Units	(a)	(b)	(c)
Physical and Organoleptic properties				
Colour		red	colourless	colourless
Conductivity at 25°C	µS/cm	811	811	813
Odour		strong	none	none
pH value	pH units	7.03	6.96	6.96
Turbidity	TE/F	1.02	0.34	0.1
Temperature	°C	11.8	11.5	11.4
Chemical properties – macro-determinants				
Ammonium as NH_4	mg/l	<0.02	<0.02	<0.02
Calcium as Ca	mg/l	98	97	96
Chloride as Cl^-	mg/l	80	80	80
Flouride as F^-	mg/l	0.2	0.16	0.18
Magnesium as Mg	mg/l	12.3	12	12.1
Nitrate as NO_3	mg/l	22.3	21.9	21.7
Nitrite as NO_2	mg/l	<0.02	<0.02	<0.02
Potassium as K	mg/l	3.8	3.8	3.7
Sodium as Na	mg/l	39	39	41
Sulphate as $SO_4^=$	mg/l	60	60	60
Zinc as Zn	mg/l	0.01	<0.005	0.006
Chemical properties – micro-determinants				
Aluminium as Al	mg/l	<0.01	<0.01	<0.01
Antimony as Sb	mg/l	<0.0015	<0.0015	<0.0015
Arsenic as As	mg/l	0.0016	0.0013	0.0013
Cadmium as Cd	mg/l	<0.0003	<0.0003	<0.0003
Chromium as Cr	mg/l	0.021	0.02	0.02
Copper as Cu	mg/l	0.011	<0.005	<0.005
Iron as Fe	mg/l	<0.07	<0.07	<0.07
Lead as Pb	mg/l	<0.001	<0.001	<0.001
Manganese as Mn	mg/l	<0.03	<0.03	<0.03
Mercury as Hg	mg/l	<0.0005	<0.0005	<0.0005
Nickel as Ni	mg/l	0.0097	0.0095	0.0095
Selenium as Se	mg/l	<0.001	<0.001	<0.001

2.2.2.5 Direct Acridine Orange Cell Count (DAOC)

The total number of microorganisms in the biofilm present on the different materials was determined by a direct fluorescence cell count with acridine orange (Sigma) as the fluorochrome. The organisms were first immobilised by filtering a series of volumes of the cell suspension through a black Nuclepore cellulose membrane filter (pore size 0.2 µm). The filters were then stained with 1 ml acridine orange (160 mg/L) for 5 minutes. The total cell count was obtained microscopically using a Zeiss microscope Laborlux (100 X magnification). A minimum of 10 different fields, were counted for each material coupon at each sampling time.

2.2.2.6 Heterotrophic plate counts

Serial dilutions were performed on the respective bacterial suspensions obtained from the different material coupons and enumerated as described in **section 2.1.2.5**.

2.2.2.7 Adenosine Tri-Phosphate concentrations

The Adenosine Tri-Phosphate (ATP) concentration was determined for the suspensions of biomass released from the respective material coupons as described in **section 2.1.2.6**.

2.2.2.8 Population Dynamics using Biolog™ Eco-Microplates

Biolog™Eco (Hayward,Ca., USA) MicroPlates were used to determine whole microbial community metabolic profiles for the respective samples from the different materials as described in **section 2.1.2.7**.

2.2.2.9 Biofilm and Chemical Analysis

Representative coupons for each test material (See **section 2.2.2.2**), as well as the flow-cell samples were collected and analysed for biofilm thickness, area coverage and cell activity using CLSM (Nikon PCM 2000 based on a Nikon Eclipse E-600FN inverted fluorescence microscope) in combination with the Baclight™ Live/Dead stain (Molecular Probes, Inc.). Samples were stained for 10 minutes with the Baclight™ stain, where after, CLSM images (300 x 300 µm) were collected at a minimum of 10 points for each coupon and material in the flow-cell system. Images obtained were further analysed with ScionImage™ to obtain a comparison of the surface coverage and cell activity over time. A water immersion lens (Nikon CFI Flour 40 x, N.A. 0.8) was used for all CLSM analysis in order to prevent distortion of the 3D-images. 3D-Projections were created by EZ_{2000} CONFOCAL Software (Nikon Inc./Coord Automatisering, Netherlands). Chemical analysis of the coupon surfaces was performed with scanning electron microscope in conjunction with energy dispersive x-ray spectroscopy (SEM/EDX) analysis.

2.2.2.10 Statistical Analyses

Statistical analyses were performed as outlined in **section 2.1.2.10**.

2.3 EVALUATION OF OXIDISING BIOCIDES FOR THE CONTROL OF BIOFILMS IN THE TEST UNITS

2.3.1 Background

Because of the small size of microorganisms, it is almost impossible to completely remove them from water by processes such as coagulation and filtration. Furthermore, while it is perceived that there is no need for treatment of good quality groundwaters, the possible presence of bacteria and viruses as well as the problems associated with their presence, should be considered (Tebbutt, 1992). Terminal disinfection of piped drinking water supplies may then be an important final barrier to the transmission of waterborne bacterial and viral diseases. Chlorine and hypochlorite are most often used for this purpose, but water may also be disinfected with chloramines, chlorine dioxide, ozone, ultraviolet irradiation and the addition of hydrogen peroxide (World Health Organisation, 1996). The presence of the disinfectant residual in the distribution system also helps to maintain water quality by preventing the regrowth of nuisance microorganisms.

Since the practise of disinfecting drinking water began in 1902, chlorine has been used as the principal disinfectant. The process of chlorination involves the addition of chlorine, usually in the form of chlorine gas, chlorine dioxide (ClO_2), sodium hypochlorite (NaOCl), and calcium hypochlorite [Ca $(OCl)_2$], directly to the water (Norman *et al.*, 1980). Chlorination is widely used for virtually all domestic waters supplied from surface water, most groundwaters used, recycled cooling and process waters and many treated wastewaters. Factors contributing to the widespread use of chlorine include its relatively low cost, ease of application, proven reliability, detectability and familiarity with its use (White, 1972). Decades of experience showed that prescribed levels of residual chlorine remaining in the water after a prescribed contact time assures the consumer that the water is safe to drink. In essence, chlorination has been shown to provide long-term protection of the water distribution network against contamination and microbial re-growth.

The efficacy of hydrogen peroxide as disinfectant is generally related to its bactericidal and sporocidal properties (Weiner *et al.,* 2000). Hydrogen peroxide acts by possibly disrupting the surface membranes of microorganisms through hydroxy radical formation (OH), as well as by oxidising protein sulphydryl groups and double bonds. Deterioration of protective polysaccharide coatings and cleavage of cell chains have also been displayed in bacterial surfaces exposed to hydrogen peroxide (Baldry & Fraser, 1988). In addition to being effective against a wide range of bacteria, hydrogen peroxide is also effective against many bacteria, such as *Pseudomonas aeruginosa,* that have become resistant to chlorine-based chemicals (Reimann, 1992). Hydrogen peroxide also has a sporocidal effect by causing lysis of spores, which includes damage to the spore coat, oxidative cortex hydrolysis or germination-like changes due to activation of cortex lytic enzymes (Foster & Johnstone, 1987).

The use of these oxidative processes may remove microorganisms, but may also enhance the formation of easily biodegradable organic substances. Microorganisms can then utilise these substances as an energy source and promote biofilm formation in distribution systems (Glaze, 1987: Gilbert, 1988). This biofilm formation could contribute to a range of costly problems within industrial environments. These interfacial microbial communities are directly involved in biofouling and biocorrosion, that cause significant reductions in system performance, and contribute to the accelerated deterioration of components within the distribution system (McFeters *et al.*, 1995). Furthermore, a significant number of waterborne disease outbreaks have been associated with public water systems (Geldreich, 1996) which may be attributed to inadequate care during repairs of the distribution system, insufficient treatment of the water before release into the network, and possibly biofilm formation on piping materials (Schwartz *et al.,* 1998; DiGiano *et al.,* 2000).

Extensive studies have been conducted on the effectiveness of chlorine as a disinfectant against biofilms in drinking water systems. Nagy *et al.* (1982) reported that the maintenance of a residual of 3 to 5 mg of chlorine per litre was necessary to reduce bacterial biofilms by more than 99.9% in aqueducts in Los Angeles, California, as bacterial levels as high as 10^4 CFU/cm^2 were present in biofilms, when a residual of 1 to 2 mg of chlorine per litre was present. Ridgway *et al.* (1984) on the other hand found that a residual of 15 to 20 mg of chlorine per litre was necessary to control biofouling of reverse osmosis membranes within water treatment systems.

Additionally, the inhibition of biofilm activity and the reduction of biofilm concentration to below 10 pg ATP/cm^2 by a concentration of 0.3 mg/L free chlorine, was demonstrated by Hallam *et al.* (2001). Momba and Binda (2001) also observed the inability of bacteria to grow on stainless steel and galvanised mild steel when 0.35 mg l^{-1} residual monochlaramine was maintained throughout the drinking water system.

The efficacy of four disinfectants (hypochlorous acid, hypochlorite, chlorine dioxide and monochlaramine) as well as several biocides (copper, zinc, sodium chlorite and alkaline pH) against free-floating and biofilm bacteria present on three types of surfaces (granular activated carbon, metal coupons, and glass slides), was investigated by LeChavellier *et al.* (1988). In general the results revealed that biofilm bacteria grown on the different types of surfaces were 2- to 100- fold more resistant to monochloramine than unattached cells, while resistance of biofilm bacteria to hypochlorous acid (free chlorine, pH7) was 150 to more than 3000 times greater to that of the unattached cells. This study confirmed that even though some of the other biocides used had some effectiveness on free-floating bacteria, the biocides and the disinfectants had a limited effect against biofilm microorganisms.

The effectiveness of disinfectants on the biofilm mode of growth is greatly influenced by various factors such as the type and concentration of disinfectant used, the surface of the material to which the biofilm is attached, etc. In this study the disinfection efficiency of sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) against biofilms present on water distribution piping materials was investigated. The concentration of disinfectant most effective against heavy biofilm contamination was also evaluated. Various concentrations of the disinfectants were initially tested on biofilms formed on three different surfaces i.e. polyethylene (PE), copper and glass, in a flow-cell system in the laboratory. The most effective concentrations as determined using the flow-cell system, as well as the concentrations typically used by the water treatment plant at the study site to disinfect the various components of the distribution and treatment facility, were then tested on material coupons of PE, polyvinylchloride (PVC), two grades of stainless steel namely, VA 1.4301 (SS 304) and VA 1.4571 (SS 316), copper, zinc-coated steel and teflon coupons. These test materials had been exposed to activated carbon treated water (treated water without corrosion inhibitors and disinfectants) in a pilot plant set-up for a period of 14 months.

2.3.2.1 *Flow-cell system*

Experiments to determine which concentrations of sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) were the most effective against the biofilm mode of growth were done using a laboratory scale flow-cell system as illustrated in **Figure 2.4**. A mixed culture of undefined species (0.5 ml) isolated from Rhine river water was inoculated into the flow-cells containing attachment coupons of polyethylene (PE), copper and glass. The nutrient medium R2A (1:100) was pumped through these flow cells with a peristaltic pump (Watson Marlow 205S) at a flow rate of 0.2 mm s^{-1} for a period of 9 days. After this time period the flow-cells were disconnected from the peristaltic pump and treated with the various concentrations of disinfectants.

The concentrations of NaOCl and H_2O_2 tested were as follows:

NaOCl: 10 mg/l, 25 mg/l, 50 mg/l (concentration used by water treatment plant Holthausen), 100 mg/l and 200 mg/l

H_2O_2: 0.15 g/l (concentration used by water treatment plant Holthausen), 1.5 g/l, 15 g/l (maximum concentration allowed by water treatment plant Holthausen), 30 g/l and 100 g/l.

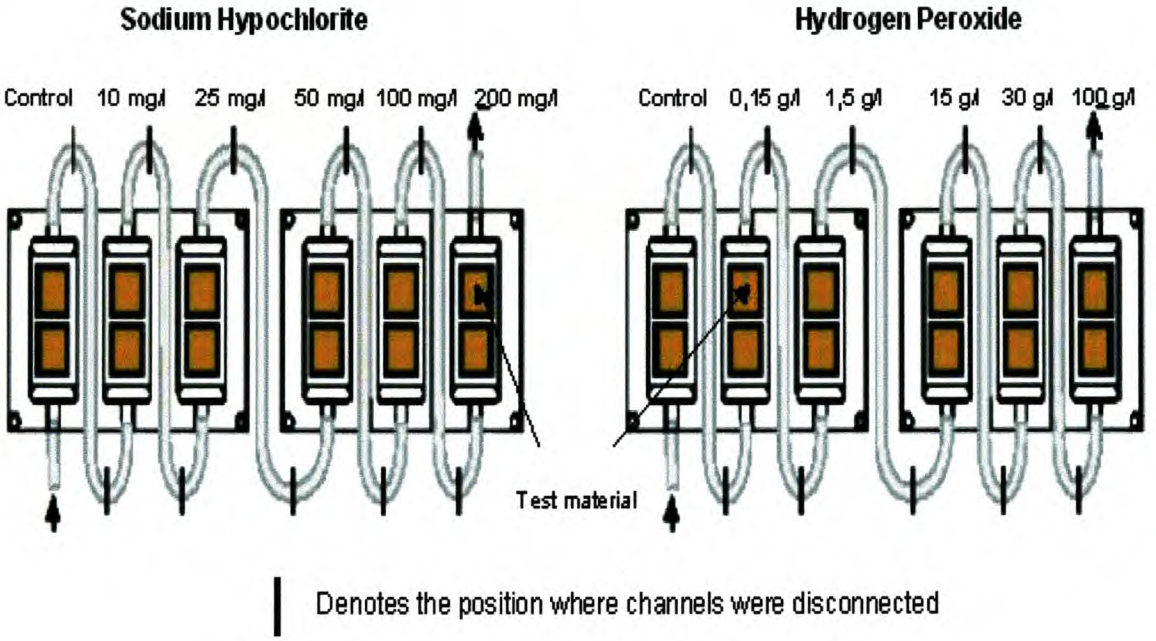


Figure 2.4 Laboratory scale flow-cell system used to analyse the different concentrations of the two disinfectants against biofilms grown on polyethylene (PE), copper and glass, respectively.

After 9 days, 5 ml of the respective concentrations of NaOCl and H_2O_2 were introduced into different flow-cell channels and left under stagnant conditions for 30 minutes. Each respective flow-cell channel was rinsed with sterile water and after an additional period of 10 minutes, stained with the Live/Dead Baclight™ stain. Surface coverage analysis of each channel was done using CLSM. Control channels to which no disinfectants were added, were included for each test material.

2.3.2.2 Pilot plant system

The most effective concentrations of the respective disinfectants, as determined using the laboratory scale flow-cell experiments were then tested on seven different materials commonly used in the water distribution systems and in laboratory experiments to monitor biofilm growth, as illustrated in **Figure 2.5**. Coupon samples of these materials, PE, polyvinylchloride (PVC), two grades of stainless steel, namely VA 1.4301 (SS 304) and VA 1.4571 (SS 316), copper, zinc-coated steel and teflon, were exposed to treated water under turbulent flow conditions in a RohrEx cylinder within the pilot plant set-up for a period of 14 months. This water had been treated with ozone and had passed through a double layer of activated carbon filters prior to the addition of the disinfectant chlorine dioxide and the corrosion inhibitors phosphate and silicate. The materials analysed in the pilot plant were either used as obtained from the manufacturer or sanded (600 grid) to determine the influence of surface roughness and damaged passivation layers on biofilm formation.

The concentrations of NaOCl and H_2O_2 tested were as follows:

NaOCl: 50 mg/l (concentration used by water treatment plant Holthausen) and 200 mg/l

H_2O_2: 0.15 g/l (concentration used by water treatment plant Holthausen), 15 g/l (maximum concentration allowed by water treatment plant Holthausen) and 100 g/l.

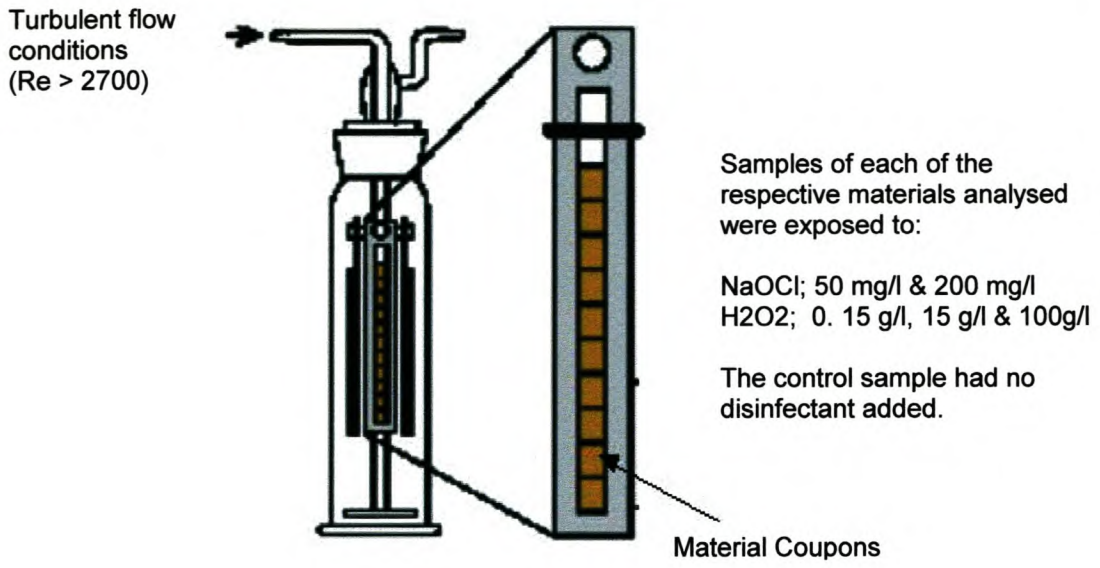


Figure 2.5 RohrEx system within pilot plant set-up used to analyse the different concentrations of the disinfectants against biofilms grown on polyethylene (PE), polyvinylchloride (PVC), two grades of stainless steel (SS 304 and SS 316), copper, zinc-coated steel and teflon, respectively.

The coupons were exposed to either NaOCl or H_2O_2 for 30 min before they were rinsed with sterile water and after a period of 10 minutes stained with the Live/Dead Baclight™ stain. Surface coverage analysis of each material coupon was done using CLSM. Untreated controls were analysed for each of the material coupons.

2.3.2.3 Statistical Analyses

Statistical analyses were performed as outlined in **section 2.1.2.10**.

3. RESULTS AND DISCUSSION

3.1 EVALUATION OF BIOFILM FORMATION CHARACTERISTICS AND BIOLOGICAL STABILITY OF DRINKING WATER

Water temperature is perhaps the most important rate-controlling factor that affects and governs microbial growth (LeChavellier *et al.,* 1990). Not only does temperature influence the microbial growth rate, but it also has an influence on treatment plant efficiency, disinfection efficiency, dissipation of disinfectant residuals, corrosion rates, distribution system hydraulics, and water velocity through customer demand. A number of investigators have observed significant microbial activity in water at temperatures of 15°C or higher (Donlan & Pipes, 1988; LeChevallier *et al.*, 1990). The Kiwa™ Biofilm Monitors were sampled over a period of 120 days from mid-autumn until early winter. The temperature of the water at the different locations remained more or less constant during the sampling period and ranged from 14°C (sites 1 and 5) to 16°C (sites 3 and 4) and 17°C (site 2) (**Figure 2.2**).

Overall analysis of the Biolog™ plates did not show any noticeable correlation between the carbon utilisation profiles of the microbial communities at the different locations, indicating that the microbes differed notably between the different sites (data not shown). In contrast, the community profiles for each site remained stable for the duration of experimentation, indicating that the bacterial communities at each site remained stable with time. Replication showed good reproducibility with the technique as applied in this study. Therefore, the Biolog™ system provided a reliable means of comparing the microbial communities at the different sampling sites, and to follow the community dynamics at the respective sites over time. Garland and Mills (1991) first used Biolog™ substrate utilisation tests to typify microbial communities of terrestrial and aquatic ecosystems. Since then this approach has repeatedly been applied in microbial ecology. The Ecolog™ system also proved useful in characterising microbial communities in drinking water biofilters (Moll and Summer, 1999).

The results for ATP analysis, colony counts and total cell count of the bacterial suspension obtained from the biofilm monitors are presented in **Figure 3.1 (A), (B)** and **(C)**, respectively.

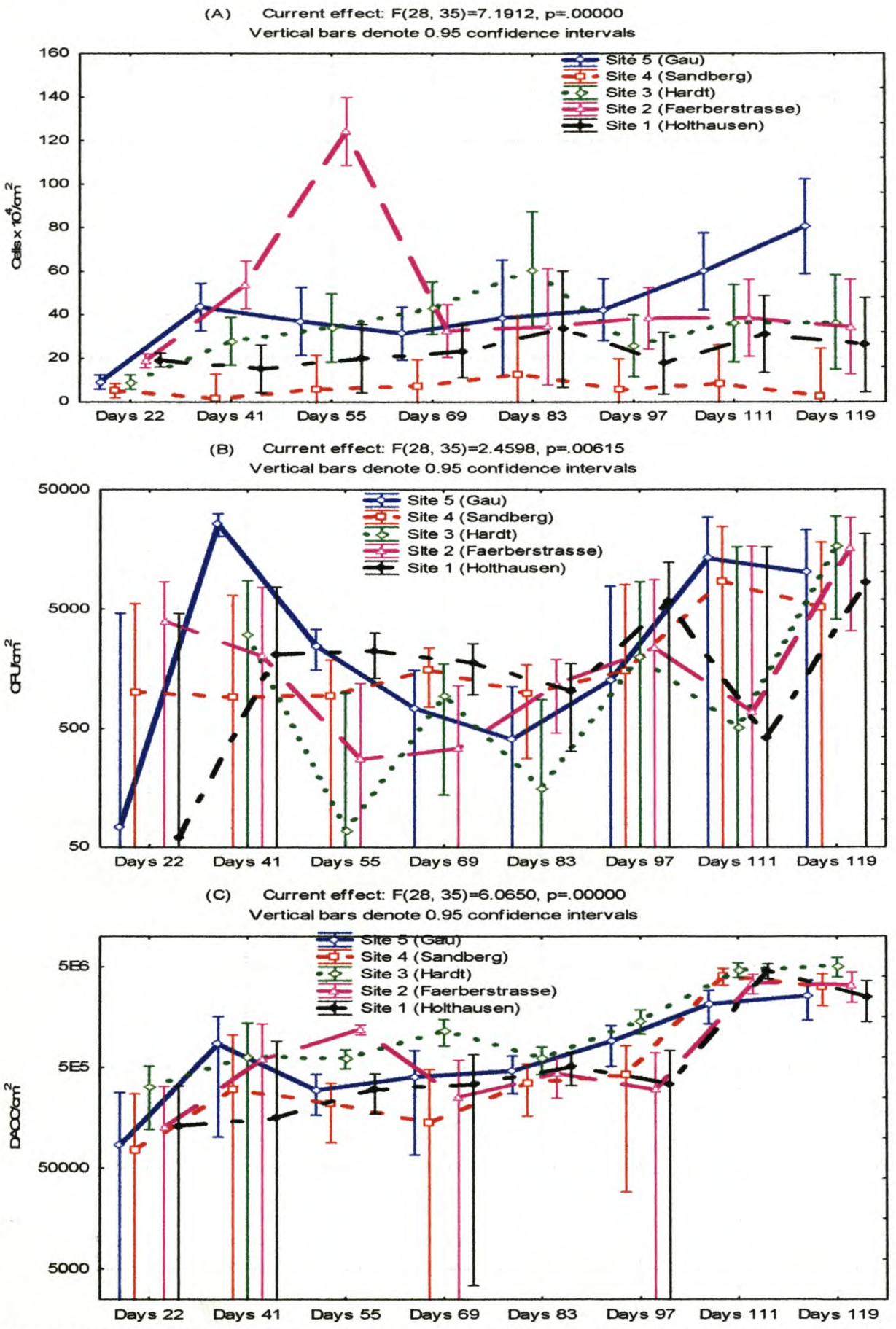


Figure 3.1 (A) Cell numbers based on ATP concentration-, **(B)** Heterotrophic plate count- and **(C)** DAOC-, over time at the different sampling points

The ATP concentrations for all the biofilm monitors [**Figure 3.1 (A)**] varied from approximately 6.3 pg/cm^2 (on average at site 4) to 46 pg/cm^2 (on average at site 2), with an increase in ATP concentration measured on Day 55 (third sampling time) for site 2. Heterotrophic plate counts [**Figure 3.1 (B)**] were determined for all samples. No significant increase in colony counts ($p > 0.05$) was observed for most of the monitors with the numbers varying from approximately 2.6 x 10^3 CFU/cm^2 (on average at site 4) to 6.7 x 10^3 CFU/cm^2 (on average at site 5). The heterotrophic plate count did however, increase significantly ($p < 0.05$) at Day 41 (second sampling time) for site 5, which has a pure groundwater source. The results for the total number of organisms [**Figure 3.1 (C)**], which was obtained by direct microscopic cell count, varied from 9.6 x 10^5 /cm^2 (on average at site 5) to 1.8 x 10^6 /cm^2 (on average at site 3). Significant increases in the total numbers of cells ($p < 0.05$) were also observed at the later sampling dates (day 111 and 119). This was accompanied by large variation between the replicate heterotrophic plate counts on these sampling dates [**Figure 3.1 (C)**], suggesting that the biofilms did not become stagnant over the experimental treatment.

In order to understand the microbial characteristics in natural environments, it is thus essential that accurate methods of measuring biomass and microbial activity for both planktonic and sessile microorganisms are employed. Adenosine tri-phosphate is an energy carrier present in all active cells and can therefore be used as a measure of active biomass present in a bacterial cell or a microbial community. One pg of ATP equals approximately 10^4 metabolically active bacterial cells, presuming an average ATP content of 10^{-16} – 10^{-15} g per active bacterial cell (Webster *et al.,* 1985; Stanley, 1989; Lehtola, 2002). To compare the relative values of the heterotrophic colony count [**Figure 3.1 (B)**] and the amount of active biomass as determined with ATP measurements [**Figure 3.1 (A)**], the percentage ratios between the heterotrophic plate counts on R2A and the cell numbers based on ATP concentration were calculated. These results showed that on average the culturable bacteria represented only a fraction (< 3%) of the active biomass in all biofilm samples (**Table 3.1**). Furthermore, on average the heterotrophic plate count measurements represented < 0.8% of DAOC [**Figure 3.1 (C)**] recorded for the biomass removed from the glass rings, whereas the ATP measurements represented < 82% of DAOC (**Table 3.1**).

Table 3.1 The average percentage ratio between the heterotrophic plate counts and the cell numbers based on ATP concentration as well as the average percentage ratio between the heterotrophic plate count, ATP and DAOC for Kiwa™ Biofilm Monitors for all sampling dates.

Parameter	Site 1	Site 2	Site 3	Site 4	Site5
CFU/ATP	0.64	0.42	0.47	2.24	0.86
CFU/DAOC	0.24	0.27	0.16	0.24	0.7
ATP/DAOC	38.33	64.65	34.43	10.51	81.39

Many water analysis laboratories worldwide still employ the classic way to enumerate bacteria in water, which is to do plate counts on media such as R2A agar, which was developed for the enumeration and cultivation of planktonic or free-floating bacteria from potable water (Reasoner & Geldreich, 1979). The ratios obtained in this study confirm the findings from previous investigations, which show the potential for potable water to produce biofilms and further emphasizes the need for biofilm based monitoring techniques to be implemented at water treatment plants (Smith, 1987; Patterson *et al.,* 1991; Coghlan, 1996; Potera, 1996; Hallam *et al.,* 2001). Various studies have also shown that the exact percentage of microorganisms culturable from the environment may range from <0.1% to < 10% (Yu *et al.,* 1995; Torsvik *et al.,* 1994). In the present study the results confirm that the principle limitation with culture based methods such as the heterotrophic plate count is that typically < 3% of the active biomass detected or identified from the environment was culturable. The ATP concentration (< 82% of the total cell count) then provided more accurate data on the actual activity within the distribution system over extended periods of time.

New techniques such as gene sequence and antibody-antigen based methods for the detection of indicator and pathogen index organisms, are constantly being developed. Results from various studies have also shown that considerably higher numbers of bacterial cells (10 to 100 more) can be detected using epifluorescence microscopy, in combination with redox dyes which enumerate active cells, than by standard plate count procedures (Yu *et al.*, 1995; Kulakov *et al.,* 2002).

The flow rate at site 4, although constantly adjusted and monitored was always extremely low (60 l/Hour) compared to the flow rate at the other sites (270 l/Hour). This could be due to construction taking place at the site during the sampling period. The low flow rate could also explain why the colony forming units, total cell count and BFP for this site was regularly of the lowest. As mentioned previously, the water supply to the sites 1, 2, 3 and 4 is generally composed of approximately 80% bank filtrated water and 20% groundwater, whereas the site 5 has a pure groundwater source. This could explain why the biofilm monitor at site 5 generated an increased BFR and BFP. Overall, the results for ATP concentrations, heterotrophic plate count and total cell count from the sites 2 and 3 compared well with the results obtained from the biofilm monitor installed at site 1. These results then indicate that the addition of the supplements phosphate and silicate to an oligotrophic system do not adversely affect the water quality, nor do they promote microbial growth.

An earlier study of the water quality was conducted in 1997 at the water treatment plants Flehe and Holthausen (corresponds to site 1 in the present study) situated in Duesseldorf (Vrouwenvelder *et al.*, 1997). While the earlier study established a basis for the present study, this study investigated the long-term stability and microbial behaviour not only at the water treatment plants, but also throughout the distribution system. For this reason monitors were installed at the water treatment plant Holthausen (site 1) and to sections of the distribution network in and around the Duesseldorf area (sites 2-5).

When compared, the results for ATP concentrations, heterotrophic plate count and total cell count obtained in the present study are marginally, although not significantly ($p > 0.05$), higher than those obtained by Vrouwenvelder *et al.* (1997). The results for the investigation of the biological stability of the drinking water at the different sites compared well to the results obtained by Vrouwenvelder *et al.* (1997), suggesting that there was no significant variation in water quality ($p > 0.05$) at the water treatment plant over the 3 years since 1997 (**Table 3.2**).

Table 3.2 Comparison of results obtained in the present study to the results obtained by Vrouwenvelder *et al.* (1997)

Kiwa™ Biofilm monitor site	Biofilm formation rate (BFR) pg ATP/(cm^2 x day)	Biofilm formation potential (BFP) pg ATP/cm^2	AOC µg C/l	*DOC mg C/l
# Flehe (1997)	1.1	225	7.8	0.45
# Holthausen (1997)	0.27 (0.01)	43 (3.6)	2.7	0.31
Site 1	0.28 (0.90)	25 (6.8)	4.2	0.25
Site 2	0.46 (0.95)	37.2 (2.3)	3.7	0.4
Site 3	0.41 (0.90)	32.8 (6.2)	2.2	0.33
Site 4	0.07 (0.01)	5.7 (2.8)	3.4	0.3
Site 5	0.6 (0.45)	61 (9.1)	2.5	0.23

* - accepted range 0.2 mg C/l to 0.5 mg C/l

- Results obtained by Vrouwenvelder *et al.*, 1997

Values in brackets show standard error

It should be pointed out that the results for the water treatment plant Flehe obtained in the 1997 study was disregarded, as it was found reconstruction work carried out during the sampling period at the water treatment plant Flehe, led to activated carbon fines being flushed into and accumulating on the glass rings in the monitor. As a result an increased BFR of 1.1 pg ATP/(cm^2 x day) and BFP of 225 pg ATP/cm^2 were recorded at this site, in comparison to the 0.27 pg ATP/(cm^2 x day) BFR and the 43 pg ATP/cm^2 BFP recorded at Holthausen.

As mentioned previously the Duesseldorf water treatment process (**Figure 2.1**) was introduced in 1961 and currently encompasses the application of ozonation and granular activated carbon (GAC) filtration of riverbank- and ground- water (Vrouwenvelder *et al.*, 1997). Additionally, after the water has passed through the activated carbon filters, phosphate and silicate (1 mg/l, respectively) are added to the water to prevent corrosion of the piping materials in the distribution system. As a final barrier chlorine dioxide is added as disinfectant at a concentration of 0.06 mg/l to prevent the regrowth of microorganisms on the way to the consumer (Vrouwenvelder *et al.*, 1997).

In order to reduce the formation of chlorine disinfection by-products (including trihalomethanes), many water treatment facilities are eliminating prechlorination as a preliminary water treatment process. The elimination of chlorine in the preliminary treatment will result in more biological growth throughout the plant including the

filters. A study conducted by DiGiano and Singer (1994) showed that GAC beds favour the growth of biofilms by providing an excellent surface for microbial growth. These results correspond to the findings of Vrouwenvelder *et al.* (1997) which showed that the presence of the activated carbon in the monitor increased the BFR and BFP indicating that the activated carbon fines provide a favourable surface for biofilm attachment. With the rising interest in biologically-active filtration or biofiltration, the results obtained in the 1997 study showed that in allowing for microbial attachment in the uppermost layer of the GAC filter, the biofilm may aid in the removal of biological material present in the water. However, care should be taken to ensure that the activated carbon fines do not seep into the rest of the distribution system as biofilm formation could be enhanced at that point.

Table 3.2 shows that the DOC concentrations of all the sites where the monitors were installed falls within the range of 0.23 mg C/l (on average at Site 5) to 0.4 mg C/l (on average at Site 2). Dissolved organic carbon values below 1 mg DOC/l are usually found in aerobic groundwater, with DOC concentrations in drinking water types in the Netherlands usually higher (Van der Kooij, 1992). As indicated, all the sites sampled in this study are supplemented with various percentages of groundwater, which possibly explains the low DOC values obtained, especially for site 5, which has a pure groundwater source. The AOC concentrations obtained over the study period fell within the range of 2.2 µg Acetate-C/l (on average at site 3) to 4.2 µg Acetate-C/l (on average at site 1). This data obtained was also comparable to that obtained by Vrouwenvelder *et al.* (1997), where it was speculated that DOC and AOC concentrations are directly related. Literature has also shown that ozonation could increase the AOC concentration in drinking water (Van der Kooij, 1992; Lehtola *et al.*, 2000), this could explain why site 1 (refer to **Figure 2.1**) has an AOC concentration slightly higher than the other sites.

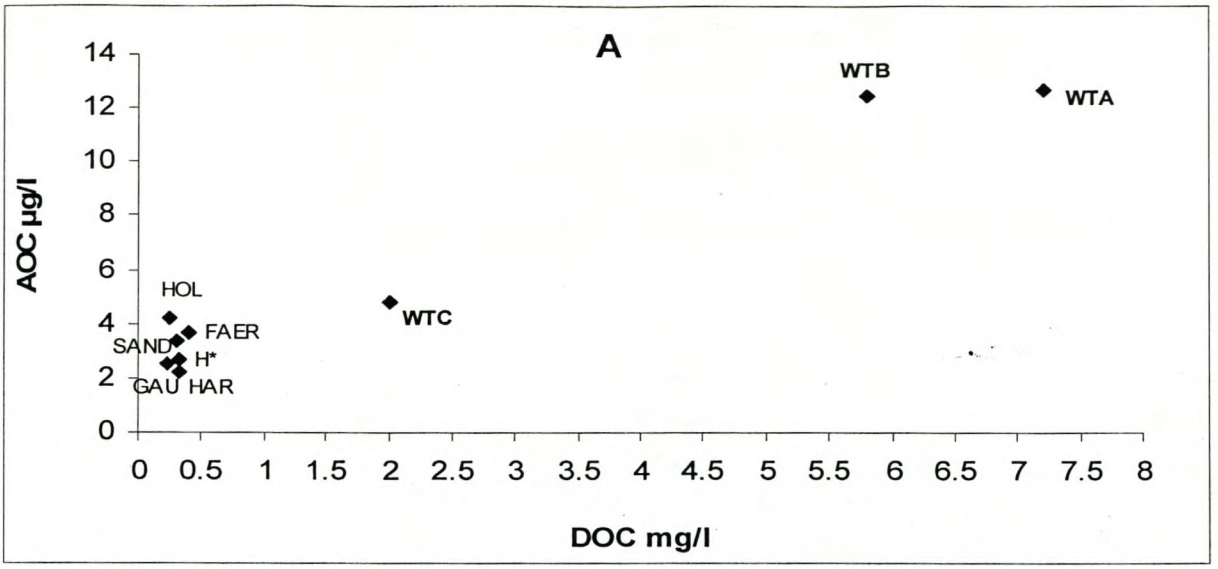
The BFR is determined by the linear increase of the ATP concentration as a function of time (Van der Kooij & Veenendal, 1993). The BFR for this study generally varied from 0.28 pg ATP/(cm^2 x day) (on average at site 1) to 0.6 pg ATP/(cm^2 x day) (on average at site 5). It is well known that the BFR depends on the amount of available biodegradable compounds in the water. Van der Kooij *et al.* (1995) showed that an acetate concentration of 10 µg C/l (for biologically stable water) results in a BFR of 360 pg ATP/(cm^2 x day). Based on this ratio the BFR observed in this study equals 0.002 (site 4) to 0.017 (site 5) µg Acetate-C/l. Extremely low BFR values, as obtained in this study, are usually observed in highly stable slow sand filtrate (e.g.

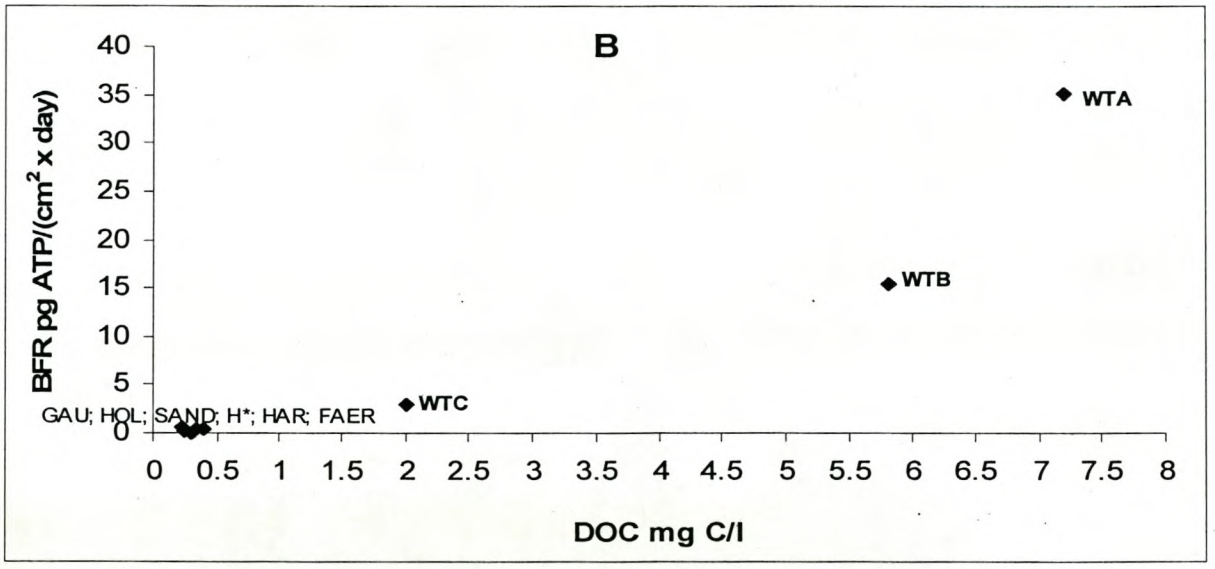
GAC procedure) and aerobic groundwater (Vrouwenvelder *et al.*, 1997). The drinking water in the Duesseldorf area is also supplemented with chlorine dioxide as free chlorine residuals may serve to protect the distribution system against regrowth. The residual chlorine dioxide concentrations below the chemical detection limit could also have been a contributing factor to the low ATP concentrations and the corresponding low BFR values observed.

The BFP is defined as the average amount of biofilm biomass accumulated during a period between 100 and 150 days of contact time, as the biofilms at the different locations were considered to reach a steady state during this time (Vrouwenvelder *et al.,* 1997). The BFP for the present study was calculated during the period between 100 and 120 days and showed that the average results varied from 5.7 pg ATP/cm^2 (site 4) to 61 pg ATP/ cm^2 (site 5). The BFP for the present study was normal for drinking water, with sites 4 and 5 being the obvious exceptions. The average BFP for the 1997 study was significantly higher than the present study however, the BFP for the 1997 study was calculated for the period between 100 and 250 days. This longer test period could explain the higher values obtained in the previous study. However, based on the assumption that a steady state is reached between days 100 to 150, the BFP for these two studies should be comparable. The fact that they are not shows that even though the biofilm may not have been growing actively, a steady state of biofilm activity has not been reached even after 100 to 150 days. This corresponds to data presented in **Figure 3.1 (C)**, where a significant increase in the total number of organisms ($p < 0.05$) for all sites was observed from Day 111 to 119 (seventh and eighth sampling time), indicating that the biofilm did not become stagnant during the period of 100 to 150 days. If indeed so (that biofilm formation in drinking water distribution systems takes longer than 150 days to reach a steady state), then this has far-reaching implications for studies of biofilms in these and other oligotrophic environments, as typically most studies observe biofilms over much shorter periods (Easton & Pitchers, 1995; Hallam, 1992; Hallam *et al.,* 2001). This may result in the development of unrealistic conceptual models of biofilm form and function under low-nutrient conditions. The implication of such incomplete description in practise, such as the control of biofouling is not known, emphasising the need for studies conducted *in situ* and over extended periods of time. Interestingly the whole-community metabolic' profiles did not suggest notable changes over time at the respective sampling sites. A possible explanation for this is that although the relative abundance of the dominant functional groups showed little

change between days 100 and 150, the low levels of organic and inorganic particles in the water resulted in a very slow rate of biofilm formation and that binding sites in the biofilms have not all been occupied to result in self-regulation of the biofilm as a unit. These results emphasises the need for the application of more than one technique to describe the formation of biofilms within the distribution system.

A comparison of the results obtained for DOC (mg C/l), AOC (µg C/l) and BFR (pg ATP/cm^2 x day) in the present study for sites 1 (HOL), 2 (FAER), 3 (HAR), 4 (SAND) and 5 (GAU) as well as for the studies conducted by Vrouwenvelder *et al.* (1997) for the water works Holthausen (H*) and Van der Kooij *et al.* (1994) for biofilm monitors exposed to treated groundwater [Water types A, B and C with the ammonia, methane, iron and manganese concentrations ranging from highest (water type A) to lowest (water type C) and treated by; intensive aeration using perforated plates for water types A and B and aeration using cascades for water type C; rapid sand filtration; CO_2 removal for water type A; and aeration and rapid sand filtration once more before distribution, with slow sand filtration as the final treatment step at some of the supplies)] in the Netherlands, respectively, is presented in **Table 3.3** and **Figure 3.2 (A-C)**. Drinking water without a disinfectant residual is distributed by most of the water suppliers in the Netherlands. The study conducted by Van der Kooij *et al.* (1994) showed that the extent of after growth of aeromonads in groundwater supplies distributed without disinfection could be predicted by the BFR-value of the water leaving the treatment plant.





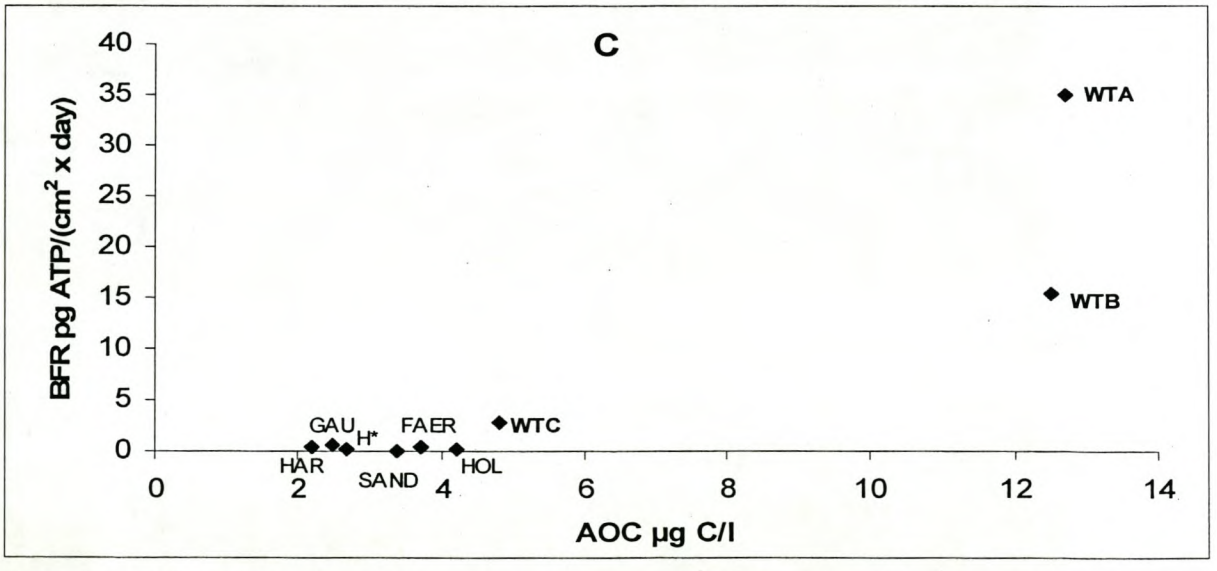


Figure 3.2 (**a**) DOC- versus AOC concentrations, (**b**) DOC versus BFR and (**c**) AOC versus BFR, for the present study as well as the studies conducted by Vrouwenvelder *et al.* (1997) and Van der Kooij *et al.* (1994)

Table 3.3 Water quality and biofilm formation properties observed for the present study and compared to the results obtained by Vrouwenvelder *et al.* (1997) and Van der Kooij *et al.* (1994)

Sample	Biofilm formation rate (BFR) pg ATP/(cm^2 x day)	AOC µg C/l	DOC mg C/l
Holthausen	0.28	4.2	0.25
Faerberstrasse	0.46	3.7	0.4
Hardt	0.41	2.2	0.33
Sandberg	0.07	3.4	0.3
Gau	0.6	2.5	0.23
^Holthausen	0.27	2.7	0.31
*Water type A (WTA)	35.1	12.7	7.2
*Water type B (WTB)	15.4	12.5	5.8
*Water type C (WTC)	2.9	4.8	2

^ - Results obtained from Vrouwenvelder *et al.* (1997)

* - Results obtained from Table 2 Van der Kooij *et al.* (1994)

Evaluation of the DOC versus AOC -results [**Figure 3.2** (**A**)] shows an almost linear relationship between the AOC and DOC concentrations, irrespective of water type. For example, the average DOC concentration in the water type A was 7.2 mg C/l with a corresponding AOC concentration of 12.7 µg C/l (Van der Kooij *et al.*, 1994). The average DOC concentration in the disinfected drinking water at site 3 was 0.33 mg C/l with a corresponding AOC concentration of 2.2 µg C/l. These results supports the findings by Vrouwenvelder *et al.* (1997) that low DOC concentrations can restrict the levels of AOC in water. The average AOC values for site 1 and water type C were similar at 4.2 and 4.8 µg C/l, respectively, as were those for water types A and B at 12.7 and 12.5 µg C/l, respectively (Van der Kooij *et al.*, 1994). Based on the theory that low DOC values restrict the AOC concentration in water it was expected that as the AOC values were low and comparable, the DOC values would similarly be low and comparable. However, the corresponding DOC values for site 1 and water type C differed with values of 0.25 and 2 mg C/l, respectively, as did those for water types A and B at 7.2 and 5.8 mg C/l, respectively (Van der Kooij *et al.*, 1994). Anaerobic groundwater contains ammonia, methane, iron and manganese, with intensive aeration and filtration performed on the water to remove these substances. Site 1 is supplied with only a 20% groundwater source whereas water types A, B and C have a pure groundwater source. Furthermore, the concentrations of ammonia, methane, iron and manganese in the respective water types range from highest concentrations present in water type A to the lowest

concentrations in water C, which could explain the difference in DOC values observed between the different water types.

Figures 3.2 (B) and **(C)** illustrate that low DOC and AOC values influence the BFR, irrespective of water type. For example, the average DOC and AOC concentrations for water type A, B and C were 7.2 mg C/l and 12.7 µg C/l, 5.8 mg C/l and 12.5 µg C/l and 2 mg C/l and 4.8 µg C/l, respectively. The BFR on glass coupons exposed to this water types for approximately 140 days was calculated as 35.1 pg ATP/(cm^2 x day) for water type A, 15.4 pg ATP/(cm^2 x day) for water type B and 2.9 pg ATP/(cm^2 x day) for water type C (Van der Kooij *et al.,* 1994). Dissolved organic carbon and AOC results for the present study and for the study conducted by Vrouwenvelder *et al.* (1997) ranged from 0.23 to 0.4 mg C/l and 2.2 to 4.2 µg C/l, respectively. The BFR for these sample sites ranged from 0.07 to 0.46 pg ATP/(cm^2 x day), indicating that a predictable trend exists between DOC or AOC and the rate of biomass accumulation.

Although no direct correlation can be made as the DOC, AOC and BFR values compared were obtained in two studies, results from the present study shows that it appears possible that a threshold value for AOC, and possibly DOC, exists above which the BFR notably increases. It is obvious that a universally acceptable BFR would be difficult to define for all water systems. However, for a given type of water system, such as the potable water distribution system in this study, it should be possible to indicate a BFR that would have a noticeable negative impact on the system, if a change in the prevailing conditions occurred. Due to the inherent difficulties in determining BFR, indices such as AOC or DOC may be a good indirect measure of BFR, and thus biofouling potential in general. Therefore, these measurements warrant due consideration when monitoring strategies for water distribution systems are developed. Although more data is necessary to accurately determine threshold values, the observations made in this study suggest that a DOC below ~0.5 mg C/l, and an AOC below ~5 µg C/l could be target values for the control of biofilm formation in drinking water systems.

3.2 BIOFILM FORMATION ON MATERIALS RELEVANT TO THE DRINKING WATER DISTRIBUTION INDUSTRY

Representative coupons of all the test materials were periodically collected over a period of 36 weeks from the different sections of the pilot plant (**Figure 2.3).** Coupons were analysed for biofilm thickness, area coverage and cell activity using confocal laser scanning microscopy (CLSM) with the Baclight™ Live/Dead stain, while a second series of coupons were sonicated and the respective effluents analysed for: (a) Adenosine Tri-Phosphate (ATP) concentrations to measure active biomass; (b) total number of organisms by direct microscopic cell count; (c) colony counts on R2A agar; and (d) whole community metabolic profiles using the Biolog™ Eco Micro Plate System.

3.2.1 Material coupons placed under turbulent flow conditions

A summary of results obtained for heterotrophic plate counts, ATP concentrations and DAOC, for the turbulent flow rate coupon system for activated carbon treated water (untreated and sanded material coupons), ozonated raw water and drinking water is presented in **Figure 3.3** to **3.5** and **Adddendum (a-j)**. All samples of the 10 materials exposed to water from three different sections of the water purification process (as illustrated in **Figures 2.2 and 2.3)**, i.e. (**A**) ozonated raw water (PE, PVC, VA 1.4301, VA 1.4571, Copper, Zinc-coated steel, and Glass), (**B**) activated carbon treated water (all 10 materials) and **(C)** potable water [activated carbon treated water with chlorine dioxide (60 µg/l), phosphate (1 mg/l) and silicate (1 mg/l)] (PE, PVC, VA 1.4301, VA 1.4571, Copper, Zinc-coated steel, and Glass), were placed under turbulent flow conditions. The materials analysed in the activated carbon treated water section were either used as obtained from the manufacturer or sanded (600 grid) to determine the influence of surface roughness and damaged passivation layers on biofilm formation

The differences in heterotrophic plate counts, ATP concentrations and direct microscopic cell counts (DAOC) for all materials, especially copper, aluminium and zinc-coated steel, placed in activated carbon treated water over time were significant ($p < 0.05$) as shown in **Figure 3.3 (A-C)**.

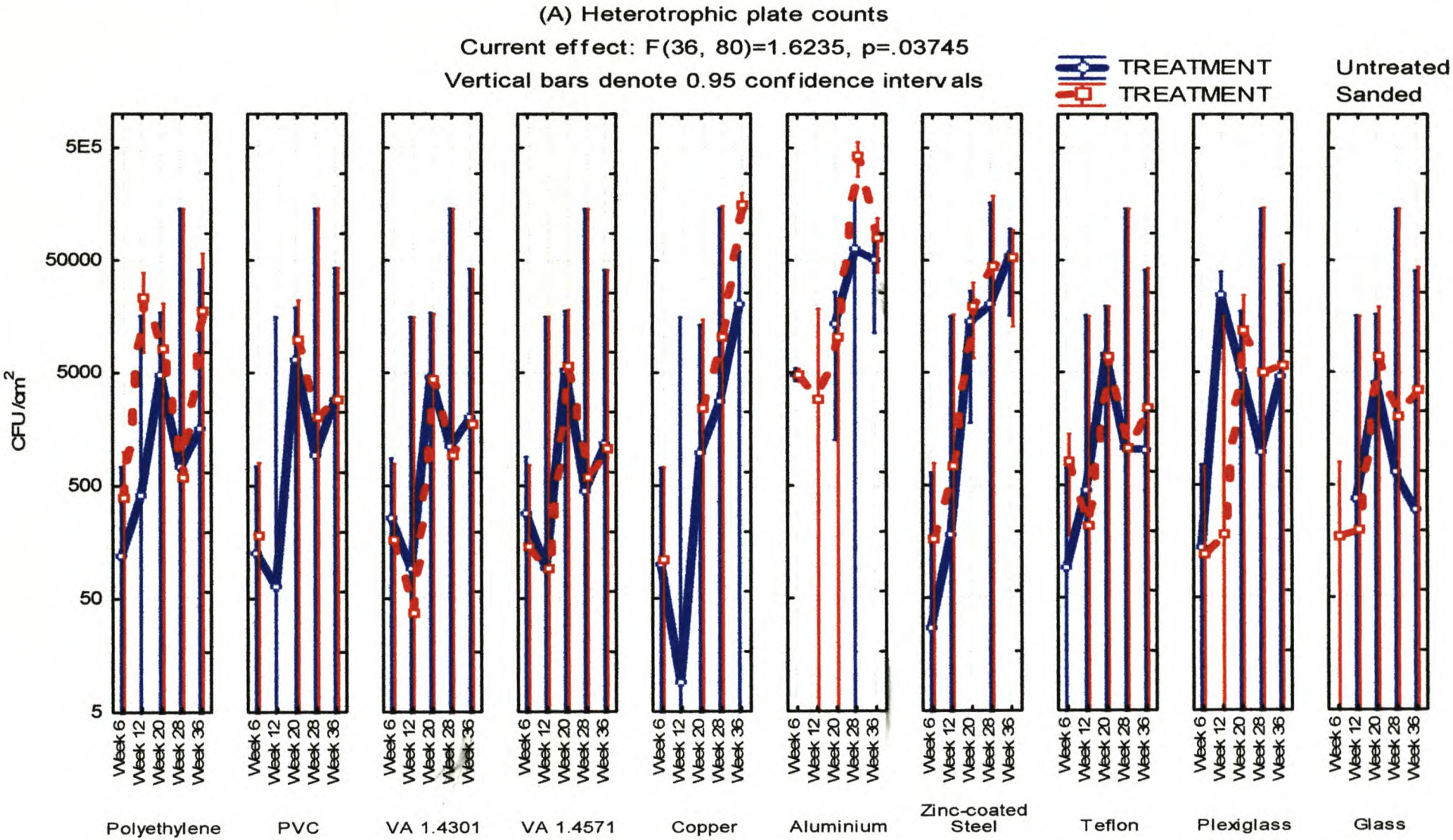


Figure 3.3 (A) Heterotrophic plate counts for untreated and sanded material coupons placed in activated carbon treated water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

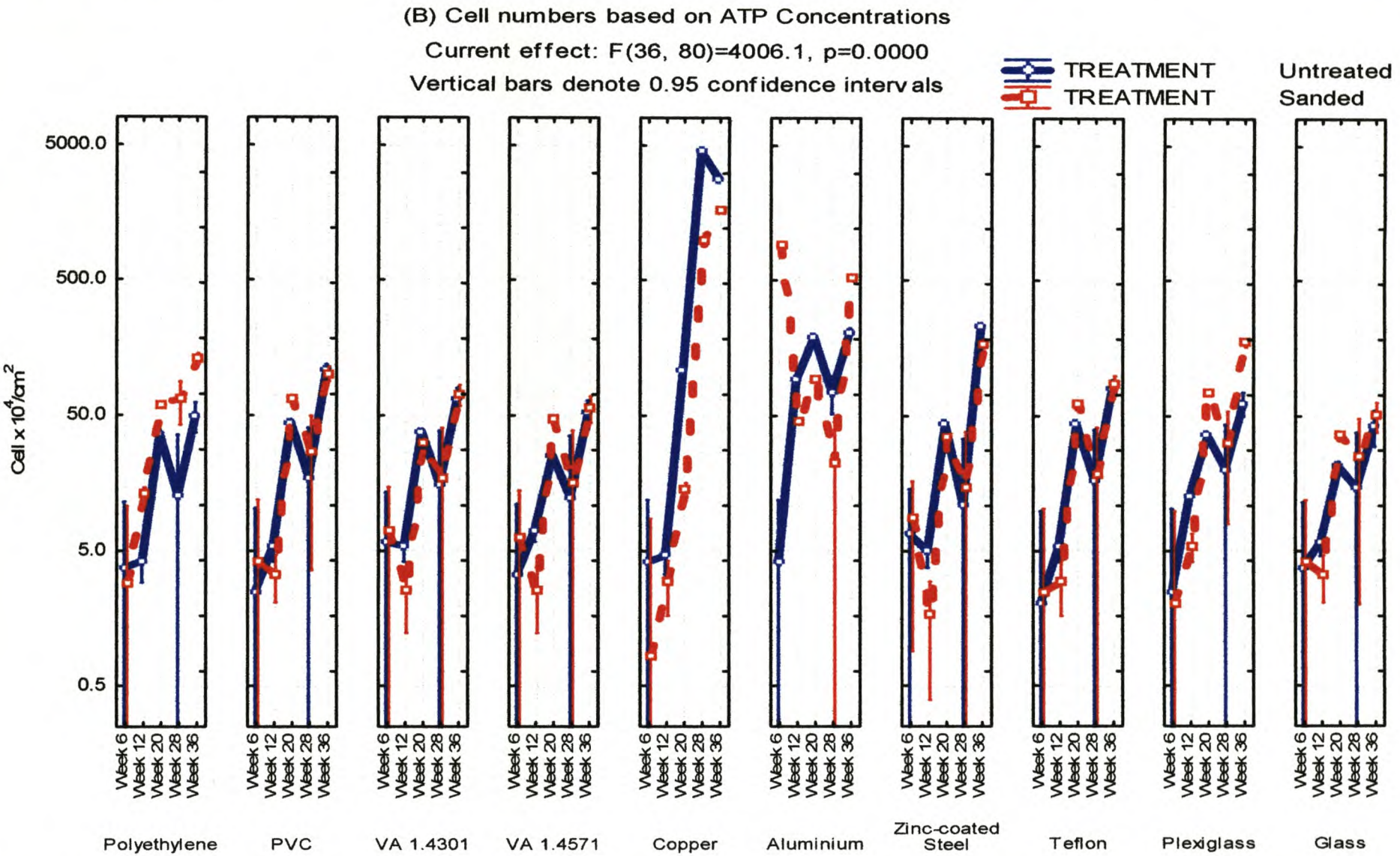


Figure 3.3 (B) Cell numbers based on ATP concentrations for untreated and sanded material coupons placed in activated carbon treated water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

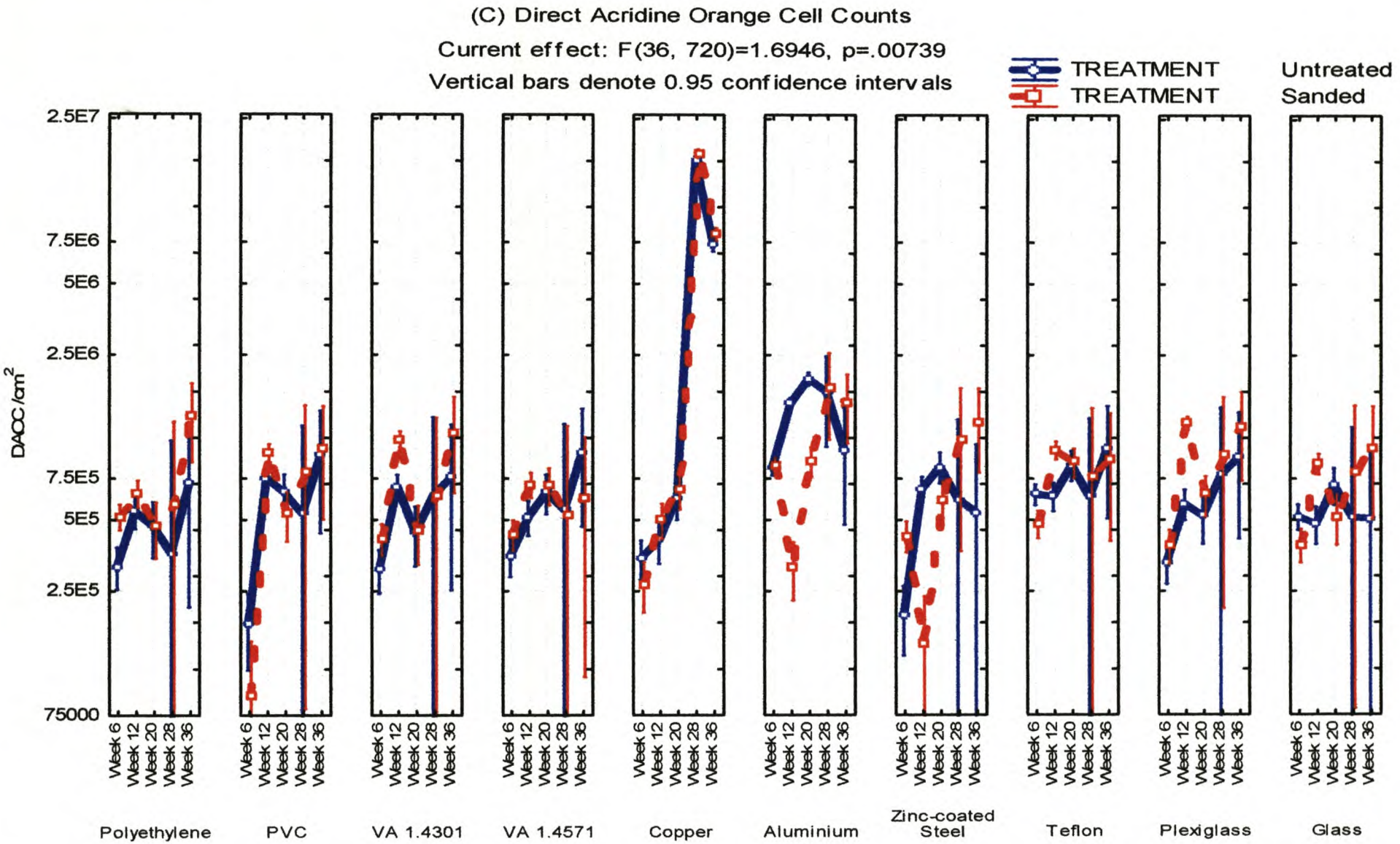


Figure 3.3 (C) Direct acridine orange cell counts for untreated and sanded material coupons placed in activated carbon treated water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

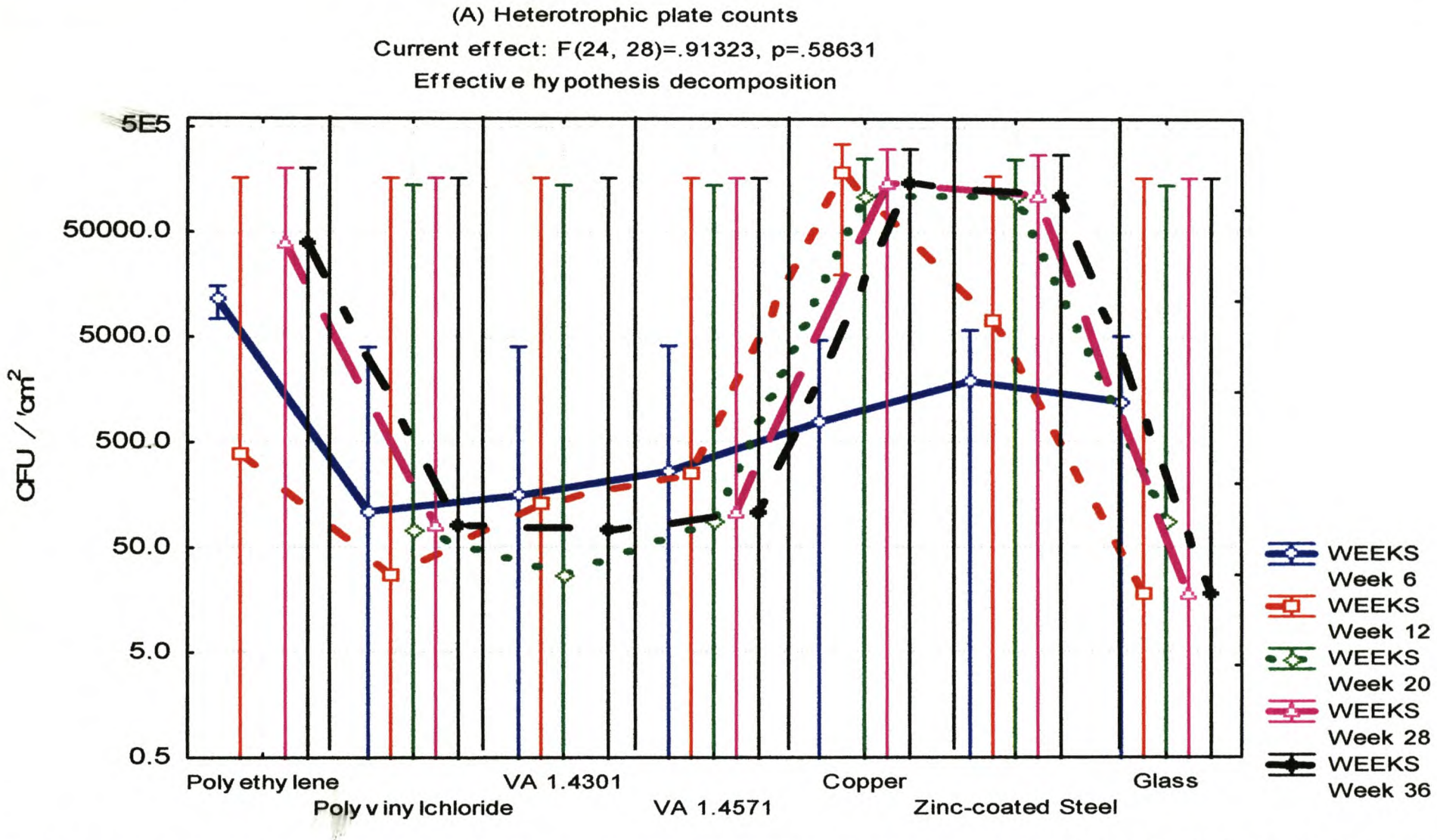


Figure 3.4 (A) Heterotrophic plate counts for untreated material coupons placed in ozonated raw water at a turbulent flow for 6, 12, 20, 28 and 36 weeks, showing high numbers for copper, zinc-coated steel and PE

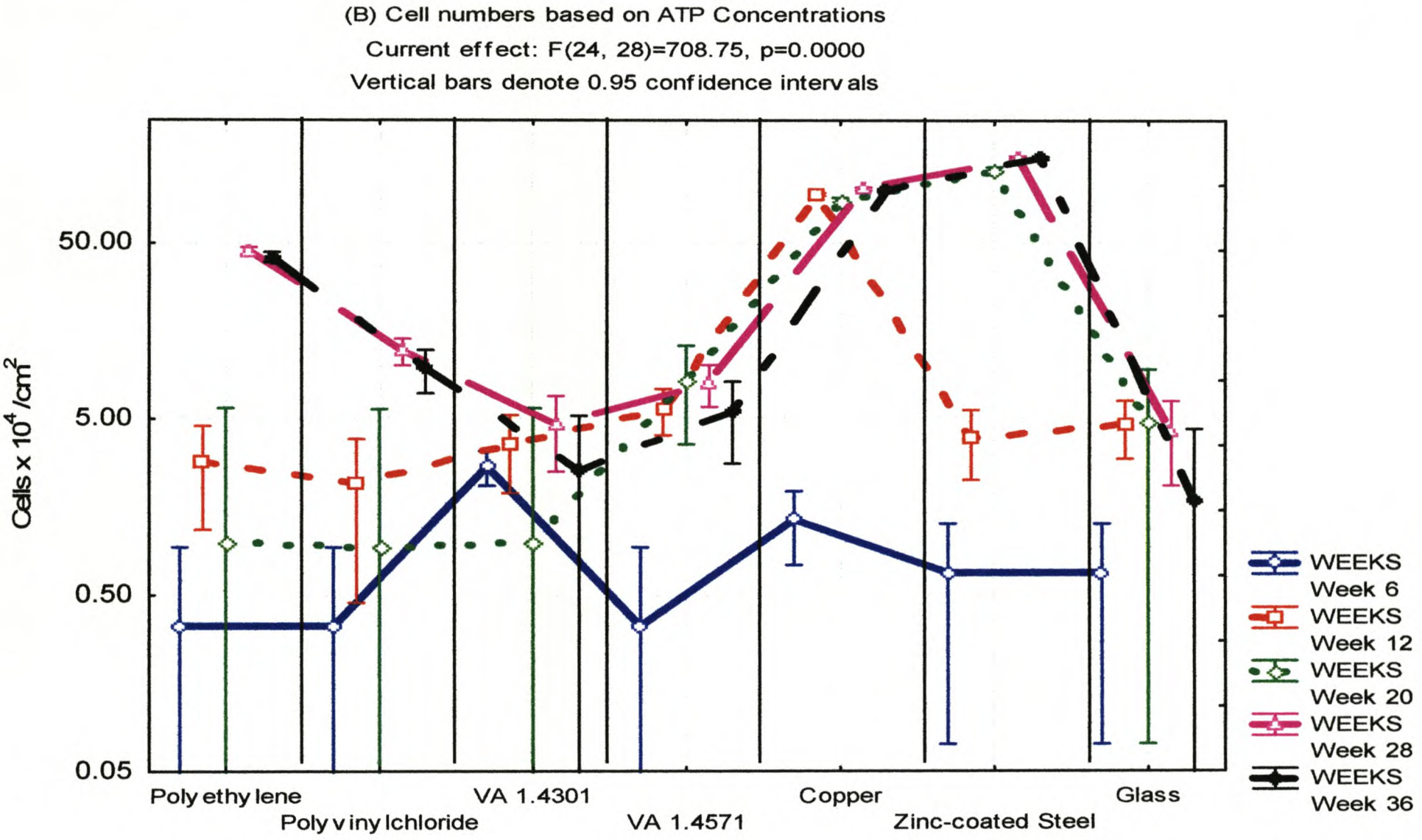


Figure 3.4 (B) Cell numbers based on ATP concentrations for untreated material coupons placed in ozonated raw water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

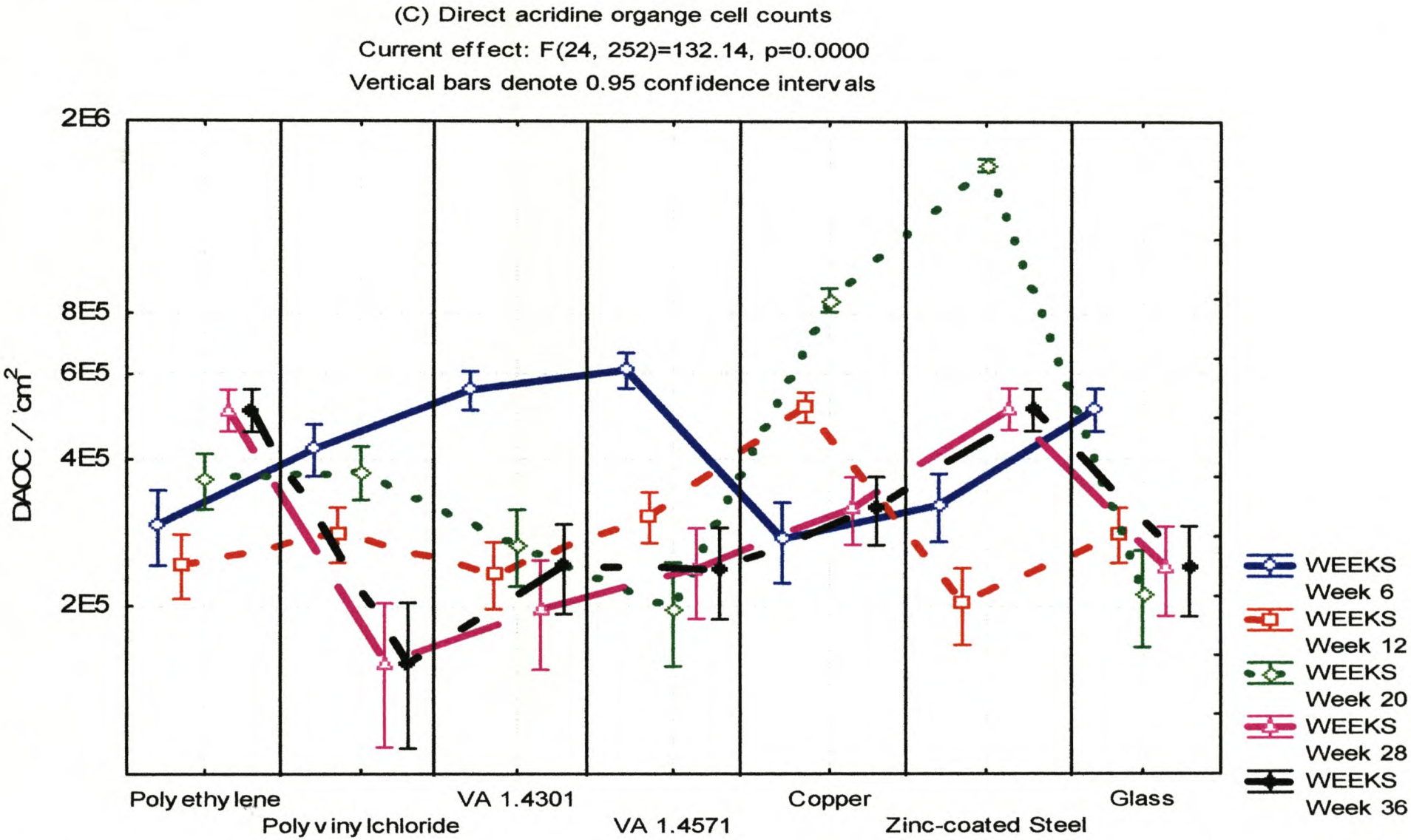


Figure 3.4 (C) Direct acridine orange cell counts for untreated material coupons placed in ozonated raw water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

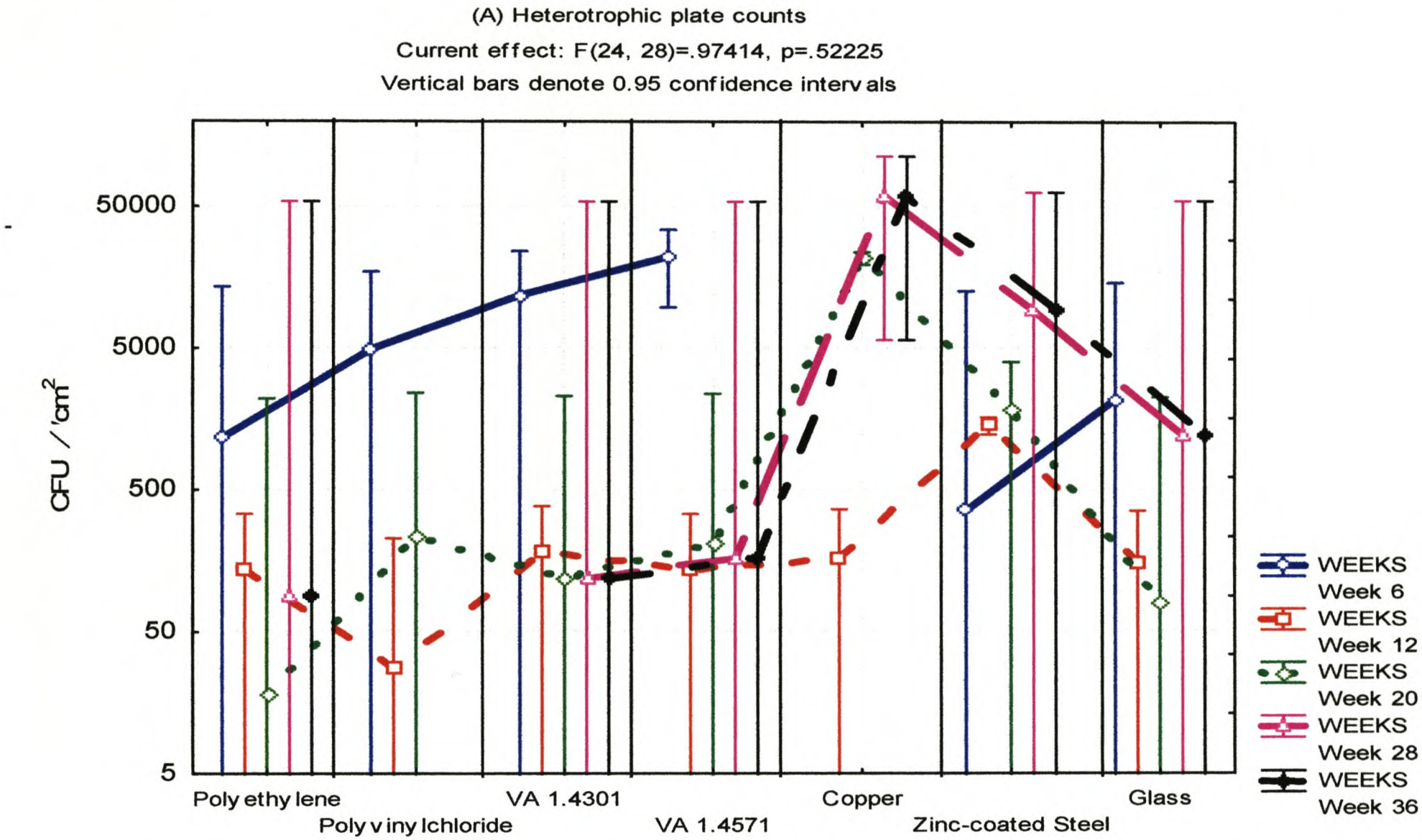


Figure 3.5 (A) Heterotrophic plate counts for untreated material coupons placed in drinking water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

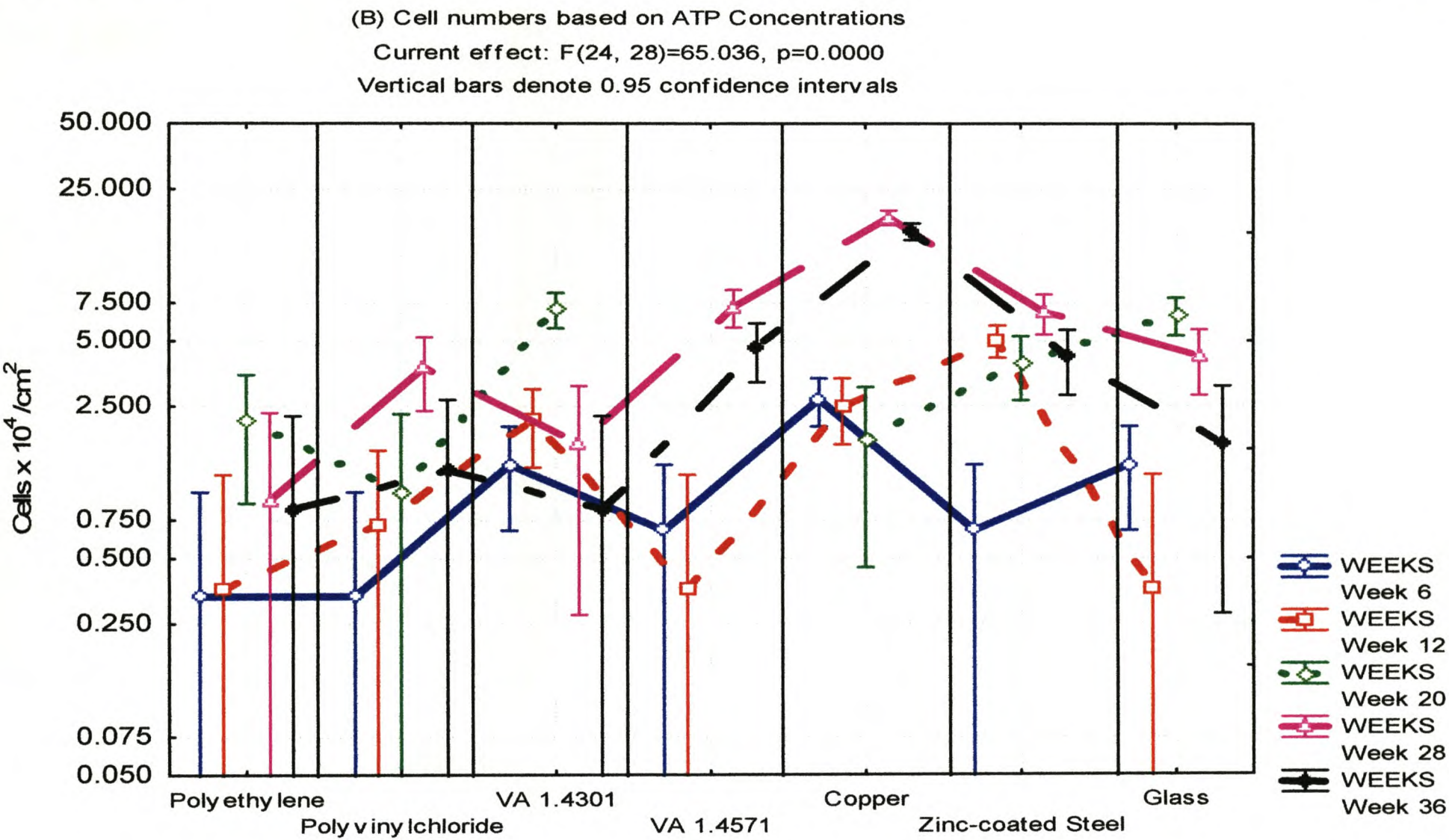


Figure 3.5 (B) Cell numbers based on ATP concentrations for untreated material coupons placed in drinking water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

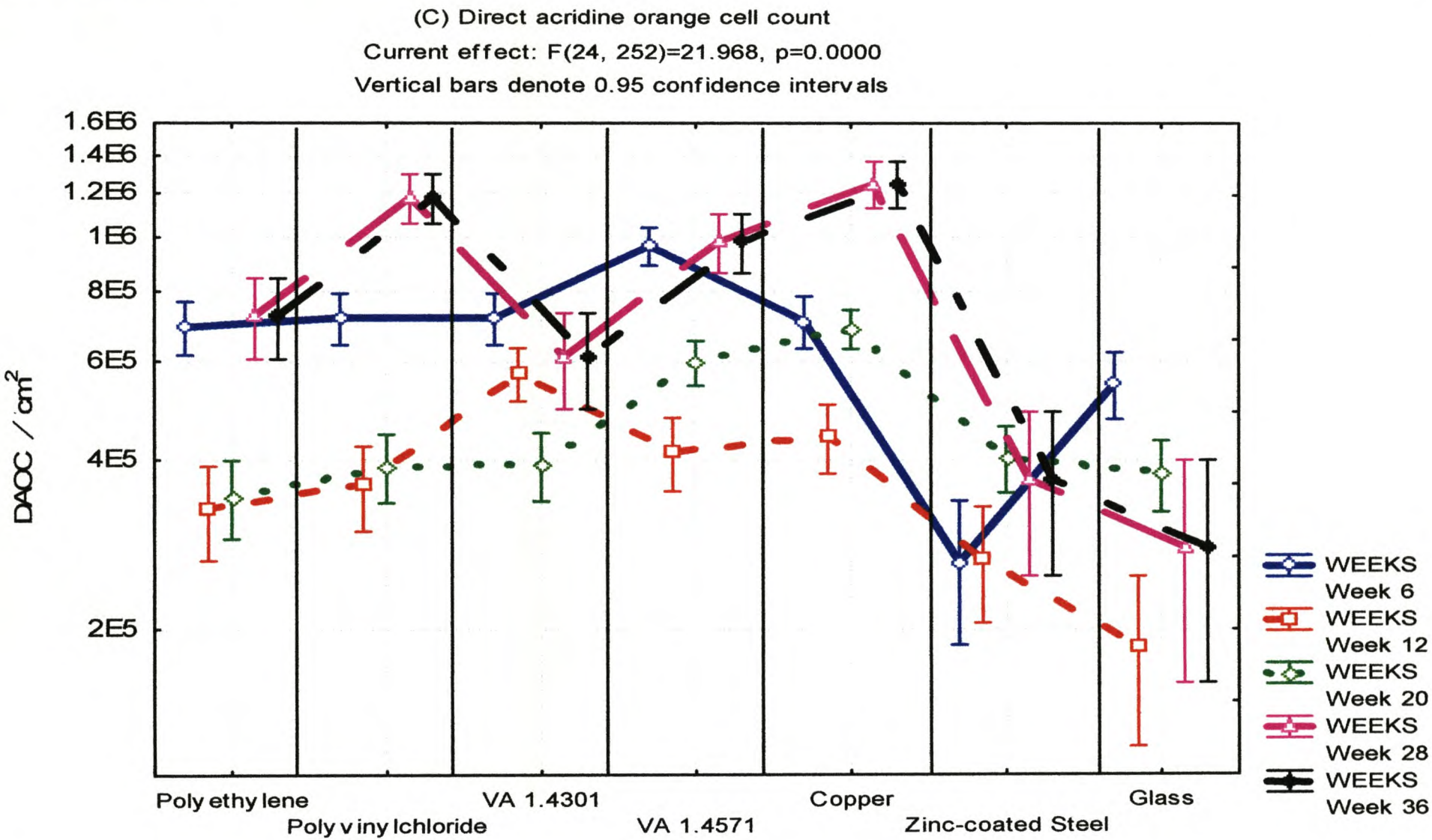


Figure 3.5 (C) Direct acridine orange cell counts for untreated material coupons placed in drinking water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

The direct cell counts for sanded polyethylene coupons exposed to activated carbon treated water also showed a significant increase ($p < 0.05$) at week 36. Scanning electron microscopy analysis of the polyethylene coupons revealed the presence of various inorganic materials, which could clarify the elevated direct cell count result obtained. The heterotrophic plate count results increased significantly for aluminium from week 28 ($p < 0.05$), and for copper and zinc-coated steel from week 36 ($p < 0.05$). Similarly the ATP concentrations and DAOC for these three actively corroding materials, as well as for the rest of the materials analysed, increased significantly from week 36 ($p < 0.05$), indicating that biofilm growth had not reached a steady state at this time. These results correspond with data obtained in **Section 3.1** where it was concluded from the heterotrophic plate counts, ATP concentrations and DAOC results obtained, that the biofilms had not yet reached a steady state between the period of 100 to 150 days. In addition, the direct cell count results for copper for secondary colonisation, both for untreated and sanded coupons were also noticeably high **[Addendum (a-j)]**. Although these changes were significant ($p < 0.05$) they were mostly relatively small (> 10x) in the context of microbiological investigations.

The heterotrophic plate counts for the bacterial suspension obtained from the materials placed in ozonated raw water **[Figure 3.4 (A)]** and drinking water **[Figure 3.5 (A)]** remained relatively constant throughout the sampling period. In contrast, the results obtained over time for ATP measurements and DAOC for the different materials placed in ozonated raw water **[Figure 3.4 (B-C)]** and drinking water **[Figure 3.5 (B-C)]** were significantly ($p < 0.05$) different. The results obtained for heterotrophic plate counts and ATP differed from each other with the ATP results indicating that the viable population present in the biofilm on the different materials fluctuated notably during the sampling period. A shortage of nutrients or a shift in the population dynamics as indicated with the Eco-Biolog™ **[Figure 3.14 (a & b)]** results could possibly explain these results. Major differences in ATP concentrations and DAOC results were however, observed for all the materials, especially PE, copper and zinc-coated steel, placed in these two water types.

The use of R2A media, which contains a relatively low concentration of nutrients in combination with a long incubation period at a lower temperature, is suitable for the isolation of stressed and chlorine-tolerant bacteria from drinking water (Reasoner & Geldreich, 1979). Higher colony counts for microbes isolated from oligotrophic conditions are generally yielded as the low concentration of yeast

extract, casein hydrolisate, peptone and glucose allows for a wide spectrum of bacteria to grow without the faster growing bacteria suppressing the slower growing or stressed species. R2A agar is suitable in standard methods for pour plate, spread plate and membrane filter methods for heterotrophic plate counts (Reasoner & Geldreich, 1979; Kelly *et al.,* 1983). The classical heterotrophic plate count procedure, however, only quantifies culturable cells, whereas the ATP method also determines bacteria not contributing to these counts (e.g. nitrifying and methane oxidising bacteria), with one pg of ATP equalling approximately 10^4 active bacteria/cells, presuming an average ATP content of 10^{-16} – 10^{-15} g per active bacterial cell (Webster *et al.,* 1985; Stanley, 1989; Van der Kooij & Veenendaal, 1993; Lehtola, 2002).

To assess the contribution of the heterotrophic colony count [**Figure 3.3 to 3.5 (A)**] to the amount of active biomass as determined with ATP measurements [**Figure 3.3 to 3.5 (B)**], the percentage ratio between the heterotrophic plate counts on R2A and the ATP concentrations was calculated (**Table 3.4**). These results showed that on average the culturable bacteria represented only a fraction (often < 1%) of the number of cells in the biofilm samples as determined by ATP analysis. Furthermore, on average the heterotrophic plate count measurements often represented < 1% of the direct acridine orange cell count [**Figure 3.3 to 3.5 (C)**] recorded for the biomass removed from the material coupons, whereas cell numbers based on ATP measurements often represented < 100% of the DAOC (**Table 3.4**). This was especially true for PE, PVC, copper, zinc-coated steel and aluminium, when exposed to activated carbon treated water and ozonated raw water. The biomass suspension obtained from these materials was turbid due to the presence of various inorganic particles (confirmed by SEM/EDX analysis) present on the PE and PVC and the active corrosion taking place on copper, aluminium and zinc-coated steel, which could have affected the ATP concentrations obtained. However, these results show that under certain conditions, such as those investigated in the present study, 1 pg of ATP may not be equal to approximately 10^4 active bacteria/cells, and that the average ATP content per active bacterial cell is indeed less than 10^{-16} – 10^{-15} g. This would then explain the percentage ratios, above the theoretical maximum limit of 100, obtained for PVC, PE, copper, aluminium and zinc-coated steel.

Table 3.4 The average percentage ratio between the heterotrophic plate counts and the cell numbers based on ATP concentration as well as the average percentage ratio between the heterotrophic plate count, ATP and DAOC for material coupons placed in activated carbon treated water (untreated and sanded materials), ozonated raw water and drinking water for all sampling dates

Parameter	PE	PVC	VA 1.4301	VA 1.4571	Copper	Zinc-coated Steel	Aluminium	Teflon	Plexiglass	Glass
Untreated coupon - Activated carbon treated water										
CFU/ATP	0.39	0.33	0.33	0.39	0.02	1.98	2.04	0.36	2.39	0.35
CFU/DAOC	0.32	0.34	0.28	0.24	0.10	2.90	3.84	0.26	1.86	0.21
ATP/DAOC	81.10	105.14	85.21	60.41	538.85	146.47	187.96	71.42	78.02	59.22
Sanded coupons - Activated carbon treated water										
CFU/ATP	1.48	0.40	0.30	0.32	0.35	2.06	3.21	0.36	0.44	0.57
CFU/DAOC	2.30	0.39	0.18	0.25	0.63	10.98	3.81	0.28	0.51	0.35
ATP/DAOC	155.70	98.72	60.62	78.56	177.04	533.72	118.66	77.99	96.06	60.56
Ozonated raw water										
CFU/ATP	5.52	0.09	0.09	0.17	16.78	Not tested	8.22	Not tested	Not tested	0.46
CFU/DAOC	4.68	0.03	0.01	0.05	51.68		21.0			0.09
ATP/DAOC	84.84	31.78	14.00	31.63	307.94		255.55			19.77
Drinking water										
CFU/ATP	2.18	4.65	5.16	9.66	34.51	Not tested	10.78	Not tested	Not tested	1.93
CFU/DAOC	0.05	0.14	0.42	0.58	6.05		2.43			0.29
ATP/DAOC	2.47	2.92	8.20	5.95	17.52		22.55			15.14

Overall this data confirms previous observations (Coghlan, 1996; Potera, 1996) that biofilm monitoring techniques need to be implemented at drinking water distribution plants as the results show that the classic plate count techniques currently performed represent only a fraction of the entire biomass, which may be present in biofilms attached to the internal surface of the water system. The results shown in **Figure 3.4 (A-C)** demonstrated that the nature of the attachment surface had a pronounced influence on microbial colonisation over extended periods of time. This implies that surface conditioning by either chemical or biological means did not mask material properties. This aspect needs to be further explored, conceptually, and by means of suitable model systems, because it is sometimes suggested that once the surface is conditioned, subsequent colonising microbes may not sense the differences specific to materials.

A summary of results obtained for the surface colonisation percentages using CLSM analysis, for the turbulent coupon system for activated carbon treated water (untreated and sanded), ozonated raw water and drinking water is represented in **Figure 3.6, 3.7** and **3.8,** respectively. Comparison of the Live/Dead ratios obtained using CLSM and the Baclight™ stain are summarised in **Addendum (a-j)**.

Strong preferences for biofilm formation on the polymeric materials polyethylene (PE), polyvinylchloride (PVC) and teflon (PTFE) was evident by results obtained for the primary colonisation of these coupon surfaces using CLSM analysis, with PE and PVC being especially amendable for biofilm formation when exposed to drinking water [activated carbon treated water with chlorine dioxide (60 µg/l), phosphate (1 mg/l) and silicate (1 mg/l)]. The metallic surfaces exhibited more distinct colonisation patterns ranging from intense surface colonisation and corrosion product production on aluminium to relatively low surface coverage on the two grades of stainless steel, namely VA 1.4301 and VA 1.4571.

Generally, the results obtained for surface coverage and live/dead ratio using CLSM analysis compared well with the microbiological results obtained for total cell count for activated carbon treated water, ozonated raw water and drinking water, as indicated in **Figure 3.3, 3.4 and 3.5 (C) and Addendum (a-j)**, respectively. For example the analysis of the surface coverage data for the two grades of stainless steel, VA 1.4301 and VA 1.4571, and glass [**Figure 3.6, 3.7 and 3.8**] revealed that the degree of surface colonisation observed on these coupons were small, irrespective of the water type they were exposed to.

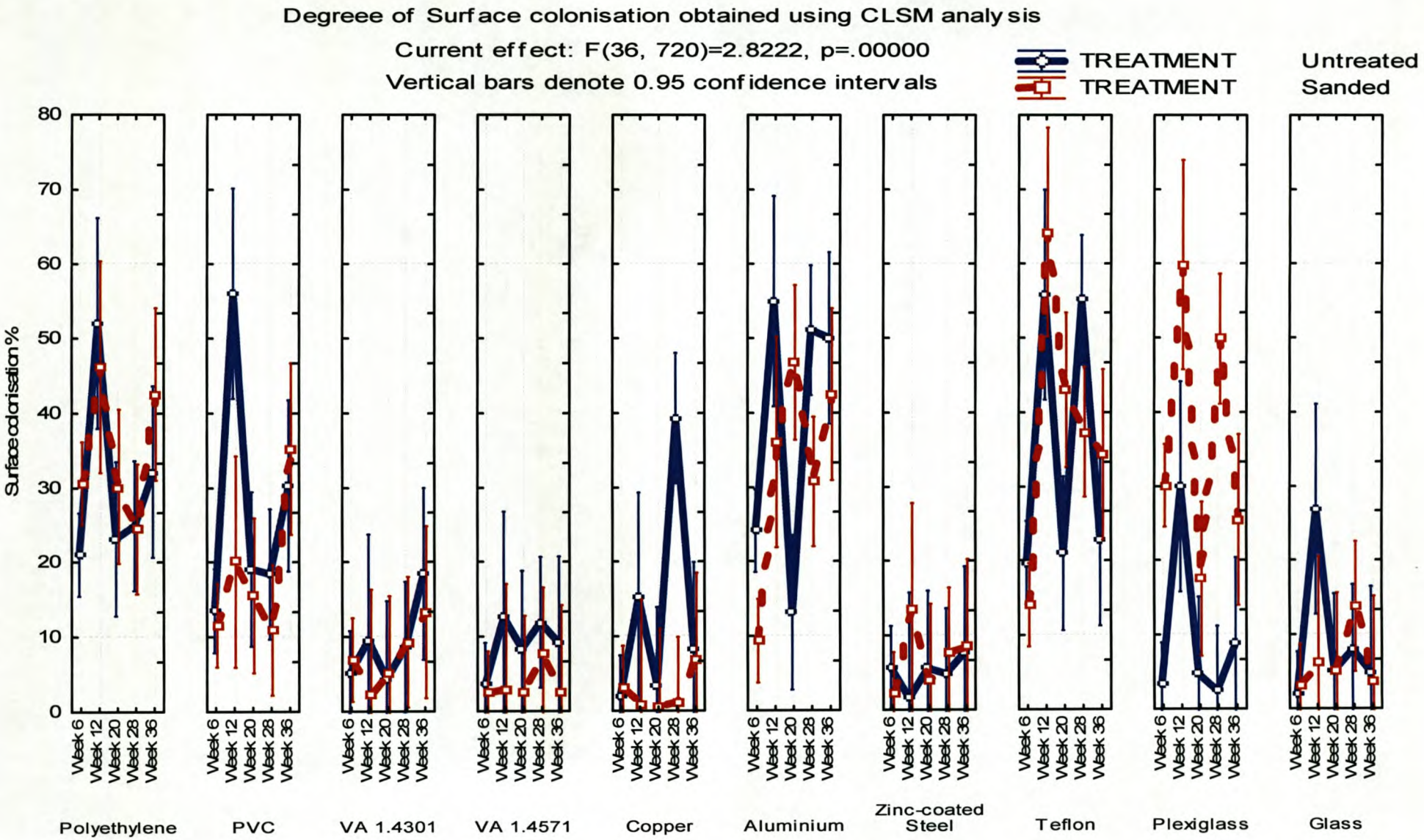


Figure 3.6 Surface colonisation percentage obtained using CLSM analysis for untreated and sanded material coupons placed in activated carbon treated water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

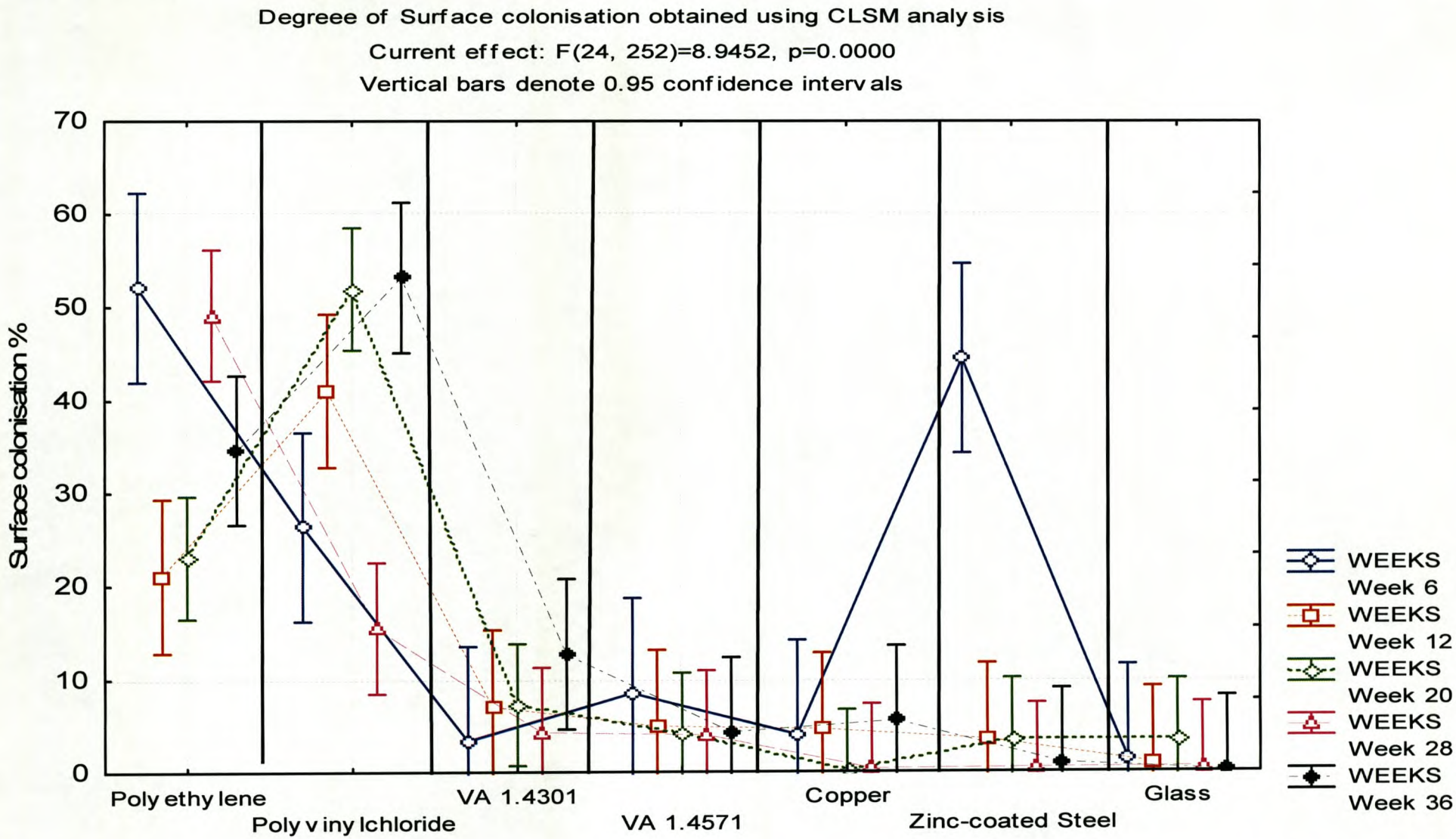


Figure 3.7 Surface colonisation percentage obtained using CLSM analysis for material coupons placed in ozonated raw water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

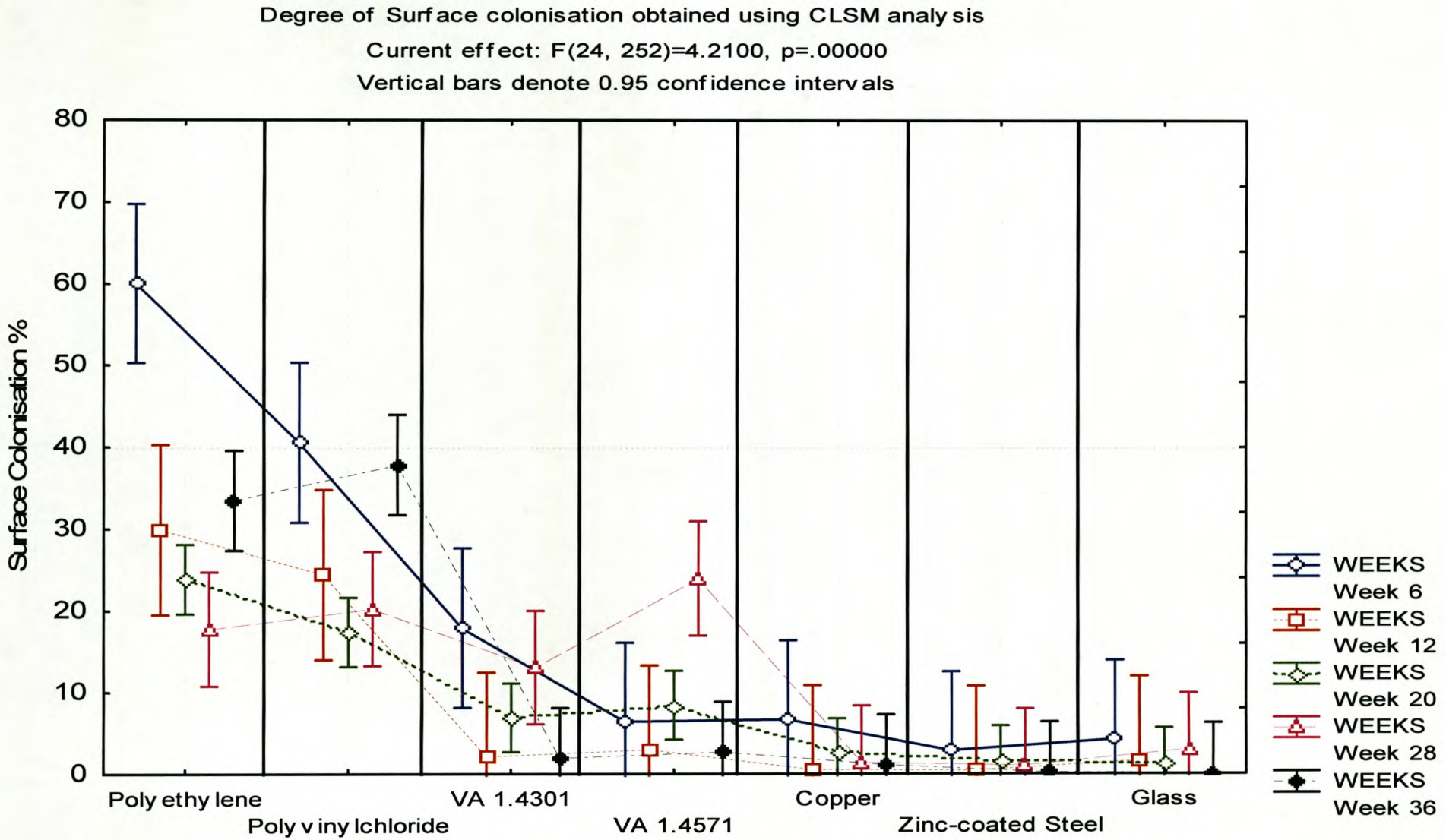


Figure 3.8 Surface colonisation percentage obtained using CLSM analysis for material coupons placed in drinking water at a turbulent flow for 6, 12, 20, 28 and 36 weeks

The low direct acridine orange cell count results obtained [**Figure 3.3 3.4 and 3.5 (C)**] for the two grades of stainless steel and glass corresponded with the surface coverage data. Similarly, the high direct acridine orange cell counts obtained for PE, PVC, teflon and aluminium [**Figure 3.3 3.4 and 3.5 (C)**] corresponded with the results obtained for CLSM analysis of the degree of colonisation on these materials. The notable exceptions were copper and zinc-coated steel. The corrosion taking place on copper and zinc-coated steel interfered with the CLSM analysis, as the results obtained for total cell count, colony forming units as well as the BFR and BFP for these two materials were continually the highest ($p < 0.05$), while on average the CLSM surface coverage data for copper and zinc-coated steel analysis was extremely low ($p > 0.05$). A presentation of the corrosion layers viewed over time on copper coupons placed in activated carbon treated water under turbulent conditions using CLSM is presented in **Figure 3.9**. Moreover, for copper and zinc-coated steel the adenosine tri-phosphate analysis was sometimes problematic, especially when exposed to ozonated raw water, as once again the active corrosion taking place on these two materials made it difficult to analyse the turbid biomass sample obtained by sonication.

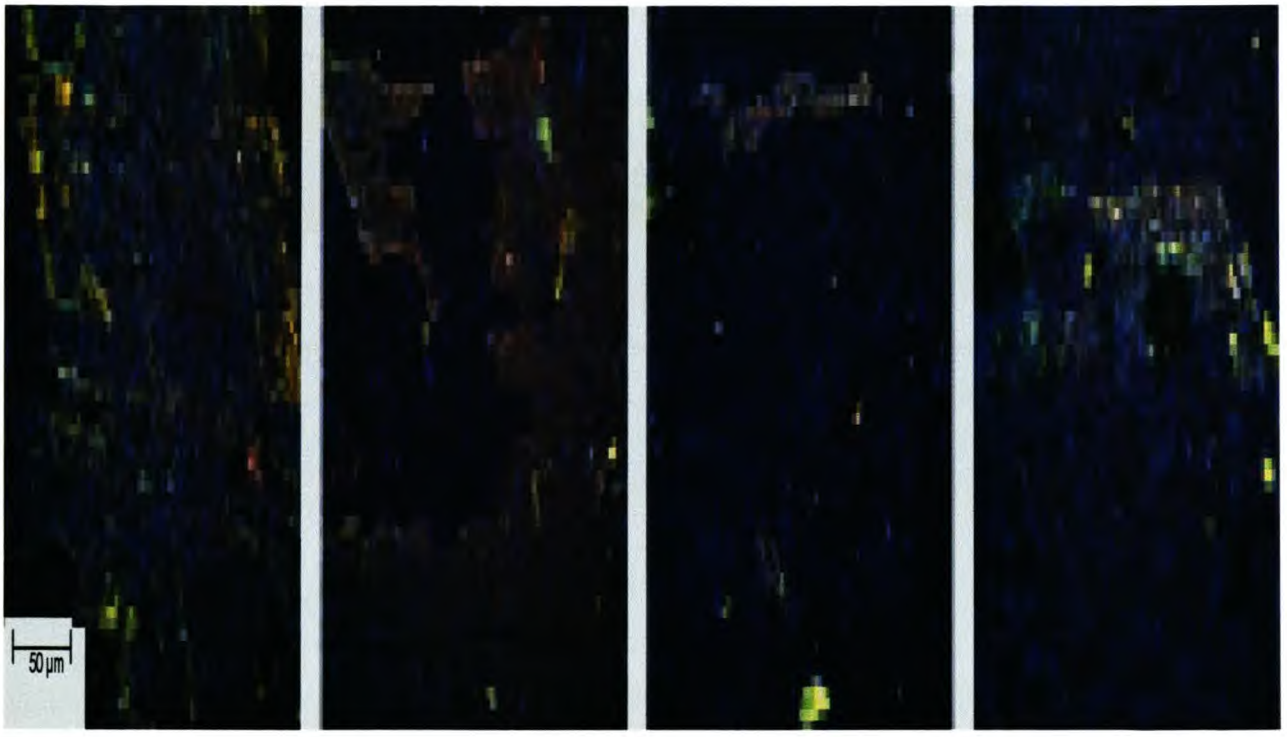


Figure 3.9 A presentation of the corrosion layers viewed over time on Copper coupons placed in activated carbon treated water under turbulent conditions using CLSM.

Changes in the degree of biofilm formation, due to surface treatment of the sample by sanding, were strongly dependent on the material as shown in **Figure 3.6**. While material preparation exhibited no significant or distinct changes for PE, PVC, VA 1.4301 and zinc-coated steel ($p > 0.05$), strong variations were observed for VA 1.4571 and copper ($p < 0.05$). The decrease in surface colonisation for these two materials could be correlated to the destruction of the passivated surface layers by sanding. Additionally, the effect of surface modification of PTFE and plexiglass allowed for a more intense colonisation of these samples.

3.2.2 Flow-cell (laminar flow) studies

The same 10 materials analysed in the pilot plant, with the exception of plexiglass and glass, were analysed in the flow-cell system exposed to activated carbon treated water under laminar flow conditions. As with the materials analysed under turbulent flow conditions, the materials were either used as obtained from the manufacturer or sanded (600 grid) to determine the influence of surface roughness and damaged passivation layers on biofilm formation. The results for surface coverage analysis for all materials [untreated and sanded] with activated carbon treated water under laminar flow conditions are illustrated in **Figure 3.10**.

When compared to turbulent exposition, the material samples exposed to laminar flow conditions exhibited less distinct, although significant ($p < 0.05$) differences in surface colonisation density. Even though PE and PVC exhibited a slightly lower degree of biofilm formation, the values for VA 1.4301 and VA 1.4571 were distinctly higher ($p < 0.05$) under the slower flow rate. Due to the lower flow rates, it appears that corrosion occurred more readily on copper, aluminium and zinc-coated steel, which had a stronger impact on the biofilm population.

Modification of the surface of the coupons by sanding produced significant differences ($p < 0.05$) under laminar flow conditions. As seen with the sanded coupons exposed to a turbulent flow, roughening of the material played a less significant role on the polymeric materials ($p > 0.05$), with the exception of plexiglass which was not examined under the slower flow rate. However, a significant increase ($p < 0.05$) in the degree of surface colonisation was observed for both grades of stainless steel (especially at week 36) and aluminium. The result for aluminium could be correlated to different intensities of corrosion, however, the high values for stainless steel colonisation need to be further investigated.

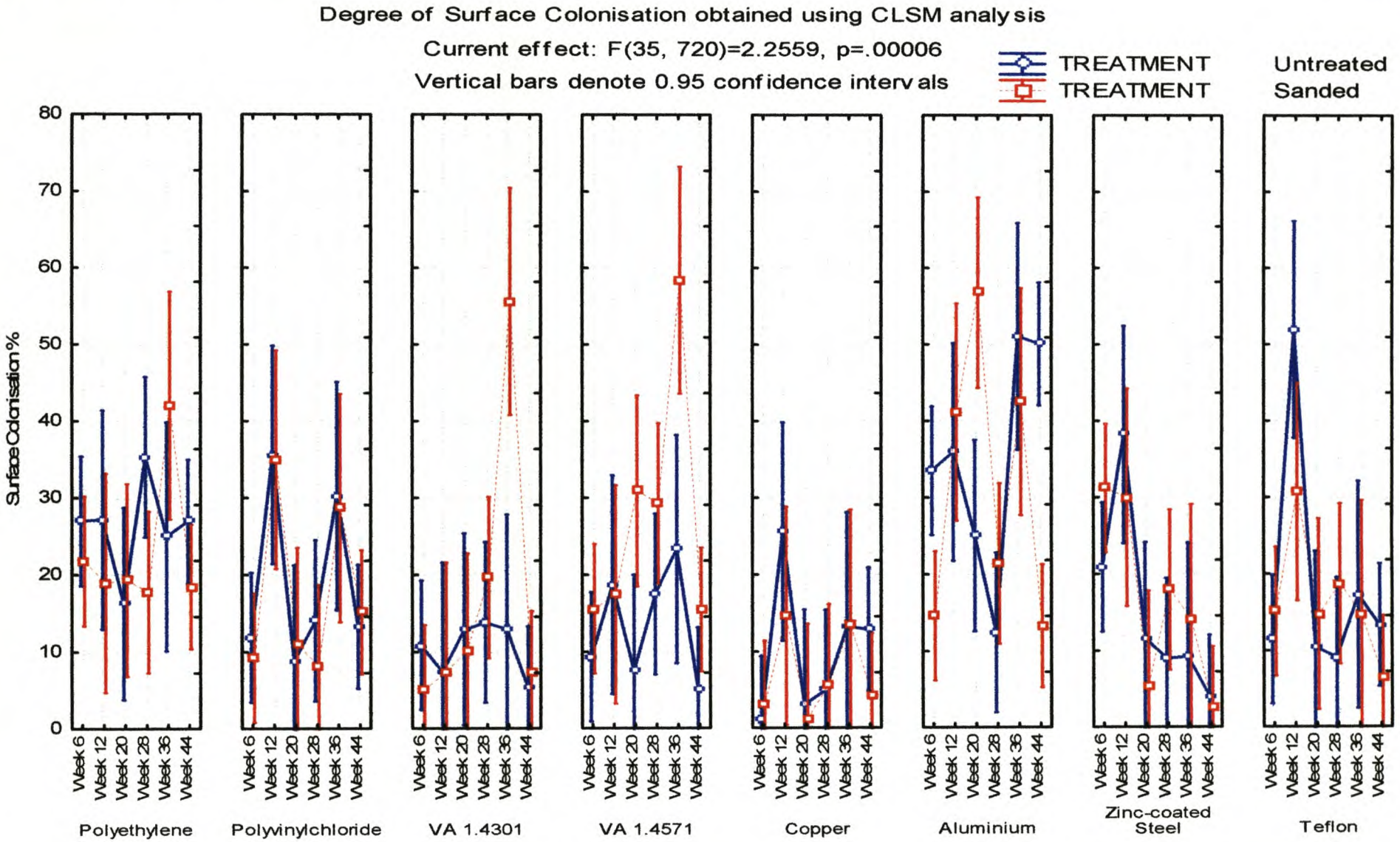


Figure 3.10 Surface colonisation percentages obtained using CLSM analysis for material coupons in a flow-cell system exposed to activated carbon treated water and laminar flow conditions for 6, 12, 20, 28, 36 and 44 weeks

Comparison of the results **[Figure 3.3 (A-C) and Figure 3.10]** obtained for turbulent and laminar flow, and surface treatment when exposed to activated carbon treated water, shows that flow conditions and surface treatment have a direct influence on surface colonisation over extended periods of time. Furthermore, differences in surface colonisation percentages obtained on the materials investigated, establish that the composition of the material may have a direct influence on microbial colonisation.

3.2.3 Secondary Colonisation:

The coupons successively introduced at each sampling time to determine the influence of an adapted microbial community on the materials exhibited a more stable colonisation pattern over time [**Addendum (a-j)**]. A comparison of the results obtained for primary colonisation versus secondary colonisation for the untreated materials showed a decrease ($p > 0.05$) in surface colonisation for the actively corroding (copper, aluminium and zinc-coated steel) materials, during secondary colonisation. When compared to the results obtained for primary colonisation (**Figure 3.6**), the surface coverage for the two grades of stainless steel namely VA 1.4301 and VA 1.4571 was also distinctly lower ($p < 0.05$) for secondary colonisation [**Addendum (a-j)**].

In contrast to the decreased colonisation of the test surfaces, a distinct increase ($p < 0.05$) in the number of viable (as determined by ATP analysis) and culturable (as determined by heterotrophic plate counts) cells was observed for most of the materials irrespective of parameter investigated [**Addendum (a-j)**]. This result could indicate that the population had adapted to the materials evaluated, and that the adapted microbial communities consisted of proportionally more viable cells. Furthermore, changes in heterotrophic plate counts and ATP concentrations indicate that the biofilm had not reached a steady state by the last sampling time (week 36). The question of how long the biofilm takes to reach a steady state, in these and other oligotrophic environments, then arises, as most studies observe biofilms over much shorter time periods (Easton & Pitchers, 1995; Hallam, 1992; Hallam *et al.,* 2001).

The influence of surface treatment on the colonisation in an adapted environment proved to be more material specific when compared to the results obtained for primary colonisation. While sanding of plexiglass and teflon resulted in

an increase of the degree of surface colonisation, the differences previously observed for copper and VA 1.4571 during primary colonisation were less distinct ($p > 0.05$), pointing to an increased ability of the biofilm population to adapt to these surfaces as well as the chemical changes during the corrosion process. Overall comparison of the results obtained for primary colonisation **(Figure 3.6)** and secondary colonisation **[Addendum (a-j)]** indicate that the different materials themselves exhibit a significant effect ($p < 0.05$) on surface colonisation and cell viability.

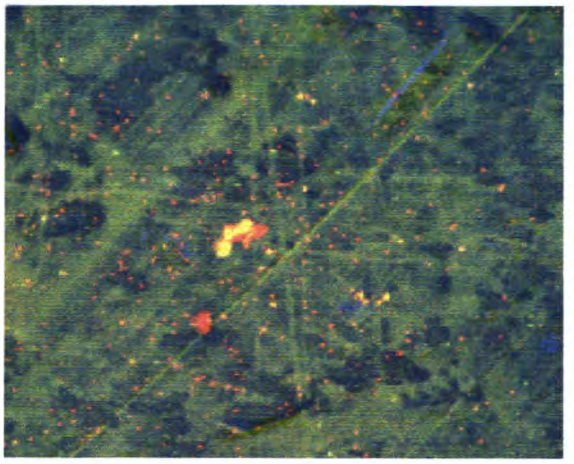
3.2.4 Imaging of Biofilm Structure:

The structure of the biofilms on the surface of the various materials examined was obtained by combining the fluorescence live/dead ratio data obtained with the Live/Dead Baclight™ stain with the reflection image (600 x magnification) of the surface of the material. Examples of the microbial colonisation patterns, for polyethylene, VA 1.4301 and zinc-coated steel to demonstrate the effect of sanding, flow conditions as well as untreated water and treated water, are represented in **Figure's 3.11 to 3.13**. In each case, the effect of surface modification by sanding of coupons, and subsequent exposure to turbulent flow and activated carbon treated water for 20 weeks are shown [**Figure 3.11 to 3.13 (A)**]. The effect of flow conditions [**Figure 3.11 to 3.13 (B)**] on the three materials, are for coupons exposed to activated carbon treated water for 20 weeks. The images of the effect of water composition [**Figure 3.11 to 3.13 (C)**] show coupons exposed to a turbulent flow for activated carbon treated water and ozonated water for 20 weeks.

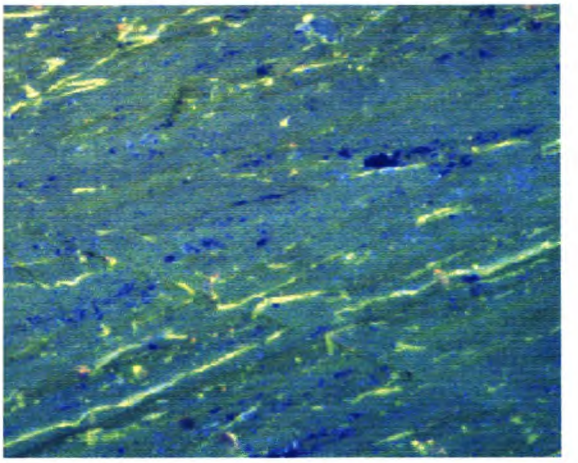
These examples provide a visual demonstration of the different behaviour of the biofilm communities on the respective materials. For instance, there was no significant difference ($p > 0.05$) between the direct acridine orange cell counts for PE and VA 1.4301 for turbulent flow. As mentioned previously the corrosion taking place on copper and zinc-coated steel interfered with the CLSM analysis as the results obtained for direct acridine orange cell count for these two materials were continually the highest ($p < 0.05$), while on average the CLSM surface coverage data for copper and zinc-coated steel analysis was extremely low ($p > 0.05$). In general, however, the CLSM images correspond well with the other data, and thus provided an internal control to verify the results obtained.

(A) Effects of Sanding on Polyethylene

Untreated Polyethylene, Coupon Surface

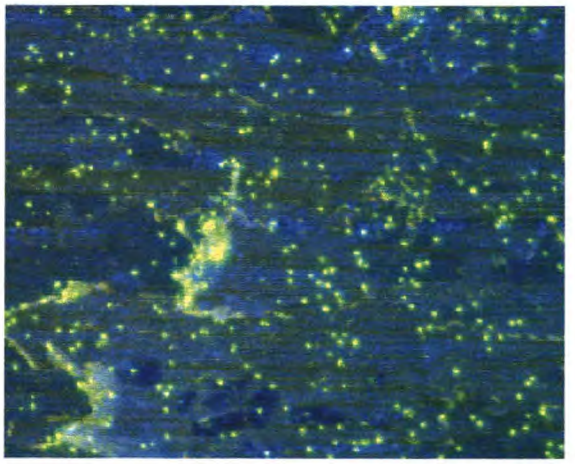


Sanded Polyethylene, Coupon Surface

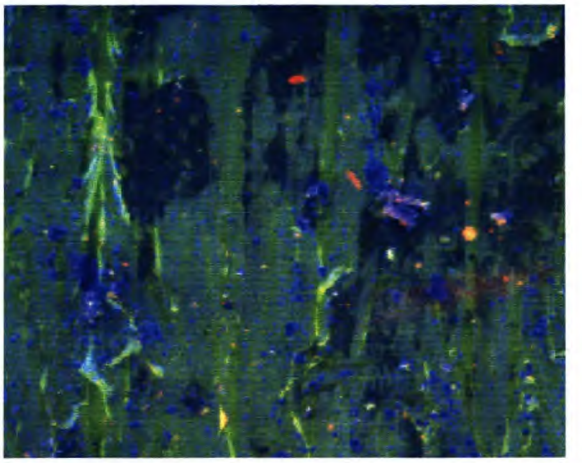


(B) Influence of Flow Conditions on Polyethylene

Sanded Polyethylene, Laminar Flow

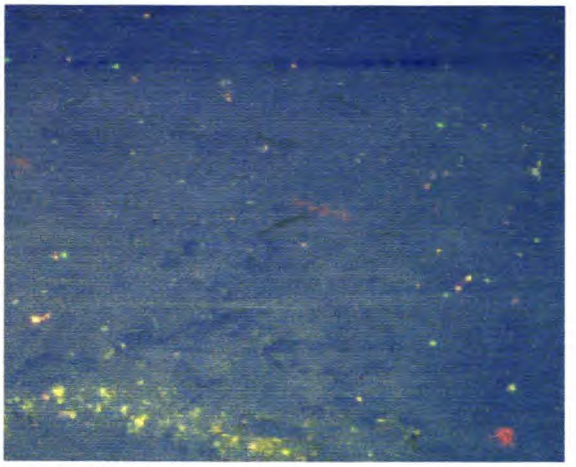


Sanded Polyethylene, Turbulent Flow



(C) Influence of Water Composition on Polyethylene

Untreated Polyethylene, Pure Water



Untreated Polyethylene, Ozonated Water

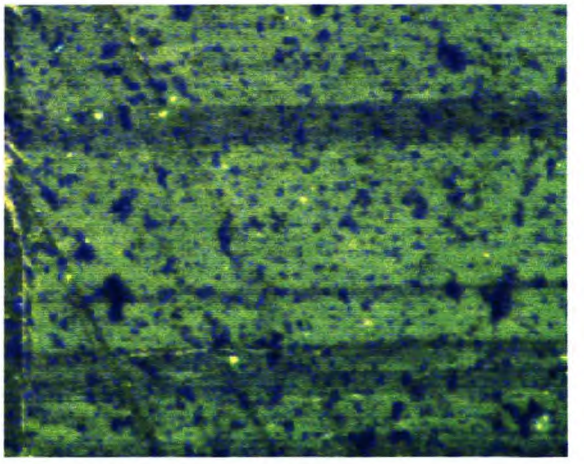
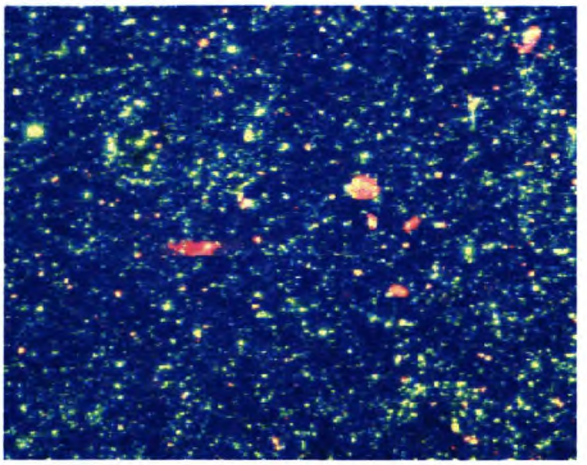


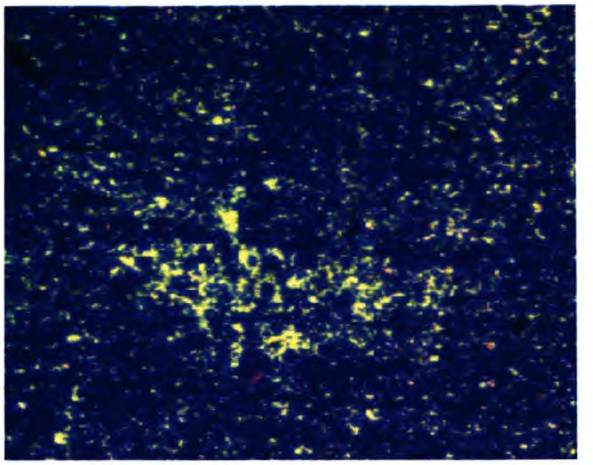
Figure 3.11 Presentation of CLSM images (600 x magnification) to determine the influence of (**A**) sanding (activated carbon treated water/ turbulent flow/20 weeks), (**B**) flow conditions (activated carbon treated water/20 weeks) and (**C**) water composition (turbulent flow/20 weeks) on Polyethylene.

(A) Effects of Sanding on VA 1.4301

Untreated VA 1.4301, Coupon Surface

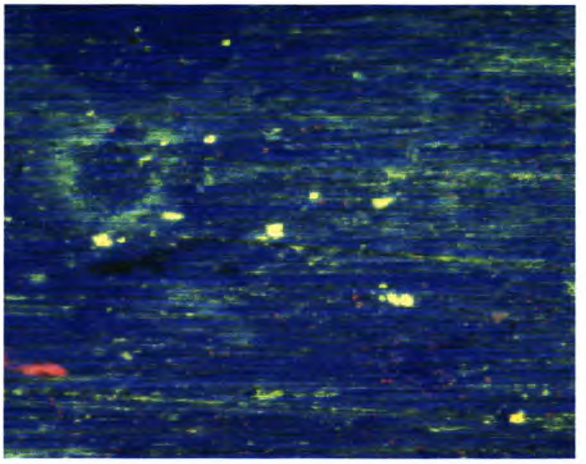


Sanded VA 1.4301, Coupon Surface

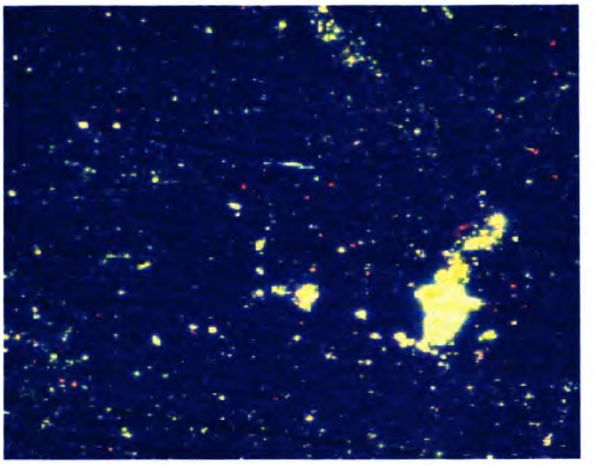


(B) Influence of Flow Conditions on VA 1.4301

Sanded VA 1.4301, Laminar Flow

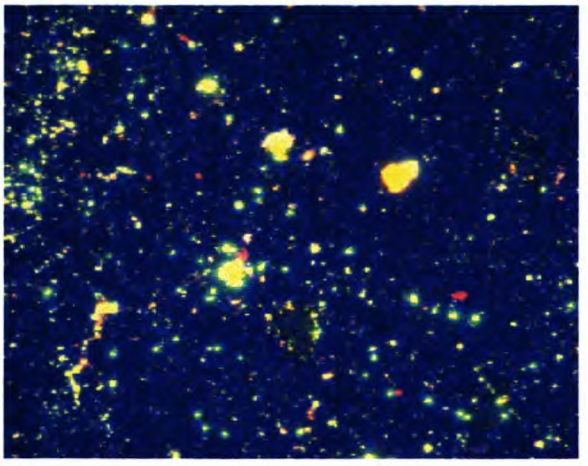


Sanded VA 1.4301, Turbulent Flow



(C) Influence of Water Composition on VA 1.4301

Untreated VA 1.4301, Pure Water



Untreated VA 1.4301, Ozonated Water

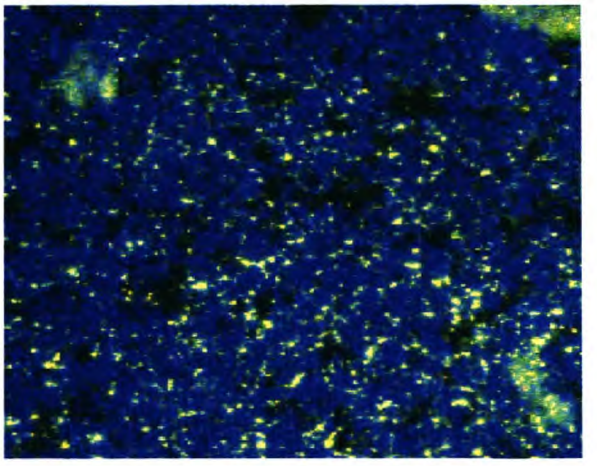
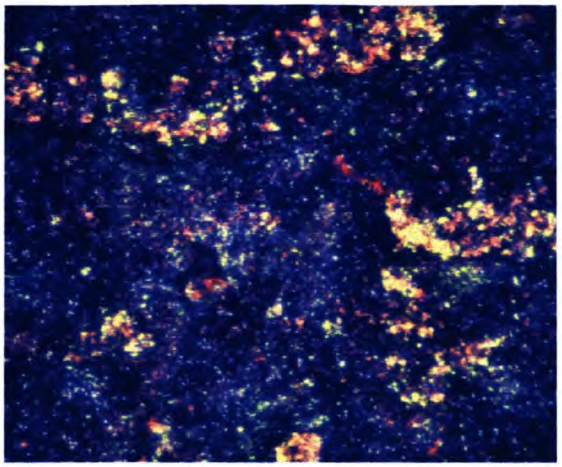


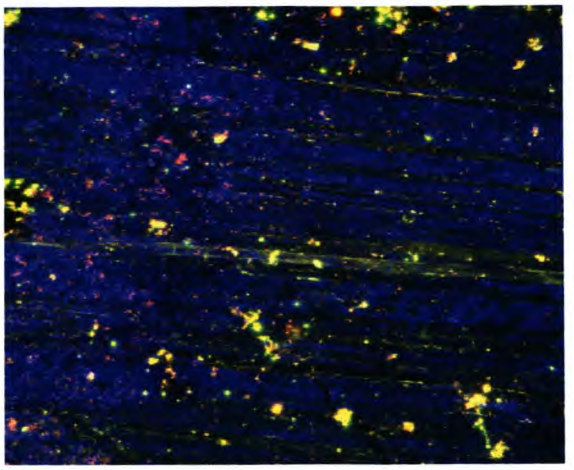
Figure 3.12 Presentation of CLSM images (600 x magnification) to determine the influence of (**A**) sanding (activated carbon treated water/ turbulent flow/20 weeks), (**B**) flow conditions (activated carbon treated water/20 weeks) and (**C**) water composition (turbulent flow/20 weeks) on VA 1.4301.

(A) Effects of Sanding on Zinc-coated Steel

· Untreated Zinc-coated Steel, Coupon Surface

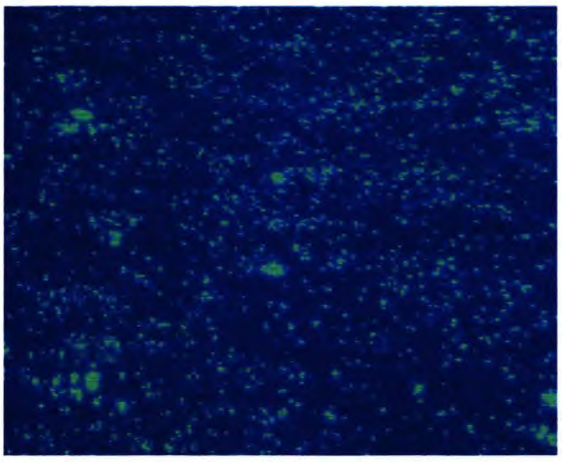


Sanded Zinc-coated Steel, Coupon Surface

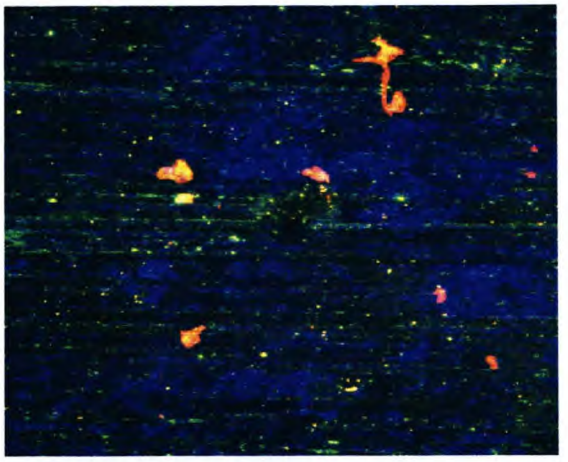


(B) Influence of Flow Conditions on Zinc-coated Steel

Sanded Zinc-coated Steel, Laminar Flow

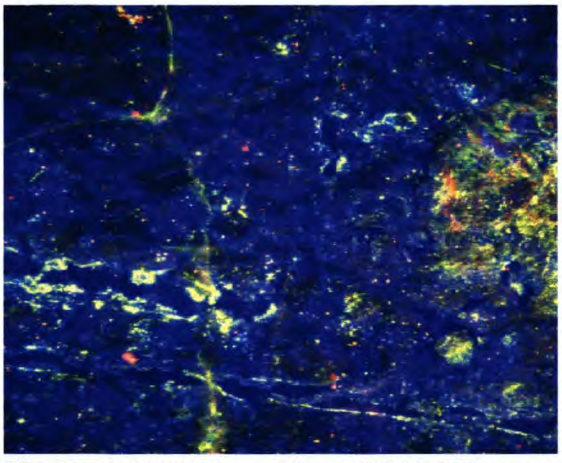


Sanded Zinc-coated Steel, Turbulent Flow



(C) Influence of Water Composition on Zinc-coated Steel

Untreated Zinc-coated Steel, Pure Water



Untreated Zinc-coated Steel, Ozonated Water

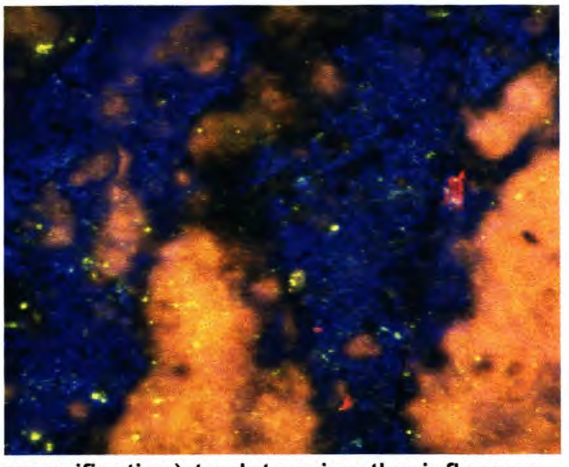


Figure 3.13 Presentation of CLSM images (600 x magnification) to determine the influence of (**A**) sanding (activated carbon treated water/ turbulent flow/20 weeks), (**B**) flow conditions (activated carbon treated water/20 weeks) and (**C**) water composition (turbulent flow/20 weeks) on Zinc-coated Steel.

3.2.5 Biolog™ MicroPlate analyses:

Biolog™ MicroPlate technology uses each microbial species ability to use a panel of carbon sources to produce a unique pattern or "fingerprint". The ECO MicroPlate technology used in the present study, contains three identical sets of 31 carbon sources, and is routinely used to monitor and study changes in microbial communities (Biolog, 1999). Usually, the microorganisms within any community cannot utilise all 31 carbon sources of the MicroPlate. The end result is a pattern of purple wells on the MicroPlate that is characteristic of that community.

The cluster analyses obtained, for the activated carbon treated water, ozonated raw water and drinking water material coupons over time are illustrated in **Figure 3.14 (a-c)**. Additionally, the influence of surface roughness on microbial colonisation was investigated in activated carbon treated water [**Figure 3.14 (c),** untreated coupons **(1-10)** and sanded coupons (**11-1010)**]. Overall the analysis of the Ecolog™ plates did not show strong correlation between the carbon utilisation profiles of the microbial communities on the different materials investigated irrespective of the water type (i.e. activated carbon treated water, filter water and drinking water) and the flow type they were exposed to. There were a few instances where meaningful clustering occurred. For example, analysis of the dendograms for drinking water for weeks 6, 28 and 36 [**Figure 3.14 (b**)] revealed that the microbial communities associated with the two materials glass and stainless steel VA 1.4301 had similar metabolic profiles. The other microbiological data (**Figure's 3.3 to 3.8**) showed that both these materials are not favourable for biofilm formation, indicating that the range of microorganisms colonising these materials might have been small.

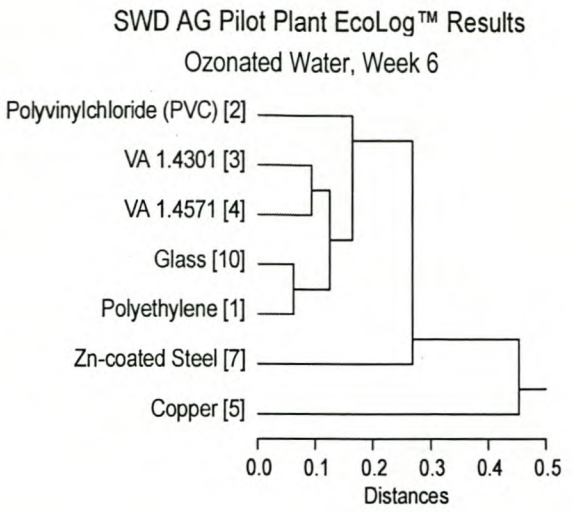
Furthermore, analysis of the dendograms for the surface treatment results (i.e. untreated materials versus sanding of the material to disrupt the surface) in the activated carbon treated water section exposed to a turbulent flow rate, initially showed no correlation in whole community metabolic profiles for any of the material investigated [**Figure 3.14 (c)**]. However, the untreated and sanded copper coupons had the same metabolic profile from week 20 to week 36, showing the strong selection pressure of copper on community composition. By week 28, VA 1.4571, zinc-coated steel and glass began to cluster together, with the sanded and untreated coupons yielding similar metabolic profiles. The last sampling time showed that sanded and untreated coupons for polyethylene, polyvinylchloride, copper, aluminium, zinc-coated steel and plexiglass grouped together, suggesting that with

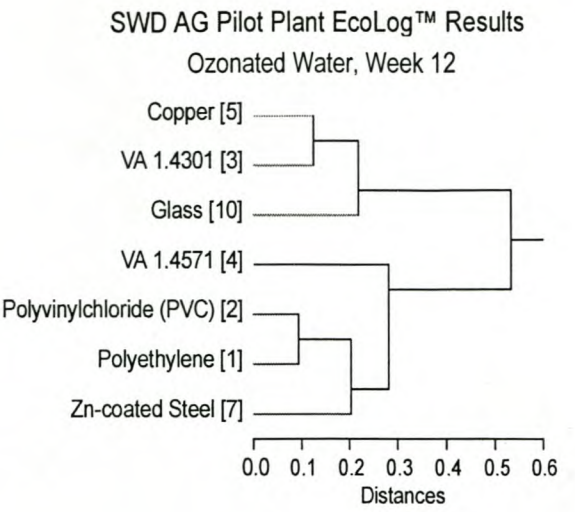
time the microbial communities on these materials had either become adapted to the respective surfaces or the mature biofilms concealed the surface of the material. It thus seems probable that, unlike the case of copper, other environmental factors than attachment material played a role in the composition of the microbial community growing on these materials.

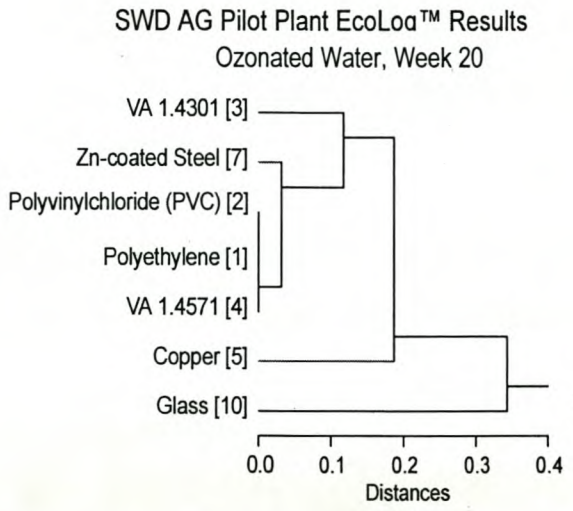
In contrast to the results for the biofilm monitors at the different locations in the distribution system where the microbial communities remained stable, there were notable changes in community composition associated with the different materials over time (data not shown). These results are not surprising considering the different parameters that the material coupons were exposed to namely, turbulent flow conditions, surface treatment or water obtained from different purification steps. Indeed, it is likely an indication that the different parameters investigated could have a direct influence on the microbial populations present on the different materials.

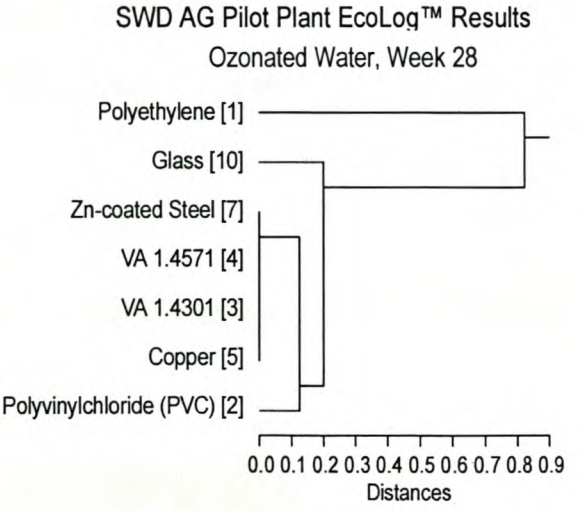
It should be pointed out that in the case of the Kiwa™ monitors the test material was glass. In the present study, untreated and sanded glass coupons began to cluster together at week 12 and then again by week 28 **[Figure 3.14 (c)]**, indicating that the glass may be a good test material to monitor biofilm formation over extended periods of time, as the composition of the material seemingly does not influence microbial colonisation. These results are then comparable to the results obtained with the Kiwa™ biofilm monitors, where it was found that the community profiles for each site remained stable for the duration of the experimentation period (data not shown).

These results show the complex nature of microbial communities, and how difficult it is to find a method to demonstrate community dynamics. It is obvious that no single method will suffice for this purpose. The Biolog™ data provided some support for the results obtained with the other techniques. However, to achieve a better description of these complex communities will require the incorporation of other techniques such as terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE). The application of these techniques was not within the scope of this project. Nevertheless, it should be pointed out that these techniques also have several limitations, including the challenge of extracting sufficient sample material from biofilms in oligotrophic environments. Furthermore, few water treatment plants have the facilities to routinely perform these analyses. In this regard, Biolog™ offers an exception.









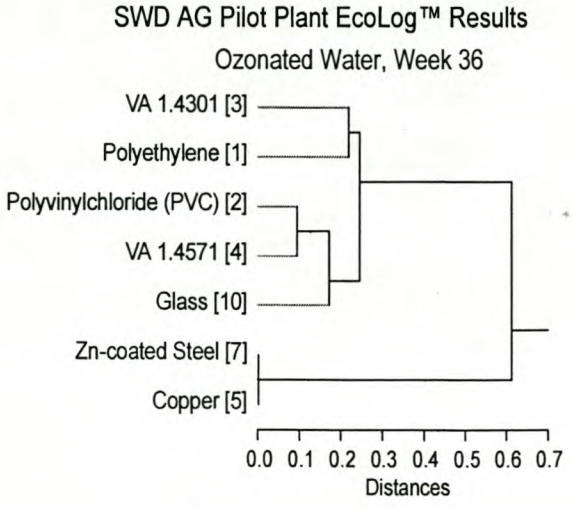
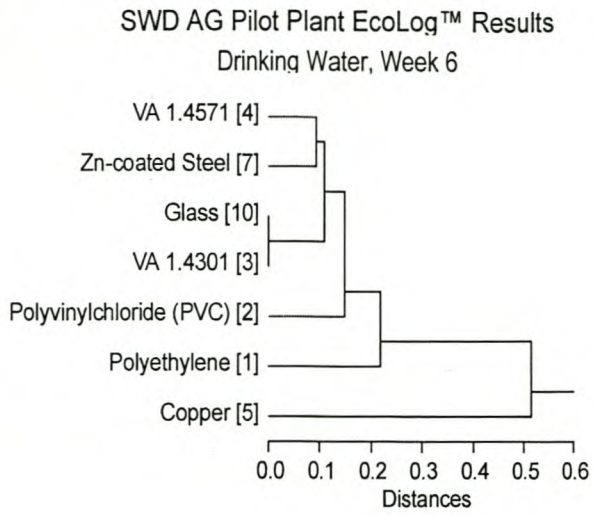
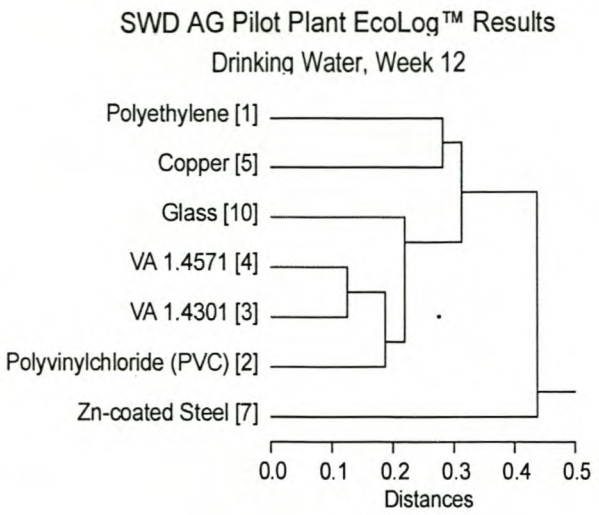
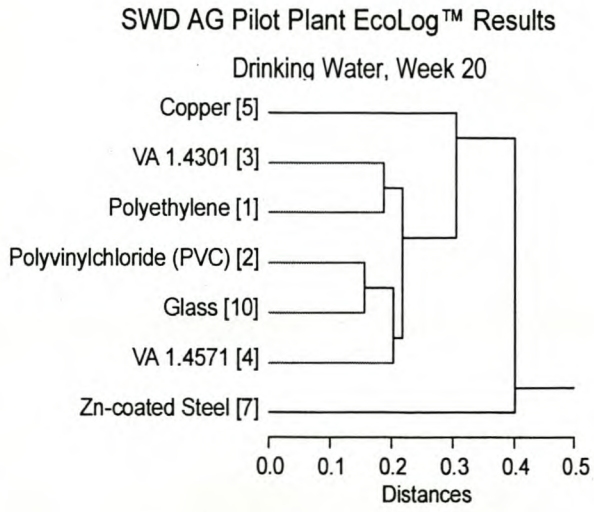
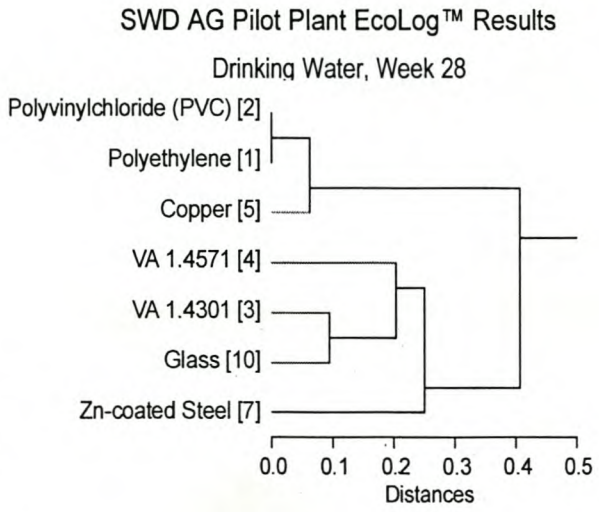


Figure 3.14 (a) Cluster analyses to compare whole community metabolic fingerprints of the microbial communities present on the seven different (untreated) materials placed under turbulent flow conditions in ozonated water for the periods of 6, 12, 20, 28 and 36 weeks.









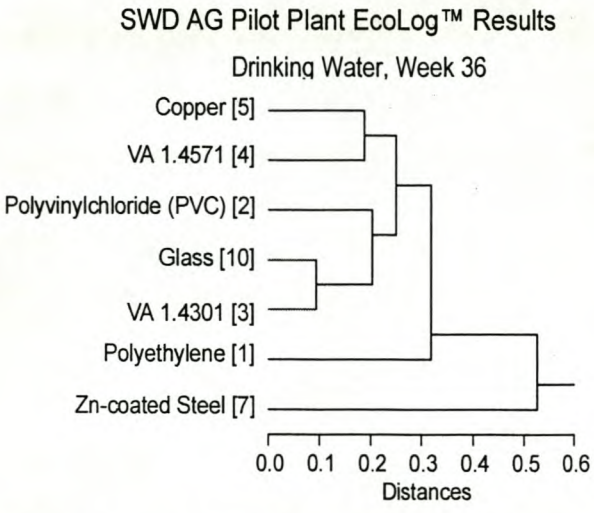
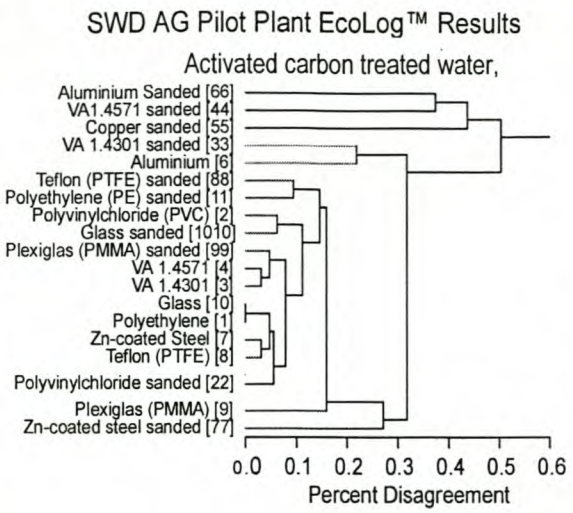
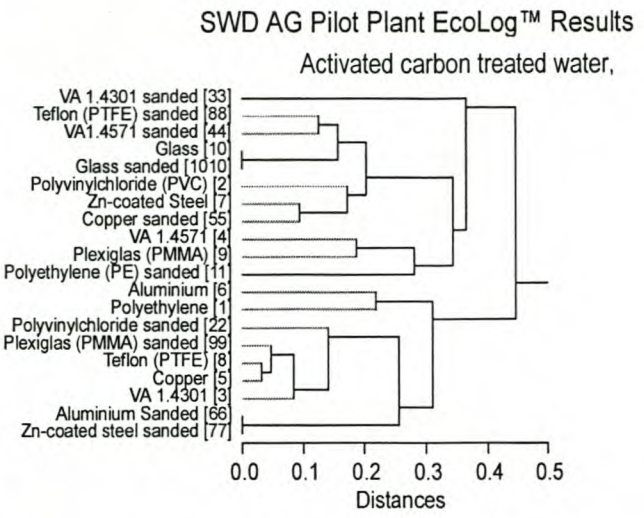
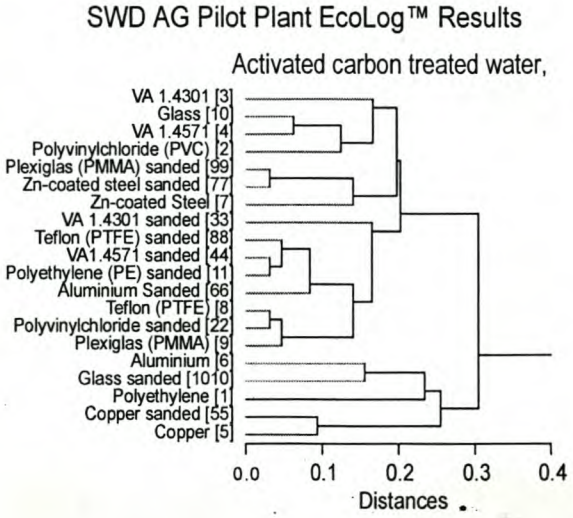
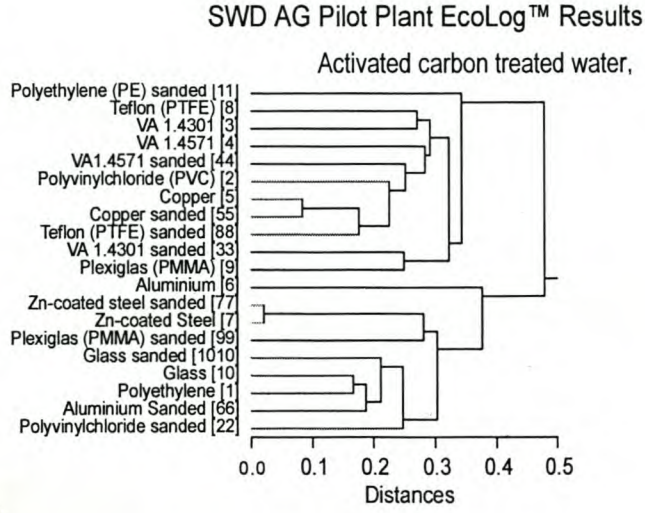


Figure 3.14 (b) Cluster analyses to compare whole community metabolic fingerprints of the microbial communities present on the seven different (untreated) materials placed under turbulent flow conditions in drinking water for the periods of 6, 12, 20, 28 and 36 weeks.









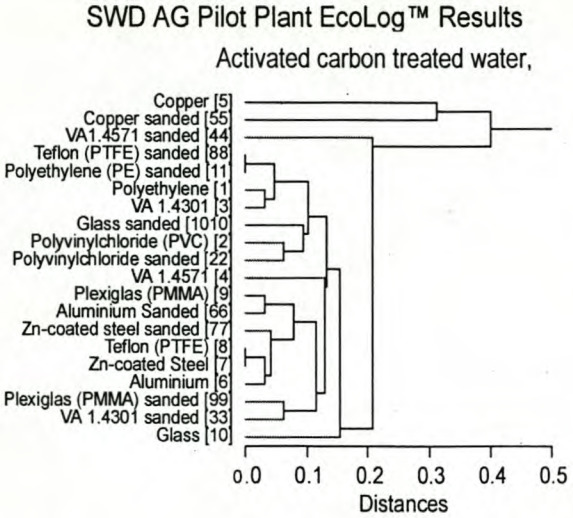


Figure 3.14 (c) Cluster analyses to compare whole community metabolic fingerprints of the microbial communities present on the ten different [untreated (1-10) and sanded(11-1010)] materials placed under turbulent flow conditions in activated carbon treated water for the periods of 6, 12, 20, 28 and 36 weeks.

3.3 EVALUATION OF OXIDISING BIOCIDES FOR THE CONTROL OF BIOFILMS IN THE TEST UNITS

Drinking water distribution systems are generally composed of a wide range of materials, which include concrete, asbestos cement, cast iron, PE, PVC, various grades of stainless steel, copper and zinc-coated steel. Irrespective of surface type, sessile microorganisms are to a large extent involved in not only the deterioration of these materials, but also in the re-introduction of planktonic microbes in disinfected water within the distribution system (Donlan & Pipes, 1988; Rogers *et al.,* 1994; Zacheus *et al.,* 2000). In this study, the efficiency of two disinfectants, sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) to kill and remove biofilm from piping material was investigated. This was done in view of a potential scenario that if a distribution system was to become heavily contaminated with microorganisms, such as in the case of technical failure or problems, it is essential to control the organisms, safely secure the system and minimise the spread of the contamination in as short a time period as possible. In this regard, the aim was thus to determine which concentration of disinfectant was the most effective in removing and killing the biofilms.

Preliminary experiments involved a laboratory scale flow-cell system to determine which concentration of comparison of sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) to employ in pilot plant experiments. The response of biofilms cultivated in the laboratory scale flow-cell system, when exposed to different concentrations of, revealed differences in disinfection efficiency by these two biocides. Irrespective of which concentration was used, NaOCl was effective in killing microorganisms present in the biofilm on polyethylene (PE) and glass, with a few viable bacteria still present on copper. In addition, NaOCl removed 50 – 95% of the biofilm mass from the PE, copper and glass coupons. The results for H_2O_2 were noticeably different. The only concentration of H_2O_2 that exhibited any noticeable effect was 100 g/l, with a 95% removal of the biofilm on copper observed. In contrast, hydrogen peroxide had no direct effect on the biofilms present on the PE and glass coupons. It was concluded from these results that sodium hypochlorite exhibited a more distinct disinfection effect on biofilms than hydrogen peroxide, irrespective of the test material.

Based on these results, NaOCl was applied at 50 mg/l (concentration used by water treatment plant Holthausen) and 200 mg/l, while H_2O_2 was applied at 0.15 g/l (concentration used by water treatment plant Holthausen), 15 g/l (maximum

concentration allowed by water treatment plant Holthausen) and 100 g/l, to the pilot plant system holding test coupons prepared from seven relevant materials.

The efficiency of these biocides at the concentrations (NaOCl 50 mg/l, and H_2O_2 0.15 g/l and 15 g/l) which are normally used by the water treatment plant Holthausen to disinfect the various components of the distribution and treatment system, was strongly influenced by the type of material as illustrated in **Figure 3.15 (a)** and (**b**). For example, 50 mg/l NaOCl was more effective on PE, VA 1.4301, zinc-coated steel and teflon, whereas, the maximum concentration of hydrogen peroxide (15 g/l) used by the water treatment plant was more effective against the biofilm present on PVC, VA 1.4571 and copper.

A presentation of CLSM images to determine the influence of the different disinfectant concentrations on VA 1.4301, copper and zinc-coated steel is presented in **Figure 3.16 (a-c)**. From the results obtained in **Section 3.2** it was clear that bacterial attachment to various surfaces is greatly influenced by the choice of material, flow conditions (laminar of turbulent water flow) and water quality. In general, the thickness and structure of the biofilms was strongly dependant on the material as well as parameters investigated. These results also show that the polymeric materials polyethylene and plolyvinylchloride, were the most favourable for biofilm formation irrespective of the water source they were exposed to. Similar results were observed in this study [**Figure 3.15 (a)** and **(b)**] for the respective control channels of these materials. Evaluation of the disinfection efficiency of these materials nevertheless revealed that at the concentrations generally used by the waterworks Holthausen to disinfect piping material, sodium hypochlorite (50 mg/l) was more effective on PE, while hydrogen peroxide (0.15 and 15 g/l) was more effective in removing and killing the biofilms present on PVC.

From **Section 3.2** it was also concluded that the biofilm growth and the surface coverage percentages were the lowest for the two grades of stainless steel (VA 1.4301 and VA 1.4571), irrespective of conditions investigated. Analysis of the control channels for these two materials [**Figure 3.15 (a)** and **(b)** revealed similar results. However, evaluating the efficiency of the standard disinfectant concentrations used by the waterworks revealed that sodium hypochlorite (50 mg/l) was more effective in removing the biofilm from VA 1.4301, whereas hydrogen peroxide (15 g/l) was more effective against biofilm growth on VA 1.4571.

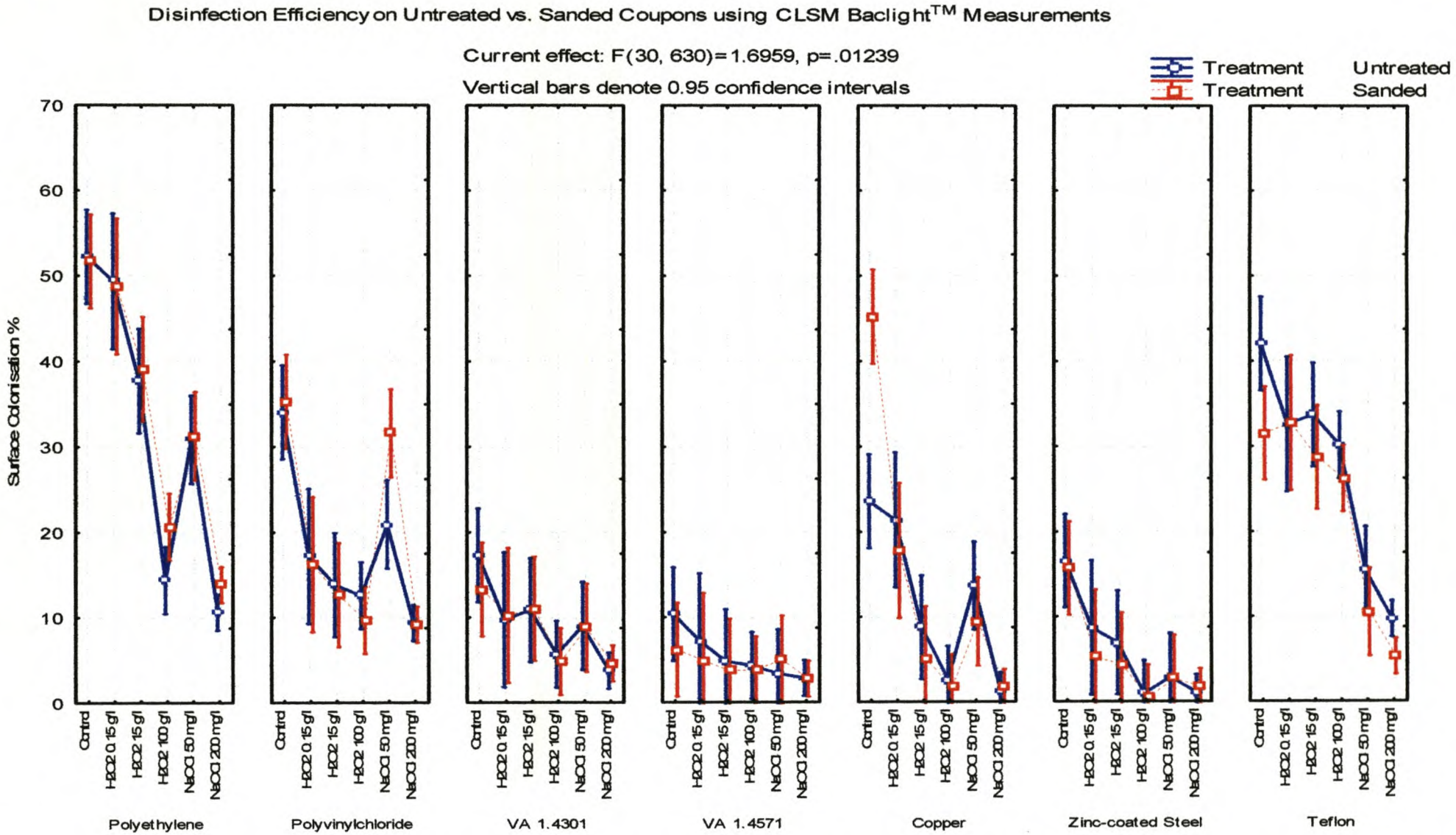


Figure 3.15 (a) Disinfection efficiency of sodium hypochlorite and hydrogen peroxide on biofilms growing in activated carbon treated water on different untreated and sanded material coupons to show the relationship between disinfectant concentration, surface treatment and type of material

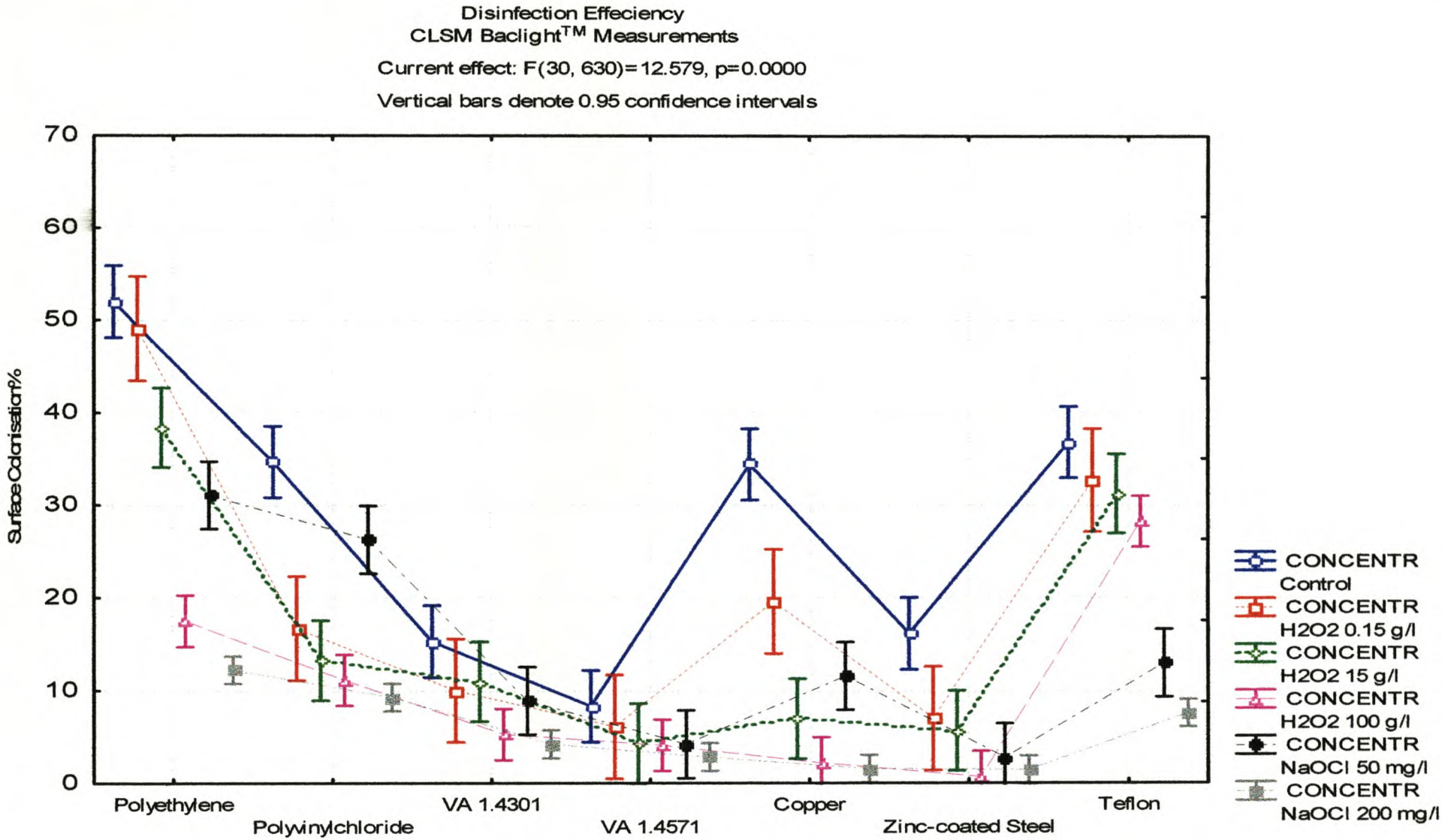


Figure 3.15 (b) Average disinfection efficiency of sodium hypochlorite and hydrogen peroxide of biofilms growing in activated carbon treated water on the respective material coupons to show the relationship between disinfectant concentration and type of material

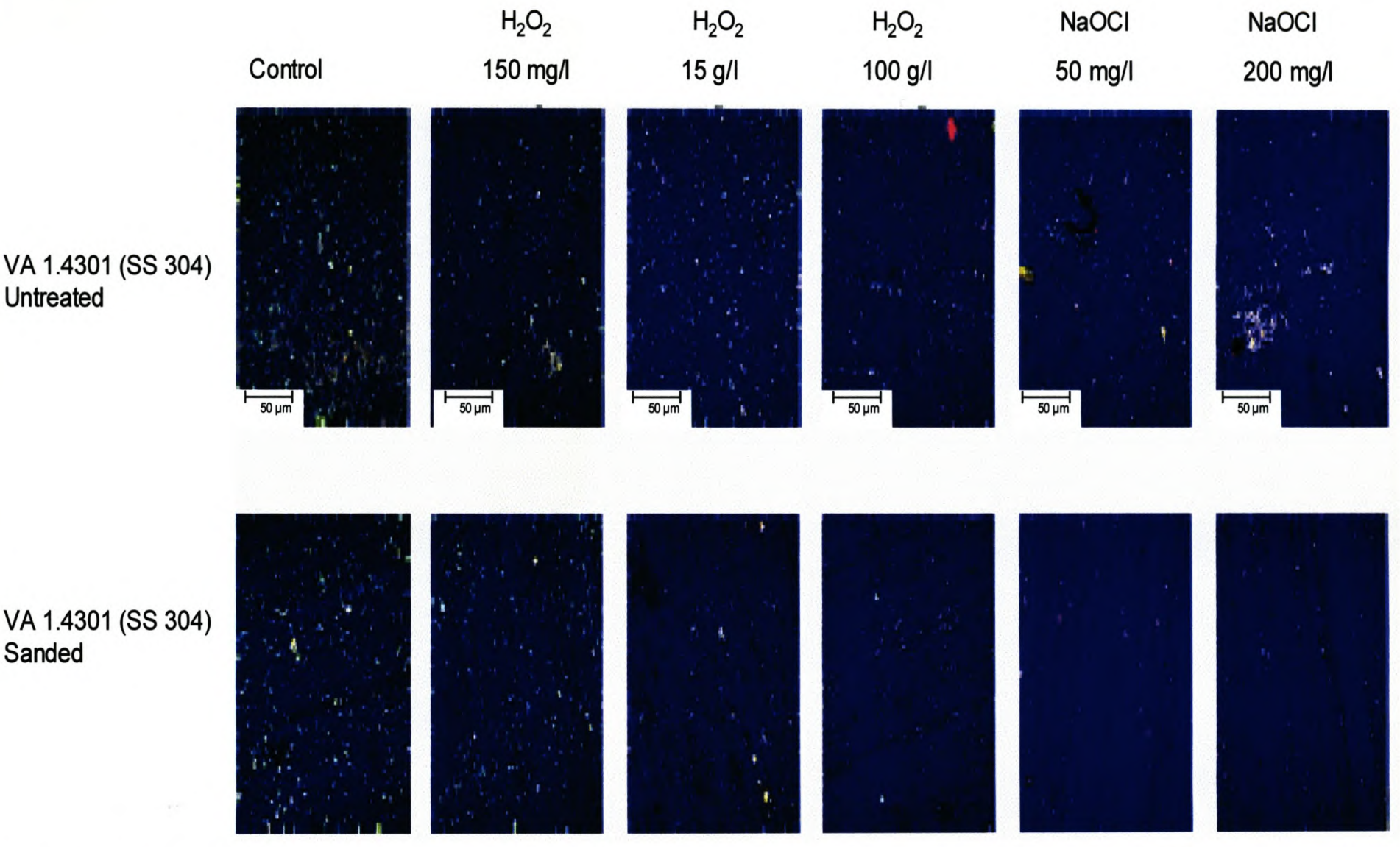


Figure 3.16 (a) Presentation of CLSM images to determine the disinfection efficiency of activated carbon treated water biofilms on untreated and sanded VA 1.4301 material coupons

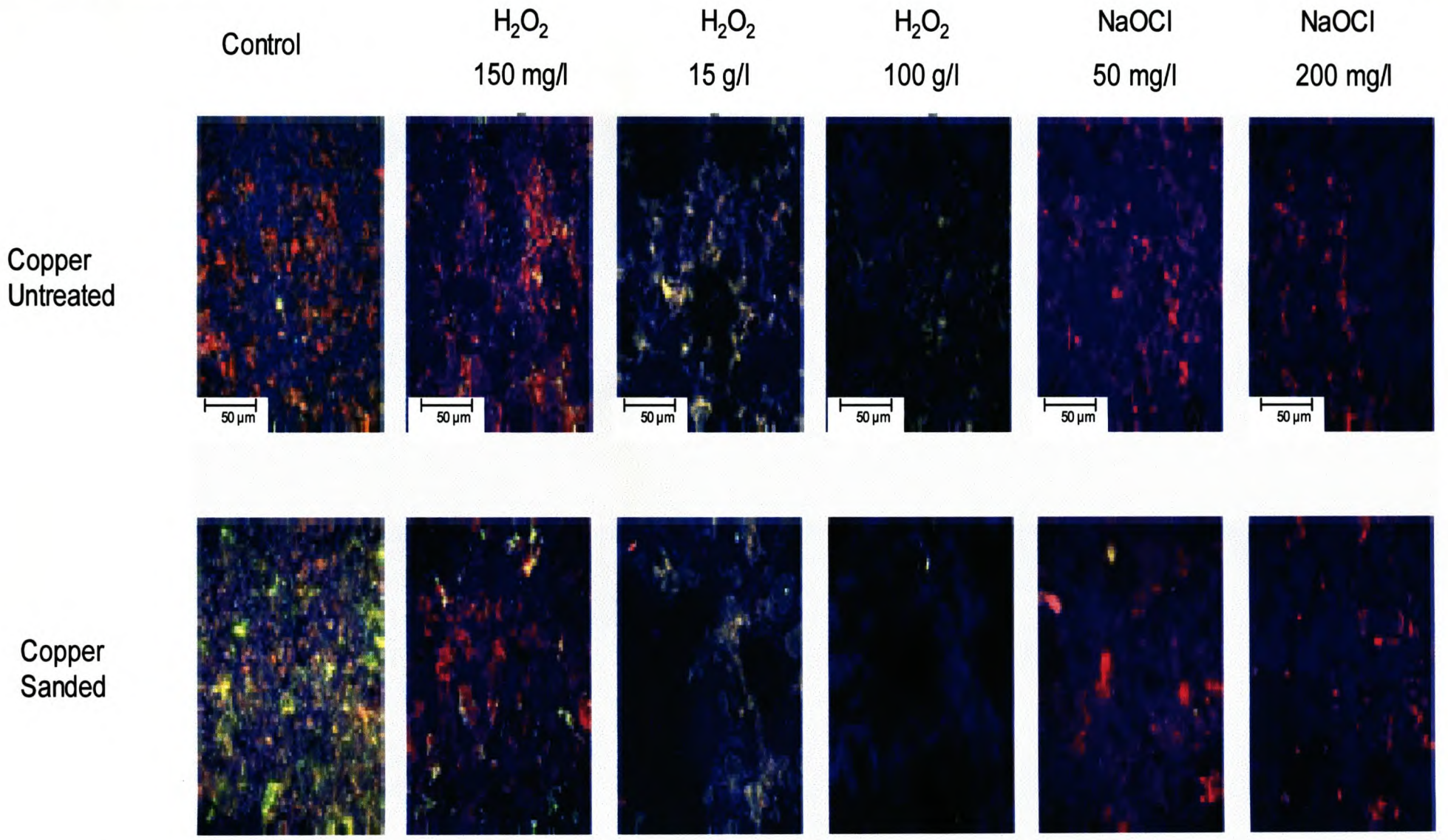


Figure 3.16 (b) Presentation of CLSM images to determine the disinfection efficiency of activated carbon treated water biofilms on untreated and sanded Copper material coupons

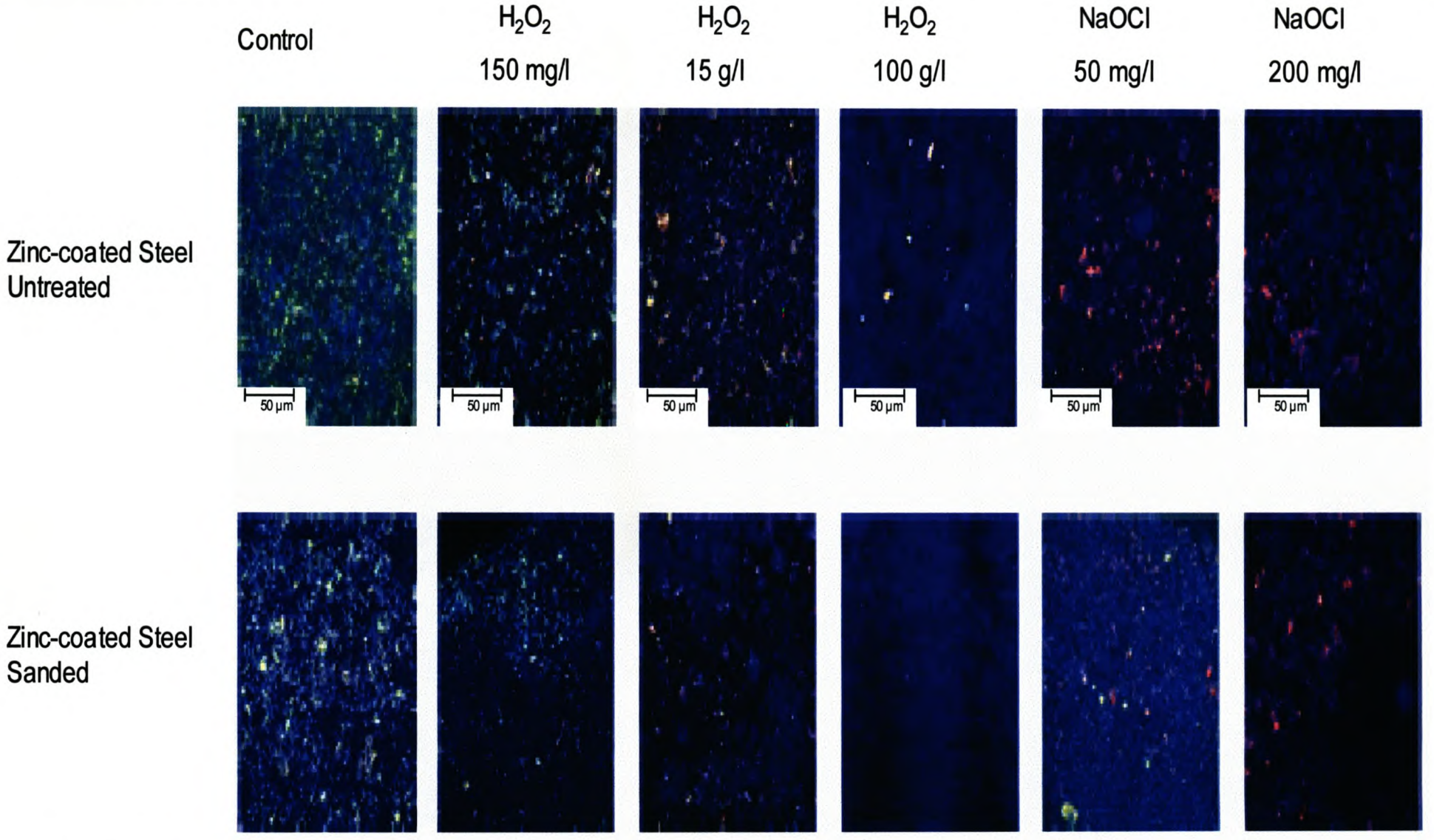


Figure 3.16 (c) Presentation of CLSM images to determine the disinfection efficiency of activated carbon treated water biofilms on untreated and sanded Zinc-coated Steel material coupons

Even though sodium hypochlorite (50 mg/l) proved to be the most effective disinfectant against the microbial accumulation on the polymeric material teflon, from **Figure 3.15 (a)** and **(b)** it is clear that the removal of the biofilm from this material proved to be the most difficult, at all the disinfectant concentrations in this study.

Analyses of the results for the maximum concentrations of sodium hypochlorite (200 mg/l) and hydrogen peroxide (100 g/l) tested, showed that the disinfection efficiency of the maximum concentrations on the different materials varied from the results obtained for the concentrations used by waterworks Holthausen. For example, of the concentrations generally used by the waterworks Holthausen to disinfect piping material, sodium hypochlorite (50 mg/l) was more effective on PE, VA 1.4301, zinc-coated steel and teflon, while hydrogen peroxide (0.15 and 15 g/l) was more effective in removing and killing the biofilms present on PVC, VA 1.4571 and copper. However, as illustrated in **Figure 3.15 (a)** and **(b)**, the maximum concentration of NaOCl (200 mg/l) tested yielded a 45-95% killing and removal efficiency of the biofilm mass present on PE, VA 1.4301 and teflon as well as on PVC, VA 1.4571 and copper, whereas the maximum concentration of H_2O_2 (100 g/l) tested yielded a 50-90% killing and removal efficiency on zinc-coated steel.

Additionally, the materials analysed in the activated carbon treated water section of the pilot plant were either used as obtained from the manufacturer or sanded (600 grid) to determine the influence of surface roughness and damaged passivation layers on biofilm formation. Analysis of the results obtained for disinfection efficiency on the untreated and sanded material coupons revealed that no significant differences ($p > 0.05$) in disinfection efficiency of the biofilm was observed for the untreated materials versus the sanded materials as illustrated in **Figure 3.17**.

Results from **Section 3.2** revealed that corrosion of copper and zinc-coated steel interfered with the CLSM analysis, as the results obtained for total cell count, colony forming units as well as the BFR and BFP for these two materials were continually the highest ($p < 0.05$), indicating that copper and zinc-coated steel are favourable for biofilm formation. The question then arises as to whether the normal shear and erosion of zinc-coated steel and copper ultimately result in a similar surface as those after sanding. The material coupons were placed in activated carbon treated water for a period of 14 months. At the time of sampling the copper and zinc-coated steel coupons were actively corroding. For both biocides tested, similar results were obtained, both for killing and removal percentages, of biofilm

results support the theory that sanding of the material resulted in a similar surface as a corroded untreated material.

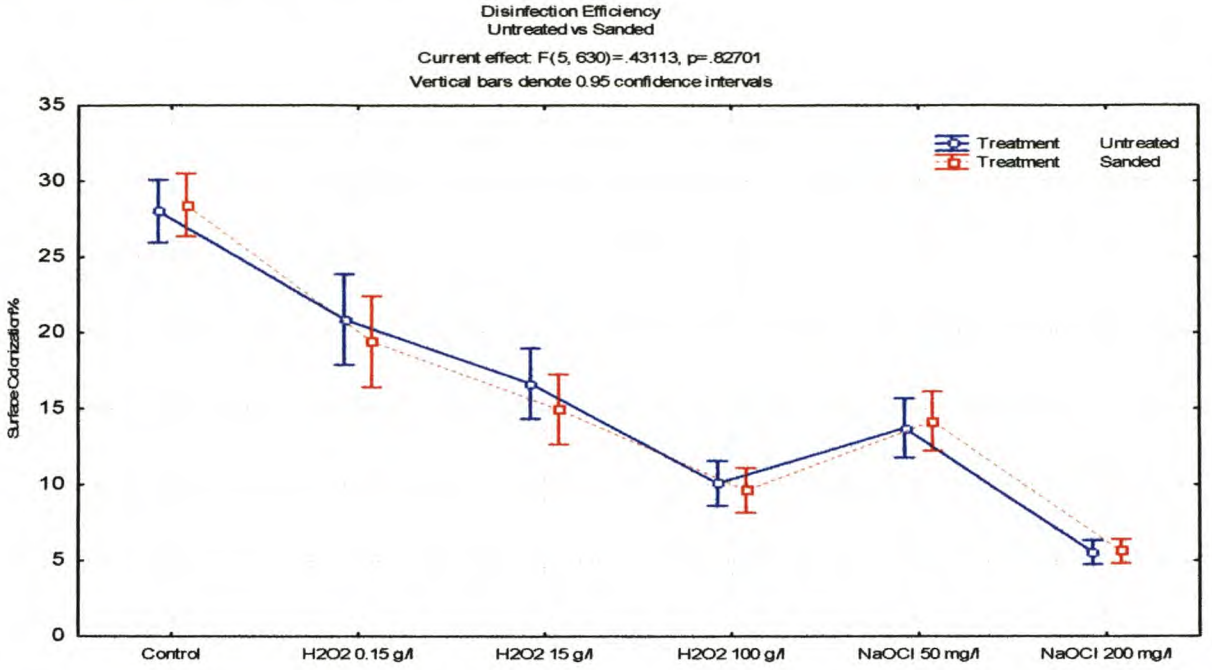


Figure 3.17 Influence of surface modification (sanding) on disinfection efficiency by various concentrations of sodium hypochlorite (NaOCl) and hygrogen peroxide (H_2O_2)

A summary of results obtained for killing (using Baclight™ Live/Dead stain and CLSM) and removal efficiency percentages of biofilm mass with the different concentrations of disinfectants used is presented in **Table 3.5.**

Table 3.5 The average killing and removal efficiency percentages of the biofilm mass on all materials tested, with the different concentrations of disinfectants used.

Disinfectact Concentration	Untreated Material Coupons		Sanded Material Coupons	
	Killing %	Removal %	Killing %	Removal %
Control	0	0	0	0
*H_2O_2 0,15 g/l	34,03 (9.92)	29,75 (20.24)	26,69 (8.77)	32,39 (17.68)
*H_2O_2 15 g/l	54,27 (6.5)	45,53 (16.93)	52,38 (6.19)	45,05 (14.34)
H_2O_2 100 g/l	74,11 (3.35)	67,55 (11.64)	67,34 (3.82)	62,31 (10.60)
*NaOCl 50 mg/l	68,80 (5.53)	51,44 (11.66)	66,57 (6.95)	50,48 (14.20)
NaOCl 200 mg/l	90,54 (1.46)	81,08 (5.31)	86,95 (1.49)	76,39 (5.02)

*- Concentrations used by the water treatment plant Holthausen for disinfecting piping and treatment components in the distribution system

Values in brackets show standard deviation

Viability percentages were obtained using the Baclight™ Live/Dead stain and CLSM **[Figure 3.18 (a)** and **(b)]**. A difference in viability percentages for untreated and sanded coupons of PE and PVC was revealed, with 100 g/l H_2O_2 yielding a greater killing efficiency of biofilms present on untreated PE than on sanded PE and 50 mg/l NaOCl yielding a greater killing efficiency of biofilms present on untreated PVC than on sanded PVC [**Figure 3.18 (a)**]. Furthermore, of the standard disinfectant concentrations used by the waterworks sodium hypochlorite (50 mg/l) was more effective in removing the biofilm from VA 1.4301, whereas hydrogen peroxide (15 g/l) was more effective against biofilm growth on VA 1.4571, revealing that results obtained for one material, even similar types of materials, cannot be used as a basis or standard for another material.

Of the concentrations presently used by the waterworks to disinfect various components of the distribution network, 50 mg/l yielded the highest killing (69%) and removal (91%) percentages on untreated and sanded coupons (**Table 3.5**). The overall analysis of killing efficiency results, however, revealed that 200 mg/l NaOCl yielded the lowest viability percentages (between 0 and 3%) on all materials tested, while the maximum concentration of hydrogen peroxide (100 g/l) tested yielded a 0 to 8% viability percentage **[Figure 3.18 (b)]**. Even though results revealed that the higher concentration of sodium hypochlorite (200 mg/l) was the most efficient in removing and killing the biofilm, high concentrations of chlorine can react with organic matter to form chloroform and trihalomethanes (Allonier *et al.,* 1999). Based on this it is recommended that the initial chlorine dose be based on its influence on the organoleptic properties of the water as well as the potential formation of trihalomethanes.

Comparison of the concentrations evaluated revealed that 100 g/l concentration of H_2O_2 was the only concentration comparable in killing and removal efficiency to the concentrations of NaOCl analysed, with 200 mg/l of NaOCl in particular having the greatest removal efficiency of biofilm on PE, PVC, the two grades of stainless steel, copper and teflon and the greatest killing efficiency of the microorganisms present in the biofilm on all of the materials analysed. The efficiency of a specific biocide concentration on the biofilm attached to the different materials is greatly dependant on the properties of the material itself, which in turn is related to factors such as the biofilm structure, thickness as well as the microbial types present.

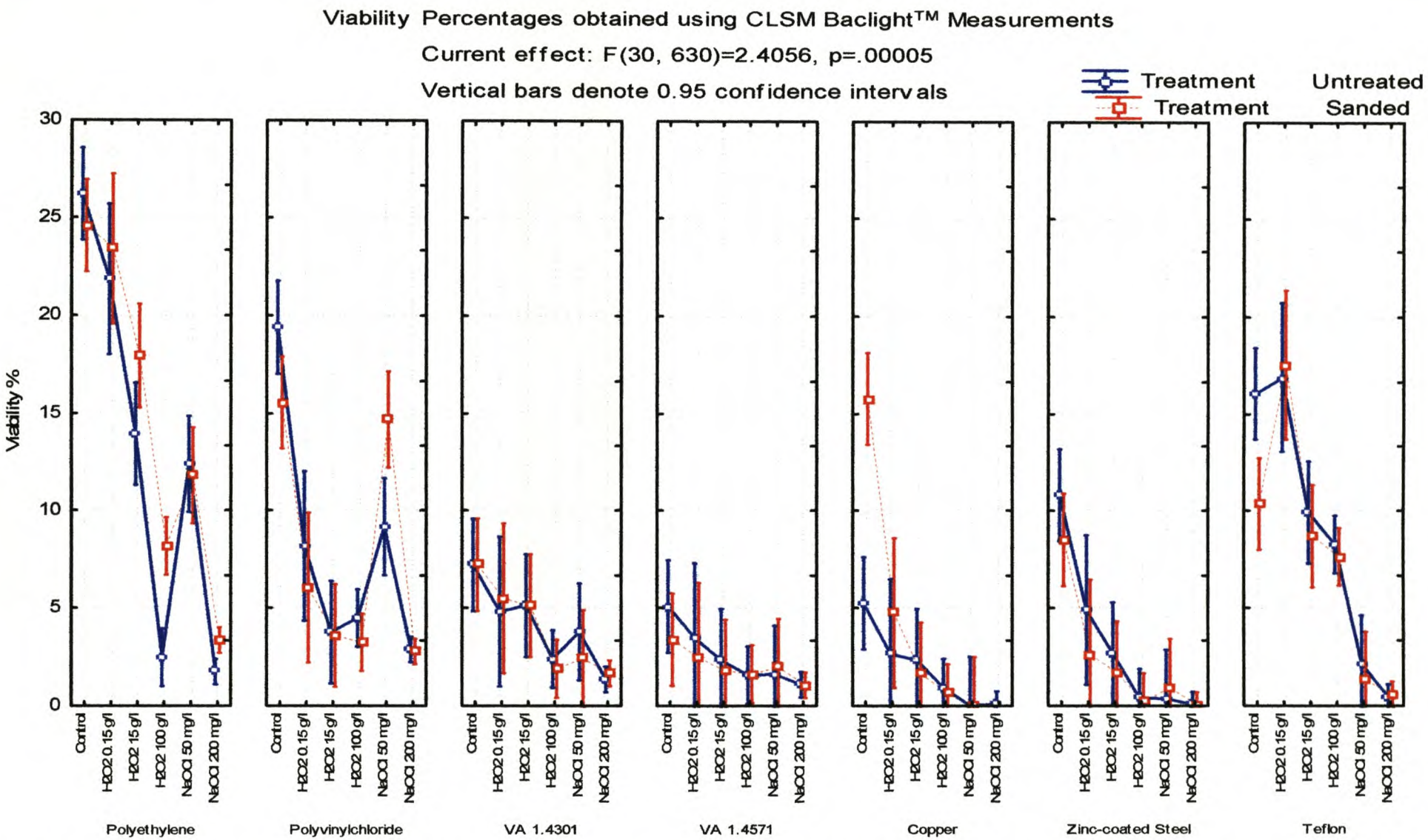


Figure 3.18 (a) Killing efficiency of sodium hypochlorite and hydrogen peroxide on biofilms growing in activated carbon treated water on untreated and sanded coupons to show the relationship between disinfectant concentration, surface treatment and type of material

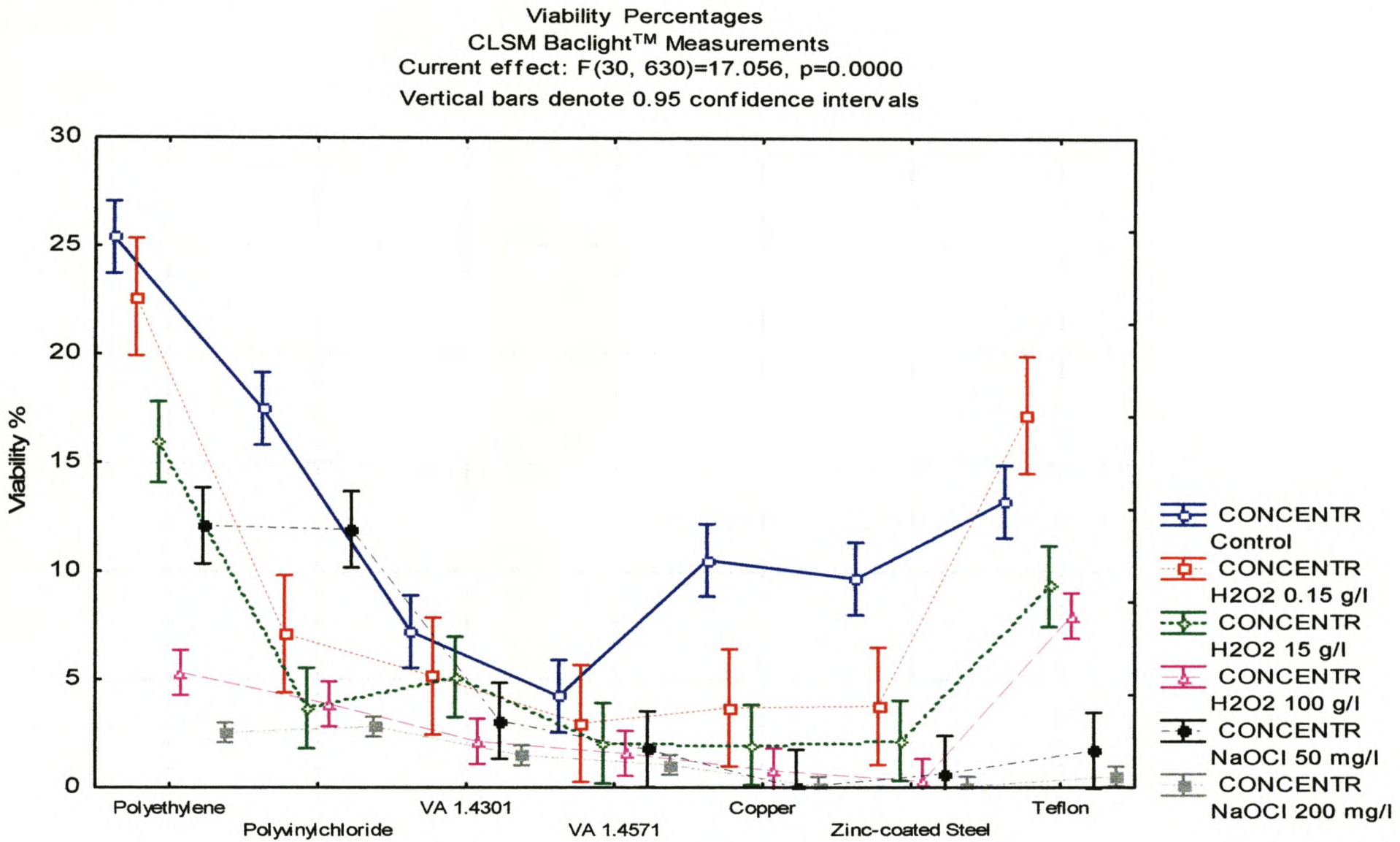


Figure 3.18 (b) Average killing efficiency of sodium hypochlorite and hydrogen peroxide on biofilms growing in activated carbon treated water on different materials to show the relationship between disinfectant concentration and type of material

4. GENERAL CONCLUSIONS

4.1 EVALUATION OF BIOFILM FORMATION CHARACTERISTICS AND BIOLOGICAL STABILITY OF DRINKING WATER AND PRACTICAL CONSIDERATIONS

The Duesseldorf water treatment process encompasses the application of ozonation and granular activated carbon (GAC) filtration on river bank- and ground- water sources. These treatment procedures provide potable water that is perceived not favourable for the growth and development of microorganisms. However, it may be possible that certain conditions, such as the regrowth of microorganisms or the presence of undesirable disinfection by-products, could result in potable water being in noncompliance with drinking water quality criteria. A programme to monitor the biofilm formation characteristics, as well as the biological stability of the drinking water in Duesseldorf was implemented by the Stadtwerke Duesseldorf AG, Germany by installing Kiwa™ biofilm monitors at the water treatment plant Holthausen (site 1) to a distribution line containing water prior to addition of chlorine dioxide, phosphate and silicate, and at four locations to lines containing potable water in the distribution network, namely; Färberstrasse (Site 2; FÄR) reservoir Hardt (Site 3; HARDT), reservoir Sandberg (Site 4; SAND) and the pumping station Gau (Site 5; GAU).

The heterotrophic plate count, total cell count and BFP for site 4 was generally of the lowest due to trouble maintaining a constant flow rate (possibly due to construction) of 270 l/h. However, the results obtained for heterotrophic plate count and total cell count for the sites 2 and 3 compared well with the results obtained for the biofilm monitor attached at the waterworks at site 1, which suggests that for the present study the addition of the supplements phosphate and silicate to the oligotrophic water system did not adversely affect the water quality. The water supply to the waterworks at site 1 and the sites 2, 3 and 4 is generally composed of 80% bank filtrated water and 20% groundwater, whereas site 5 has a pure groundwater source. This could clarify why the biofilm monitor installed at site 5 generated an increased BFR and BFP. The results suggest that the determining factor influencing biofilm formation in the distribution system evaluated in this study is limited nutrient availability and not temperature.

Data obtained for the contribution of the heterotrophic colony count [**Figure 3.1** (**B**)] to the amount of active biomass as determined with cell numbers based on

ATP measurements [**Figure 3.1** (**A**)] showed that on average the culturable bacteria represented only a fraction (< 3%) of the active microorganisms present in the biomass suspension samples. Even though new techniques to monitor drinking water quality are being developed (Fewtrell & Bartram, 2001), the classic drinking water monitoring techniques employed by many water treatment plants generally encompasses either the most probable number (MPN) and/or membrane filtration methods as the standard procedures to enumerate planktonic microorganisms present in drinking water samples. Results obtained emphasize the need for new biomass enumeration techniques to be employed in the routine drinking water monitoring. Furthermore, on average the heterotrophic plate count measurements represented < 0.8% of the direct acridine orange count [**Figure 3.1** (**C**)] recorded for the biomass removed from the glass rings, whereas the cell numbers based on ATP measurements represented < 82% of the direct acridine orange count (**Table 3.1**). The need for biofilm based monitoring techniques to be implemented at water treatment plants was further accentuated by this data, which corresponds to data obtained from previous investigations which showed that most of the microorganisms present in water systems are likely to be in biofilms attached to internal surfaces (Coghlan, 1996; Potera, 1996).

The results obtained in the present study compared well with the results of an earlier study of the water quality, conducted in 1997 at the water treatment plants Flehe and Holthausen situated in Duesseldorf (Vrouwenvelder *et al.*, 1997), suggesting that there was no significant variation in water quality at the water treatment plant over the 3 years since 1997 (**Table 3.2**). Furthermore, the installation of the Kiwa™ biofilm monitors at the water treatment plant at site 1 and to sections of the distribution network in and around the Duesseldorf area (sites 2-5) allowed for the investigation into the long-term stability and microbial behaviour not only at the water treatment plants, but also throughout the distribution system. It could be deduced that the biological stability and quality of the potable water remain constant from the water treatment plant, throughout the distribution system, to the consumer.

Results obtained in the present study and the study conducted by Vrouwenvelder *et al.* (1997) (especially the low BFR and AOC values) supported the hypothesis that the drinking water in the Duesseldorf area does not support notable biofilm formation. Moreover, by evaluating the results obtained for DOC, AOC and BFR in the present study and those obtained by Vrouwenvelder *et al.* (1997) and

Van der Kooij *et al.* (1994) it was concluded that low AOC and DOC values influence the BFR, irrespective of water type, and that a possible threshold value for AOC and DOC exists above which the BFR notably increases. This implies that even though a fixed acceptable BFR would be difficult to define for all water systems, for a given type of water system, such as the potable water distribution system in this study, it should be possible to indicate a BFR that would have a noticeable negative impact on the system, if a change in the existing conditions within the distribution system occurred. If difficulties in determining BFR occurred, indices such as AOC may then prove to be a good indirect measure of BFR, and thus biofouling potential in general. Although more data is necessary to accurately determine threshold values, the observations made in this study suggest that a DOC below ~0.5 mg C/l, and an AOC below ~5 µg C/l could be target values for the control of biofilm formation in drinking water systems.

4.2 BIOFILM FORMATION ON MATERIALS RELEVANT TO THE DRINKING WATER DISTRIBUTION INDUSTRY

An interesting question concerning bacterial attachment to surfaces relates to the effect of solid surfaces on bacterial activity. Even though it is known that the activity of the bacteria is enhanced at surfaces, particularly in low nutrient environments, this concept is not clearly understood. A pilot plant was designed to investigate biofilm formation on ten different materials relevant to a large drinking water distribution industry and generally used within the laboratory to evaluate biofilm formation. The materials used in the pilot plant set-up were; polyethylene (PE), polyvinylchloride (PVC), VA 1.4301 (SS 304), VA 1.4571 (SS 316), copper, aluminium (AlMgSi 0,5), zinc-coated steel, teflon (PTFE), plexiglass (PMMA) and glass. The effect of material surface preparation, flow conditions and water obtained from different purification steps were studied.

To enumerate the viable (culturable and non- culturable) and non-viable microorganisms in this oligotrophic environment, heterotrophic plate counts, cell numbers based on ATP concentrations and direct acridine orange counts were measured. Enumerating the contribution of the heterotrophic colony count [**Figure 3.3 to 3.5 (A)**] to the amount of active biomass as determined with ATP measurements [**Figure 3.3 to 3.5 (B)**], revealed a similar trend to results obtained in **Section 3.1**, with the culturable bacteria on average representing only a fraction

(often < 1%) of the active biomass in all biofilm samples. Furthermore, on average the heterotrophic plate count measurements often presented < 1% of the direct acridine orange cell count (**Table 3.4**) recorded for the biomass removed from the material coupons, whereas the cell numbers based on ATP measurements often presented < 100% of the direct acridine orange count. The biomass suspension obtained from PE, PVC, copper, zinc-coated steel and aluminium was turbid due to the presence of various inorganic particles (confirmed by SEM/EDX analysis) present on the PE and PVC and the active corrosion taking place on copper, aluminium and zinc-coated steel, which could have affected the ATP concentrations obtained. However, these results could show that under certain conditions, such as those investigated in the present study, 1 pg of ATP may not be equal to approximately 10^4 active bacteria/cells, and that the average ATP content per active bacterial cell is indeed less than $10^{-16} – 10^{-15}$ g. This hypothesis would explain the percentage ratios, above the theoretical maximum limit of 100, obtained for PVC, PE, copper, aluminium and zinc-coated steel.

This data supports the notion that techniques which enumerates total biomass for viable microorganisms and total cell counts, such as the ATP and DAOC techniques, need to be incorporated into routine drinking water monitoring, not only in water samples but also for evaluating the biofilms attached to the internal surface of the drinking water distribution system, as the classic plate count techniques currently performed enumerate only a fraction of the entire biomass which may be present. Biomass suspension obtained for PE, PVC, copper, zinc-coated steel and aluminium were extremely turbid especially during the later sampling stages, which influenced ATP concentration analyses. Gene sequenced based techniques such as the polymerase chain reaction (PCR) and *fluorescence in situ hybridisation* (*FISH*), which are currently being developed for their application in drinking water monitoring, should eventually serve as an alternative to culture based techniques, as results are usually obtained in a short period of time and the target organisms do no need to be culturable (Meier *et al.,* 1997; Fuchs *et al.,* 1998; Patel *et al.,* 1998; Fewtrell & Bartram, 2001; Straub & Chandler, 2003).

The microbiological and CLSM data indicate that the ten materials exhibit distinct and diverse biofilm colonisation patterns. Analysis of the samples showed that PE, PVC, teflon, plexiglass and aluminium were favourable for primary colonisation and ultimately biofilm formation. Polyethylene and plolyvinylchloride in particular had the highest surface coverage percentage irrespective of the water

source that they were exposed to. The two grades of stainless steel (VA 1.4301 and VA 1.4571) were the least favourable materials for biofilm formation under all conditions investigated, yielding the lowest surface colonisation percentages.

For PE, PVC, and teflon, biofilm formation occurred at a higher rate under conditions of turbulent flow, whereas biofilm formation on the two grades of stainless steel, VA 1.4301 and VA 1.4571 was highest under laminar flow conditions. Furthermore, treatment of potable water with disinfectant (chlorine dioxide) and corrosion inhibitors (phosphate and silicate) notably decreased microbial colonisation of the different materials. Generally the results obtained for surface coverage and live/dead ratio using CLSM analysis compared well with the microbiological results obtained for total cell count. Although the active corrosion of copper and zinc-coated steel negatively influenced the surface coverage data, results obtained for BFR and BFP, total cell count and viable colony count on R2A agar for these two materials were constantly significantly higher ($p < 0.05$) than results for all the other materials, indicating that copper and zinc-coated steel are favourable for biofilm formation.

Copper piping is generally used as the plumbing material of potable systems in larger institutional buildings (e.g. hospitals) or in domestic set-ups. However, the failure of this piping system due to abnormal forms of pitting corrosion has been reported within the United Kingdom, Germany, Saudi Arabia and Japan (McEvoy & Colbourne, 1988; Fischer *et al.*, 1992). This corresponds to data obtained in the present study where in general strong colonisation changes were observed for copper, aluminium and zinc-coated steel, correlating to the breakdown of the protection layers by active corrosion. The two grades of stainless steel, which are used as an alternative to the corroding materials, as well as glass, generally exhibited a low BFP as observed by the microbiological results as well as CLSM analysis. In contrast the polymeric materials, PE, PVC and teflon were found to offer favourable surfaces for biofilm formation. Others have reported similarities between the formation of biofilms on the two grades of stainless steel analysed, i.e. VA 1.4301 (SS 304) and VA 1.4571 (SS 316), even though the grade SS 316 is the more corrosion resistant grade (Percival *et al.,* 1998). Studies conducted by Zacheus *et al.* (2000), however revealed no clear difference in biofilm formation on the stainless steel grade VA 1.4301, PE and PVC. Data obtained in the present study corresponds to data obtained by Rogers *et al.* (1994) where higher bacterial numbers were reported on PE and PVC than on stainless steel. A study conducted by Van der Kooij *et al.* (1995a) revealed great similarities in biofilm accumulation rate

and amount on Teflon and glass when exposed to a specific water type. Even though similarities in the microbiological analysis for these two materials were observed, CLSM analysis showed that the surface colonisation rates for teflon was considerably higher than those obtained for glass.

Analysis of the whole community metabolic profiles after surface treatment (i.e. untreated materials versus sanding of the material to disrupt the surface) in the activated carbon treated water section exposed to a turbulent flow rate, initially showed no correlation for any of the materials investigated. However, with time a distinct correlation in the population dynamics on the sanded and untreated coupons for polyethylene, polyvinylchloride, copper, aluminium, zinc-coated steel and plexiglass was observed, indicating that the microbial population on these materials had either become adapted to the respective surfaces or the mature biofilm masked the surface of the material. These results showed that biofilm formation on different materials used within the drinking water distribution industry is greatly influenced by the choice of material, flow conditions and water quality.

4.3 EVALUATION OF OXIDISING BIOCIDES FOR THE CONTROL OF BIOFILMS IN THE TEST UNITS

The magnitude and ubiquitous character of biofilm formation emphasize the significance of controlling their growth in industry, with extensive research being conducted into the disinfection of biofilms in distribution systems. The inactivation of biofilm bacteria and characterisation of the interaction of biocides with microbial interfaces was investigated by LeChavellier *et al.* (1988b). Results showed that unattached bacteria were susceptible to a variety of disinfectants (hypochlorous acid, hypochlorite, chlorine dioxide and monochloramine) tested. Biofilm bacteria grown on the different types of surfaces were 150 to more than 3000 times more resistant to hypochlorous acid (free chlorine, pH 7) than were unattached cells. Various studies have found that the limited penetration of chlorine in the biofilm matrix could be an important factor influencing the reduced efficacy of this biocide against biofilms as compared with its action against planktonic cells (Paquin *et al.*, 1992; De Beer *et al.*, 1994).

The present study evaluated the disinfection efficiency of sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) on biofilms present on water distribution piping materials. Furthermore, if a distribution system was to become heavily

contaminated with microorganisms, such as in the case of technical failure, it is essential to control the organisms, safely secure the system and minimise the spread of the contamination in as short a time period as possible. A further aim was thus to determine which concentration of disinfectant was the most effective in removing and killing the biofilms.

Five different concentrations of the disinfectants were initially tested on biofilms formed on PE, copper and glass in a flow-cell system in the laboratory. Thereafter, based on the results obtained for the preliminary experiments in the laboratory flow-cell system, NaOCl was applied at 50 mg/l (concentration used by water treatment plant Holthausen) and 200 mg/l, while H_2O_2 was applied at 0.15 g/l (concentration used by water treatment plant Holthausen), 15 g/l (maximum concentration allowed by water treatment plant Holthausen) and 100 g/l, to the pilot plant system holding test PE, PVC, two grades of stainless steel namely, VA 1.4301 (SS 304) and VA 1.4571 (SS 316), copper, zinc-coated steel as well as teflon coupons. The test coupons were exposed to activated carbon treated water (treated water without corrosion inhibitors and disinfectants) in a pilot plant set-up for a period of 14 months. The materials analysed in the 'pure' water section of the pilot plant were either used as obtained from the manufacturer or sanded (600 grid) to determine the influence of surface roughness and damaged passivation layers on biofilm formation.

In general, the efficacy of the different concentrations of biocides at the concentrations (NaOCl 50 mg/l, and H_2O_2 0.15 g/l and 15 g/l) that are normally used by the water treatment plant Holthausen to disinfect the various components of the distribution and treatment system, revealed a strong dependence and influence on the type of material as illustrated in **Figure 3.15 a** and **b**. For instance, NaOCl (50 mg/l) yielded a greater disinfection efficiency on PE, VA 1.4301, zinc-coated steel and teflon, whereas, H_2O_2 (15g/l) was more effective on PVC, VA 1.4571 and copper. No distinct differences in disinfection efficiency of the biofilm were observed for the untreated materials versus the sanded materials as illustrated in **Figure 3.17**.

The results obtained in **Section 3.2** generally showed that polymeric materials polyethylene and plolyvinylchloride, were the most favourable for biofilm formation irrespective of the water source they were exposed to. Similar results were observed in this study [**Figure 3.15 (a)** and **(b)**] for the respective control channels of these materials. Evaluation of the disinfection efficiency of these materials nevertheless revealed that at the concentrations generally used by the waterworks Holthausen to disinfect piping material, sodium hypochlorite (50 mg/l) was more effective on PE,

while hydrogen peroxide (0.15 and 15 g/l) was more effective in removing and killing the biofilms present on PVC. Similarly, analysis of the control channels revealed low surface coverage percentages for the two grades of stainless steel (VA 1.4301 and VA 1.4571) [**Figure 3.15 (a)** and **(b)**]. However, evaluating the efficiency of the standard disinfectant concentrations used by the waterworks revealed that sodium hypochlorite (50 mg/l) was more effective in removing the biofilm from VA 1.4301, whereas hydrogen peroxide (15 g/l) was more effective against biofilm growth on VA 1.4571. Of the concentrations presently used by the waterworks to disinfect various components of the distribution network, 50 mg/l yielded the highest killing (69%) and removal (91%) percentages on untreated and sanded coupons (**Table 3.5**).

The maximum concentration of NaOCl (200 mg/l) analysed yielded a 45-95% killing and removal efficiency of the biofilm mass present on PE, VA 1.4301 and teflon as well as on PVC, VA 1.4571 and copper, whereas the maximum tested concentration of H_2O_2 (100 g/l) yielded a 50-90% killing and removal efficiency on zinc-coated steel. Additionally, analysis of the killing efficiency results revealed [**Figure 3.18 (b)**] that 200 mg/l sodium hypochlorite (NaOCl) yielded the lowest viability percentages (between 0 and 3%) on all materials tested, while when testing the maximum concentration of hydrogen peroxide (100 g/l) 0 to 8% viable cells remained. These results show that factors such as the concentration of biocide, as well as the material to which the biofilm is attached, greatly influence how effective a specific biocide will be on the biofilm mode of growth. Consequently, from the overall analysis it can also be deduced that sodium hypochlorite has a greater efficacy against activated carbon treated water biofilms than hydrogen peroxide irrespective of material or material treatment, and it is recommended that 200 mg/l NaOCl will possibly be the most effective disinfectant concentration to use against heavy biofilm contamination. However, high concentrations of chlorine can react with organic matter to form chloroform and trihalomethanes (Allonier *et al.,* 1999). Based on this it is recommended that the initial chlorine dose be based on its influence on the organoleptic properties of the water as well as the potential formation of trihalomethanes.

4.4 MAJOR FINDINGS OF THE STUDY

The significant results from this study were as follows:

1. The BFP is defined as the average amount of biofilm density during a period between 100 and 150 days of contact time, as the biofilms at the different locations were considered to reach a steady state during this time. Results from this study suggest that even though the biofilm may not have been growing actively, a steady state of biofilm activity was not reached after 100 to 150 days. If indeed so (that biofilm formation in drinking water distribution systems takes longer than 150 days to reach a steady state), then this has far-reaching implications for studies of biofilms in these and other oligotrophic environments, as typically most studies observe biofilms over much shorter periods.
2. Typically it has been reported that < 10% of the microorganisms from the environment are culturable. The oligotrophic environment evaluated in this study, showed that the relative values of the heterotrophic colony count to the active biomass as determined with cell numbers based on ATP measurements were often < 1%. The ratio of heterotrophic plate counts on R2A and direct acridine orange counts were often <1%, while the ratio between cell numbers based on ATP measurements and direct acridine orange counts were often < 100%.
3. Results in **2** above also showed that under certain conditions, such as those investigated in the present study, 1 pg of ATP may not be equal to approximately 10^4 active bacteria/cells, as stipulated by previous investigations, and that the average ATP content per active bacterial cell is indeed less than $10^{-16} – 10^{-15}$ g. This hypothesis would explain the percentage ratios, above the theoretical maximum limit of 100, obtained for PVC, PE, copper, aluminium and zinc-coated steel, which then directly influenced the average percentage ratios calculated for all ten materials.
4. From this study it appears possible that a threshold value for AOC, and possibly DOC, exists above which the BFR notably increases. Although more data is necessary to accurately determine threshold values, the observations made in this study show that a DOC below ~0.5 mg C/l, and an AOC below

~5 µg C/l should be target values for the control of biofilm formation in drinking water systems.

5. By installing the Kiwa™ biofilm monitors at the water treatment plant and at various locations of a large distribution network it was possible to investigate the long-term stability and microbial behaviour not only at the water treatment plants, but also throughout the distribution system. As the monitor provided a suitable physical model to study biofilm phenomena in drinking water systems, it could be deduced that the biological stability and quality of the potable water remained constant.
6. Analysis of the material samples showed that PE, PVC, teflon, plexiglass and aluminium were favourable for primary colonisation and ultimately biofilm formation. The corrosion taking place on copper and zinc-coated steel interfered with the CLSM analysis ($p > 0.05$), as the results obtained for total cell count, colony forming units as well as the BFR and BFP for these two materials were continually the highest ($p < 0.05$), showing that they are favourable for biofilm formation. The two grades of stainless steel (VA 1.4301 and VA 1.4571) were the least favourable materials for biofilm formation under all conditions investigated, yielding the lowest surface colonisation percentages.
7. Comparison of the results obtained for turbulent and laminar flow, and surface treatment when exposed to activated carbon treated water, shows that flow conditions and surface treatment have a direct influence on surface colonisation over extended periods of time. Furthermore, even after extended periods of time, significant ($p < 0.05$) differences in surface colonisation on the materials were observed, indicating that the composition of the material may have a direct influence on microbial colonisation.
8. Modification of the surface by sanding did not result in significant differences ($p > 0.05$) in disinfection efficiency of the biofilms attached to the untreated and sanded materials. It was further shown that factors such as the concentration of biocide, as well as the material to which the biofilm is attached, greatly influence how effective a specific biocide will be on the biofilm mode of growth.

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MIGRATION AND LEVEL OF PROTECTION OFFERED BY BIOFILMS TO GFP TAGGED ORGANISMS WITHIN DRINKING WATER DISTRIBUTION SYSTEMS

Background

Water-related diseases are generally not associated with the developed world, as most of these countries have efficient water distribution- and wastewater disposal-systems. Contamination of water distribution systems in these countries can be caused via cross-connections, back-siphonage, corrosion, or construction and repairs of the distribution system. Waterborne epidemics can also be caused by contaminated groundwater (Ronchi, 2003). As many as 1.1 billion people or 18% of the world's population lack access to safe drinking water and over 2.4 billion people in the developing world lack access to adequate sanitation. Furthermore, in these developing countries between 90 and 95% of sewage and 70% of industrial wastes are dumped untreated into waters where they pollute the usable water supply (World Summit on Sustainable Development, 2002).

The Millennium Development Goal for water is aimed at halving the proportion of people worldwide without access to safe drinking water and sanitation by 2015 (UNICEF, 2003). This implies that the key water quality issues that undermine the safety of drinking water, and that affect the quality of lives around the world will have to be addressed. One of the major problems associated with protecting the water supplies in the developed, and especially developing countries is the potential source of contamination by pathogens and toxicants.

Human pathogenic agents, such as bacteria (e.g. pathogenic *Escherichia coli* strains, *Campylobacter* and *Salmonella* spp.), viruses (e.g. adenoviruses, enteroviruses and rotaviruses) and protozoa (e.g. *Cryptosporidium parvum* and *Giardia lamblia*), which can contaminate and be transmitted in drinking water, may cause diseases varying in severity from mild gastroenteritis to severe and sometimes fatal diarrhoea, dysentery, hepatitis or typhoid fever. Faecal contamination of drinking water is only one of several faeco-oral mechanisms by which these pathogens can be transmitted from person to person or in some cases from animals to people. However, not all potentially waterborne human pathogens are of equal public health significance. Furthermore the likelihood of ingesting very large numbers of a pathogen on a single occasion from drinking water is relatively small,

as the enteric pathogens cannot normally multiply in water and because water tends to disperse them. If the polluted water however, comes into contact with food, the bacterial pathogens, initially present in small numbers, can multiply to produce very large doses (World Health Organisation, 1996; UNICEF, 2003).

Probably the best-known and most deadly case of contamination in the United States in recent years happened in Milwaukee in 1993. *Cryptosporidium*, an intestinal protozoan, contaminated the municipal water supply. At least 50 people died and approximately 400 000 people became ill, 4 000 serious enough to be hospitalised (Hoxie *et al.*, 1997). Contamination of drinking water by the deadly strain of intestinal bacterium *Escherichia coli* 0157:H7 killed 7 people and made more than 2 500 people ill in Walkerton, Ontario in 2000 (Jones, 2000).

From 1980 to 1999, 116 outbreaks of waterborne diseases were reported in Sweden, affecting about 58,000 people. An organism that was often detected in these cases was *Campylobacter*, although in about 70% of outbreaks of gastrointestinal illness no known agents were detected. Between 1991 and 2000, 41 outbreaks were reported in the United Kingdom, with more than 3,768 reported cases of illness. Most of these outbreaks were due to *Campylobacter* and *Cryptosporidium*, an emerging pathogen of notable concern in many water supply systems (Ronchi, 2003).

Microbial biofilms constitute one of the most important issues in public health microbiology, requiring attention in such diverse areas as fouling in the food and beverage industry, to biofilm accumulation on prostheses and catheters in medicine (Errampalli *et al.*, 1999). A biofilm is the cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin. In other words, in a water distribution system, a biofilm is the patchy, slime layer that lines the inside of the pipes and consists of corrosion products from the pipes and colonies of bacteria, viruses, and algae and their products. This biofilm mode of growth could provide microenvironments that can protect pathogens from the physical, chemical and biological stresses of conventional water and wastewater treatment processes. Furthermore, biofilms are a potential cause of disease because their microorganisms exhibit increased resistance to antibiotics and disinfectants. It has been suggested that *Escherichia coli 0157:H7*, for instance, is 2000 times more resistant to chlorine in biofilms than when floating free in water (Percival *et al.,* 2000).

An effective way of observing the migration of waterborne pathogens through the biofilm environment and examining the measure of protection this environment

affords them would be to tag these organisms with a reporter gene encoding a protein that is either fluorescent itself or which catalyses a reaction that leads to a fluorescent product. These easily identifiable markers can effectively be used to monitor, detect and enumerate microorganisms after their introduction into the environment (Errampali *et al.,* 1999; Molecular Microbial Ecology Group, 2000). In 1994 the *gfp* gene encoding the green fluorescent protein (Gfp), originally isolated from the jellyfish *Aequorea victoria*, became available as an important visual, molecular marker of gene expression in eukaryotic organisms. The *gfp* gene requires no special cofactors for detection, meaning it fluoresces without the addition of any external substrate, apart from low levels of oxygen (Chalfie, 1994; Chalfie *et al.,* 1994; Errampalli *et al.,* 1999).

The environmental applications of *gfp*-based marker systems started in 1996, and the versatility of the protein is illustrated by the diversity of applications described to date. The dynamics and distribution of bacteria in water systems, soils, rhizospheres, root systems and biofilms has been investigated using *gfp*. As *gfp*-labelled bacteria can be viewed by epifluorescent- or scanning confocal laser-microscopy, single cells of the labelled microorganism can be detected in various samples and under various conditions, providing information about location, survival and gene expression (Errampalli *et al.,* 1999). An investigation by Moller *et al.* 1998 showed that *gfp*-labelled *Pseudomonas putida* RI preferred to colonise the outermost layer of a mixed-species biofilm while and *Acinetobacter* sp strain C6 primarily attached to the substratum.

A group of molecular microbial ecologists under the leadership of Molin *et al.* (2000), have designed a set of general cloning vectors for construction of various tagging and reporter systems involving e.g. *gfp* and *lacZ*. Use of this toolbox of vectors allows for the combination of a variety of promoter of interest with the most convenient marker gene for a given application (Molecular Microbial Ecology Group, 2000). Bao *et al.* 1991 demonstrated the functionality of a Tn-7 Gfp based system for single-copy insertion of cloned genes into the chromosome of *Rhodospirillum rubrum*. Similarily a panel of Tn-7 based vectors was developed by Koch *et al.* 2001 for the insertion of the *gfp* marker gene or for the delivery of cloned DNA into gram-negative bacteria at a neutral chromosomal site.

Drinking water comes from two types of sources: surface waters and groundwater. Surface waters are exposed to environmental elements and require extensive treatment. Groundwater on the other hand has been assumed to be safe

and usually does not require treatment to kill microorganisms. Layers of soil act as a natural filter, removing microbes and other particles as water seeps through it. Most water distribution systems are fed with both surface- and groundwater, consequently this water may require various purifying and treatment procedures. Before reaching the consumer the water goes through a long treatment process that may involve many steps. Even though the water is presumed free of microbial cells when it leaves the treatment plant, re-contamination of the water may occur. The possibility exists that pathogens, which might enter the distribution system, will be taken up and protected by biofilm communities. To test this hypothesis, the extent to which biofilms offer protection to naturally isolated waterborne organisms within the water distribution industry was examined. This was accomplished by tagging an enterobacterium, which had been isolated from a river water biofilm, with *gfp*. Additionally, the interaction of this organism with biofilms on materials relevant to the drinking water distribution system was examined.

Experimental

Bacterial strains, plasmids and growth conditions

The bacterial strain used in this experiment was isolated from a river contaminated with human waste in the Stellenbosch, South Africa, region. Sampling entailed collection of biofilm-covered stones, which were placed in airtight sterile plastic bags and sealed until sonication. The microbial suspension was obtained by removing the biofilm from the rock sample using the method described in **Section 2.1.2.3.** The suspension was then immediately transferred on ice to the laboratory, where pure cultures were obtained and preliminary identification of this strain was done using physiological cultivation and biochemical techniques. Molecular typing of this organism as outlined below, presumably identified it as *Escherichia coli*. The plasmids used for tagging with the Tn7-system were; pUX-BF13 and pBK-miniTn7-*gfp*2. The undefined mixed culture used to grow the biofilms in the experiments, was isolated from an aerated water source (prior to final disinfection) obtained from the Water Works in Paradyskloof, Stellenbosch. The biofilm was allowed to develop on glass slides suspended in the water sample for a period of two weeks. The biofilm was then removed from the slides using the method described in **Section 2.1.2.3**. The *Escherichia coli* strain was cultured at 37°C in Luria Bertani medium (LB)

(Sambrook *et al.,* 1989) at 37 °C. The media was solidified by 1.5% agar (Difco) when appropriate. The undefined biofilm community was cultured in R2A medium (100%) at 30°C. Antibiotics were used for *E. coli* at the following concentrations (in µg/ml): ampicillin, 100; kanamycin (Km), 25; streptomycin (Sm) 25.

Molecular Typing of naturally isolated and GFP –tagged strains

Genomic DNA was extracted from the presumptive positive naturally isolated *Escherichia coli* strain using the procedure as outlined in Current Protocols in Molecular Biology (Ausubel *et al.*, 1993). The polymerase chain reaction was performed in a 50 µl volume containing 1 U Taq polymerase (Promega, Promega Corporation, Madison, USA) in a GeneAmp PCR System 2400. PCR conditions were 2 min at 94°C (initial denaturation) followed by 30 cycles of 60 s at 94 °C, 60 s at 52 °C and 120 s at 72 °C. Finally the mixture was heated at 72°C for 10 min and subsequently cooled to 4 °C. The oligonucleotide primers used for the PCR reactions were rPP2 (primer sequence: 5'CCAAGCTTCTAGACGGITACCTTGTTACGACTT3') and fDD2 (primer sequence: 5' CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG3'. Agarose gel electrophoresis was carried out using standard methods (Sambrook *et al.,* 1989). PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Roche Diagnostics GmbH, USA). DNA sequencing was done by the dideoxy chain termination method, using an ABI PRISM™ 377 automated DNA sequencer. The sequence was analysed using the PC-based DNAMAN (version 4.1) package from Lynnon Biosoft, with comparison serches performed using the gapped-blast program at the National Centre for Biotechnology Information (NCBI) [http://ncbi.nlm.nih.gov/BLAST/] (Altschul *et al.,* 1997).

Construction of Tn7 vectors

The plasmids pUX-BF13 and pBK-miniTn7-*gfp*2 were constructed as outlined in Bao *et al.* 1991 and Koch *et a*l. 2001, respectively.

Plasmid extraction and GFP-tagging of the naturally isolated Escherichia coli strain

The plasmids pUX-BF13 and pBK-miniTn7-*gfp*2 were extracted using the High Pure Plasmid Isolation Kit (Roche, Roche Diagnostics GmbH, USA). Competent cells (100 µl suspension) were mixed in an Eppendorf tube with 0.5 µg of plasmid DNA (pBK-miniTn7-*gfp*2) in 5 µl of TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA] and 0.5 µg of pUX-BF13 in 5 µl of TE buffer. Plasmid pUX-BF13 carries the genes encoding the transposition proteins necessary for insertion of the Tn7 cassette into the genomic target site (Bao *et al.,* 1991). Transformations were carried out using standard heat-shock methods (Sambrook *et al.,* 1989). After the incubation period, 100 µl aliquots of the culture were spread plated in triplicate on selective LB plates containing the respective antibiotics. The plates were incubated overnight at 37°C. Integration of mini-Tn7 from pBK-miniTn7-*gfp*2 produced the strain *E.c.g.*

Migration of GFP-tagged organism through the biofilm

The migration through and level of protection offered to the GFP tagged test strain by the biofilm was determined using a laboratory scale flow-cell system as illustrated in **Figure 1**. Two scenarios were investigated using this flow-cell system; (1) the introduction of the GFP labelled organism into an existing mixed species biofilm, and (2) the introduction of the labelled organism first to form a biofilm, followed by the introduction of the mixed community. For scenario one 0.5 ml of a mixed culture of undefined species isolated from an aerated water source (prior to final disinfection) obtained from the Water Works in Paradyskloof, Stellenbosch, was first inoculated into the flow-cells containing material samples (1 x 1 cm) of polyethylene (PE), VA 1.4301 (SS 304), copper and zinc-coated steel. For scenario two 0.5 ml of the GFP-labelled organism was first inoculated into the flow-cells containing the respective material samples. The flow-cells were irrigated with R2A broth (1:100) using a peristaltic pump (Watson Marlow 205S) at a flow rate of 0.2 mm s^{-1} throughout the experimental period of two weeks.

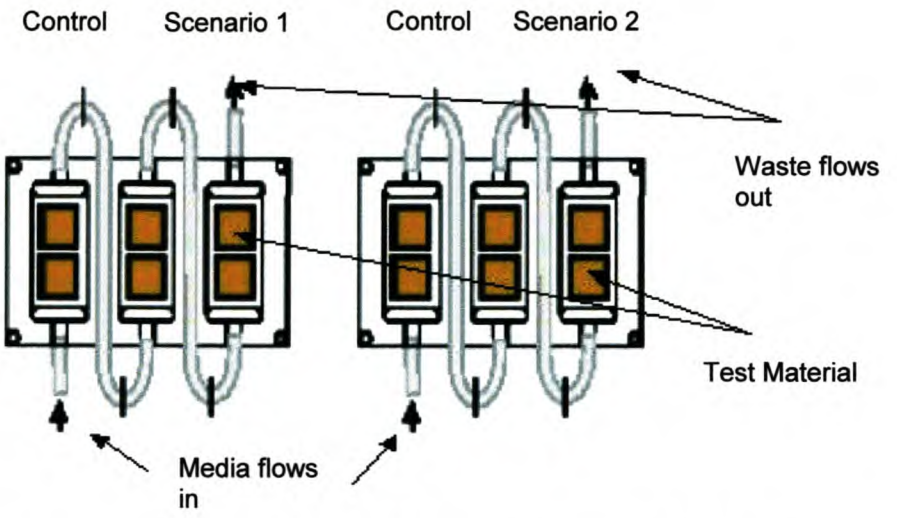


Figure 1 Flow-cell systems used to study the migration and level of protection offered to GFP-tagged organisms by biofilms. Two scenarios were investigated (1) introduction of a GFP labelled *E.coli* into an existing mixed species biofilm, and (2) introduction of the labelled organism first to form a biofilm, followed by the introduction of mixed community. The test materials used in the flow-cells were polyethylene, VA 1.4301, copper and zinc-coated steel.

After the initial two day incubation period, either the 0.5 ml of the GFP labelled organism or 0.5 ml of the undefined community was inoculated into the respective flow-cells with the pump turned off for one hour before flow was resumed for a further two days. After this time period, flow channels were positively stained with Nile Red (Eastman Kodak Co., Rochester, NY), a hydrophobic-specific benzophenoxazinone dye (Lamont *et al.,* 1987). A staining solution at final concentration of 5 μg ml^{-1} Nile red in 50% aqueous glycerol, was prepared from a 1 mg ml^{-1} stock solution of Nile red dissolved in acetone. Each respective coupon was then analysed using Epifluorescent microscopy (Nikon Eclipse E400), every two days for a period of two weeks, with the images captured using a Nikon Coolpix 990 Digital Camera. Control channels to which no GFP-labelled organism was added, were included for each test material. These control coupons were then analysed for live/dead ratio as well as surface coverage analysis using a combination of Epifluorescent microscopy (Nikon Eclipse E400) and the Baclight™ Live/Dead stain by collecting a minimum of 10 points for each control material in the flow-cell system. This was followed by analyses with ScionImage™ to obtain a comparison of the surface coverage and cell activity over time.

Growth measurements

To demonstrate that insertion of the GFP protein did not affect the bacterial growth rate, growth curve experiments were carried out over a period of 24 hours for the wild type *E. coli* strain and the *E.c.g.* strain, prior to the pathogen-biofilm experiments. Overnight cultures of the *Escherichia coli* isolated from the river biofilm as well as the *E.c.g* strain grown in LB broth were used to inoculate 100 ml LB broth in 1L Erlenmeyer flasks, respectively. Cultures were incubated on a rotary shaker (300rpm) at 37°C and the OD_{600} was monitored for 24 h.

Results and Discussion

The physiological and biochemical characterisation techniques, as well as the molecular typing of the organism isolated from the river biofilm positively identified it as an *Escherichia coli* strain. After transformation, the bacteria that were tagged with the mini-Tn7 transposon grew on the selective antibiotic plates. Analysis of these colonies with epifluorescent microscopy then confirmed that the mini-Tn7 from pBK-miniTn7-*gfp*2 was integrated into the strain, which was subsequently denoted as *E.c.g.*

Important characteristics of the mini-Tn7-transposon system are among others that it inserts as one copy into one chromosomal site, the inserted sequences are stably maintained during growth and insertion into this site does not affect bacterial growth (Bao *et al.,* 1991; Koch *et al.,* 2001). Growth curve experiments were carried out over a period of 24 hours for the wild type *E. co*li and the *E.c.g.* strains, before the pathogen-biofilm experiments were performed. This investigation demonstrated that insertion of the GFP protein did not affect the bacterial growth rate, as the growth curves of the *E. coli* and the *E.c.g* strains were identical.

Tagging of a bacterium requires that both the delivery plasmid (pBK-miniTn7-*gfp*2) and helper plasmid (pUX-BF13) be present in the bacterium at the same time. The transposase genes will then be expressed and the mini-Tn7 transposon (the DNA flanked by Tn7L and Tn7R) will be transposed into a specific site on the chromosome (Barth *et al.*, 1976; Rogers, *et al.,* 1986). If the plasmids are unable to replicate in the tagged bacterium, the bacteria will maintain the inserted sequence stably, while the two plasmids will be lost from the strain when it grows. The delivery plasmid used, pBK-miniTn7-*gfp*2 is able to replicate in *E. coli* (Koch *et al.*, 2001).

This however, did not influence or negatively affect our experiments, as the primary aim of tagging the organism with the GFP visual marker was to observe it *in situ* within the biofilm environment. Experiments to ensure that the mini-Tn7 transposon was indeed inserted into a specific site on the bacterium's chromosome are however, currently being performed.

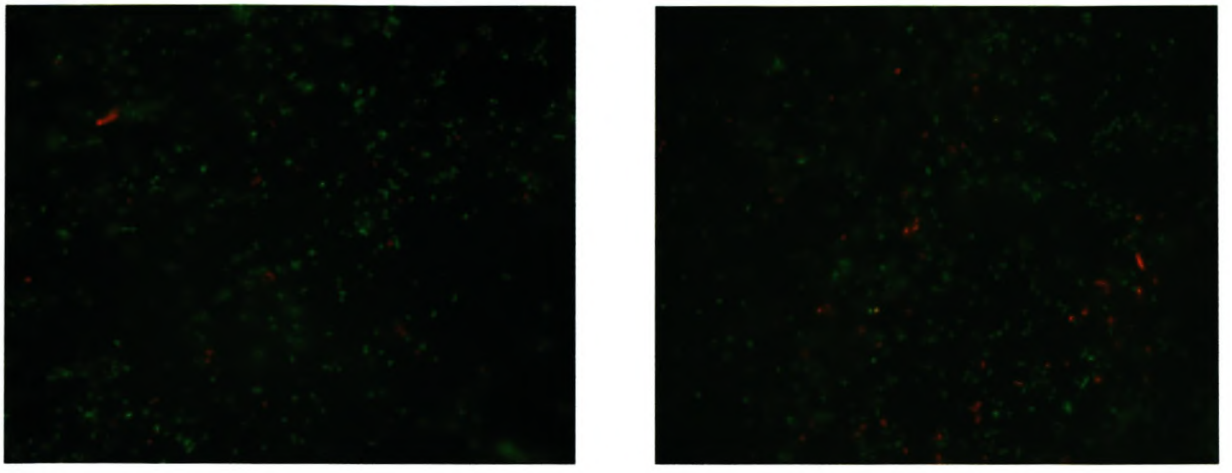
By inserting the *gfp* gene into an enterobacterium isolated from a river water biofilm, it was possible to evaluate how pathogens, which may enter the distribution system, will be taken up and offered a habitat by biofilm communities. Results for the first scenario, which investigated the introduction of the GFP labelled organism into an existing mixed species biofilm on PE, VA 1.4301, copper and zinc-coated steel, showed that after the initial incorporation of the tagged organism into the mixed community, it could be detected until the 8th day of sampling on copper and zinc-coated steel and until the end of the sampling period on PE and VA 1.4301 [results obtained for polyethylene is presented in **Figure 2** (**A-F**)]. Even though it cannot be concluded that other organisms will behave similarly, these results suggest that other organisms or pathogens entering the water distribution system after treatment may persist and be offered a habitat by the biofilm community, possibly forming part of a micro-habitat for other waterborne organisms, which may enter the system.

Interestingly, results for the second scenario investigated, namely the introduction of the GFP labelled organism first to form a biofilm, followed by the introduction of the mixed community, revealed that the tagged organism could typically only be detected until the 8th sampling day, irrespective of material analysed [results obtained for copper is presented in **Figure 3** (**A-F**)]. Distribution systems generally consist of kilometres of piping, so even though the tagged organism detached from the surface, the possibility exists that it will re-attach further down in the system to form a biofilm.

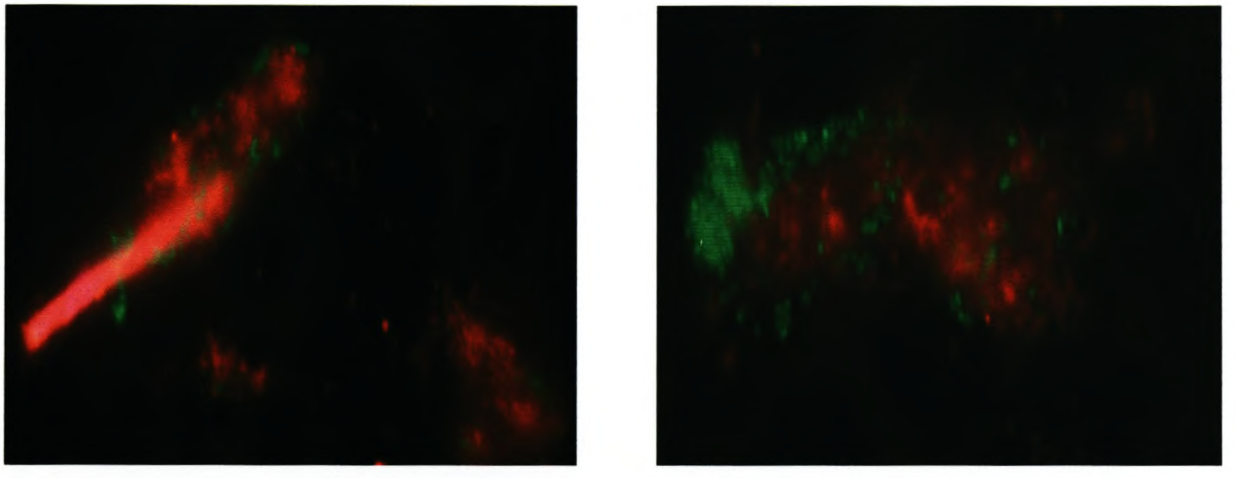
Gilbert *et al.* (1997) challenged steady-state biofilms of *Enterococcus faecalis* (JH2), with planktonic strains of the same species (JH2-2). This study was done to investigate the immigration of the planktonic strains into the mature *E. faecalis* biofilms. The data obtained suggested that when provided with a metabolisable carbon substrate and cell-cell contact, an active interaction occurred between biofilm and planktonic phenotypes of *E. faecalis*, resulting in the death or lysis of the biofilm cells. Similarly, analysis of the data in the present study showed that for both the scenarios investigated, after the introduction of the second inoculum into the flow-cell, the organism inoculated first could not be detected after 6-8 days.

POLYETHYLENE

(A) Biofilm coverage after 4 days of inoculation: Stain Baclight™



(B) Biofilm pre-covered coupon after GFP-inoculation: 2 days



(C) Biofilm pre-covered coupon after GFP-inoculation: 4 days

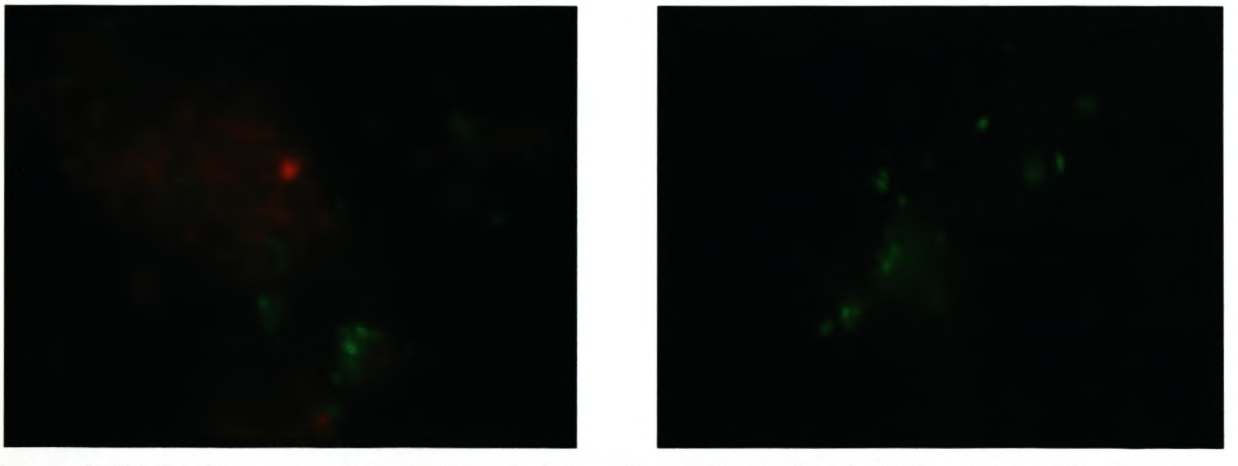
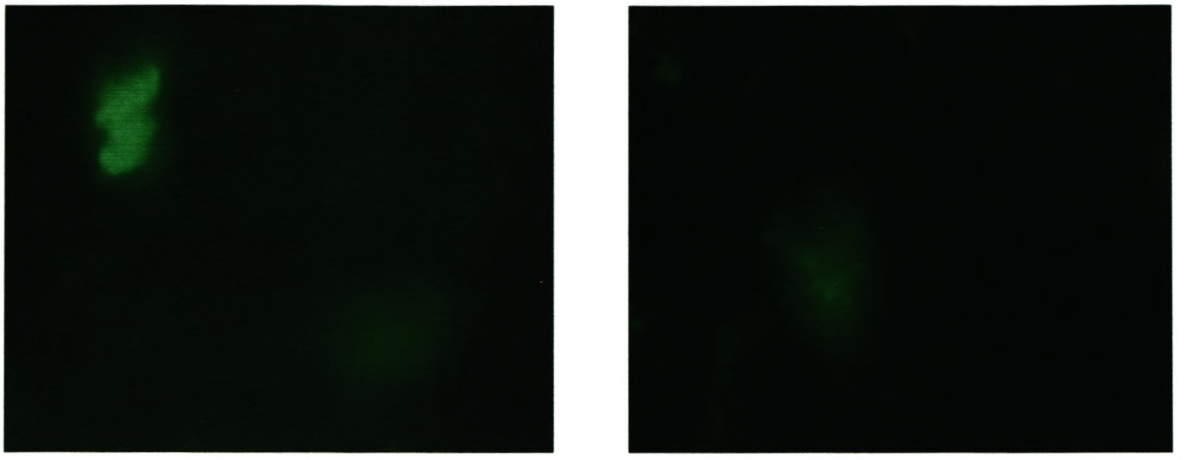


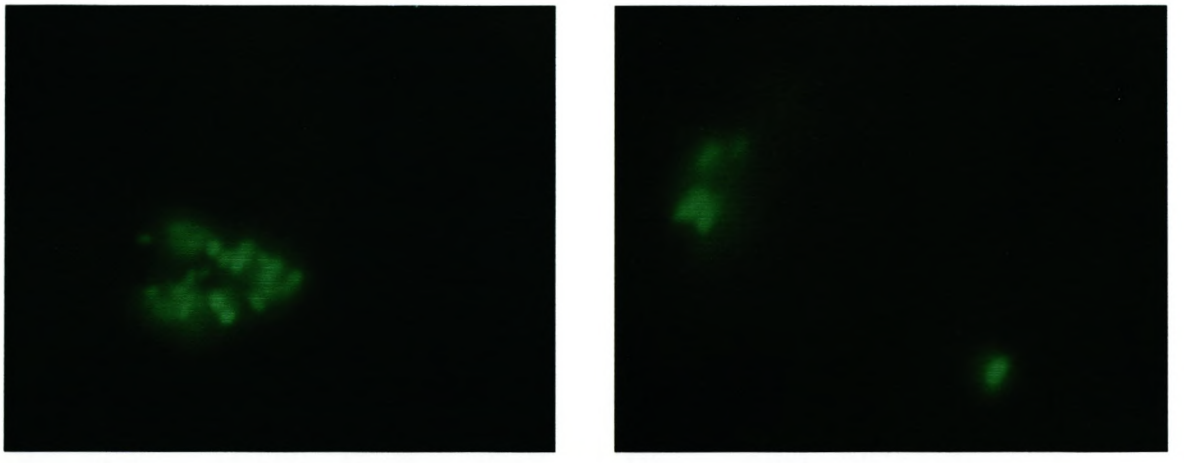
Figure 2 (A) Biofilm coverage on polyethylene after 4 days of inoculation (stain: Live/Dead Baclight™) **(B)** and **(C)** the introduction of the GFP labelled organism into an existing mixed species biofilm, after 2 and 4 days, respectively

POLYETHYLENE

(D) Biofilm pre-covered coupon after GFP-inoculation: 6 days



(E) Biofilm pre-covered coupon after GFP-inoculation: 8 days



(F) Biofilm pre-covered coupon after GFP-inoculation: 12 days

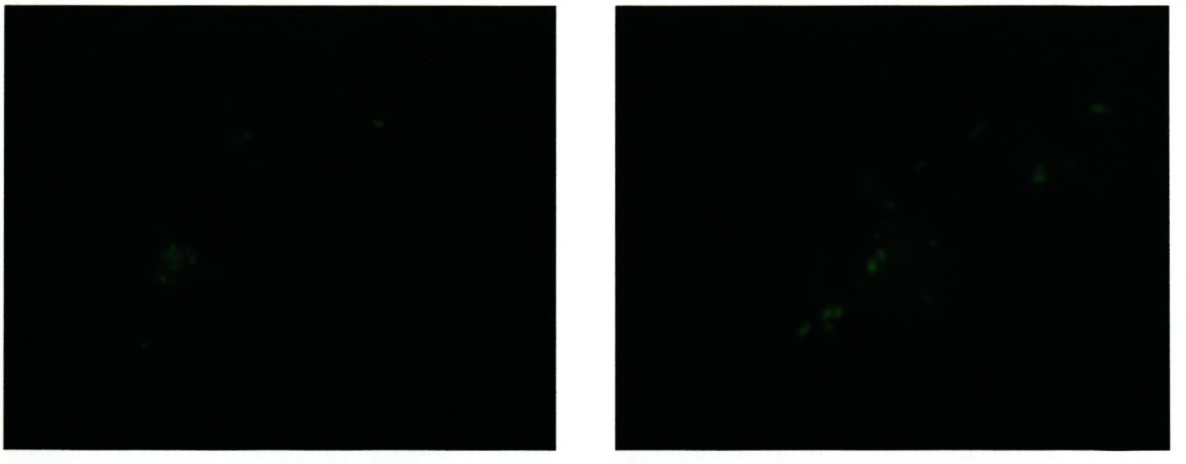
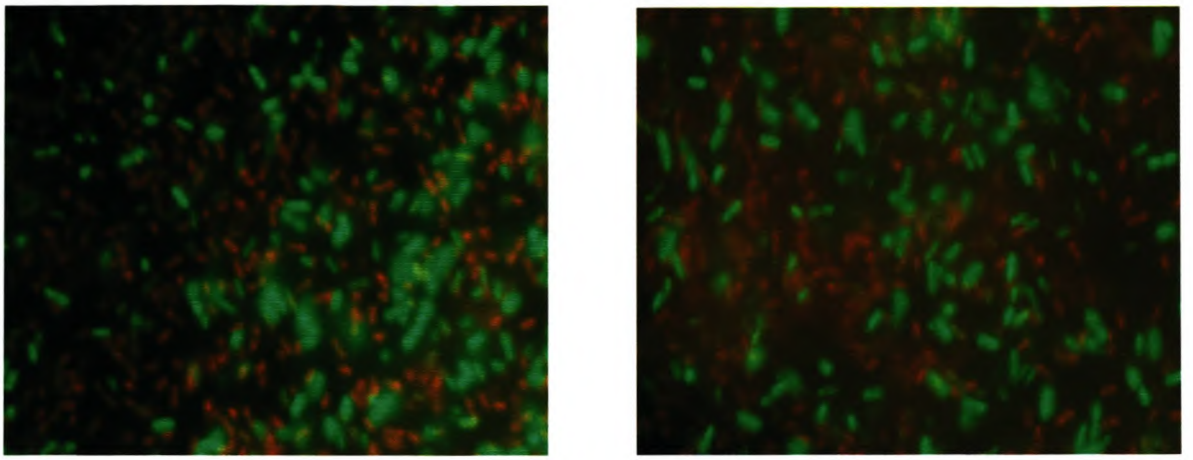


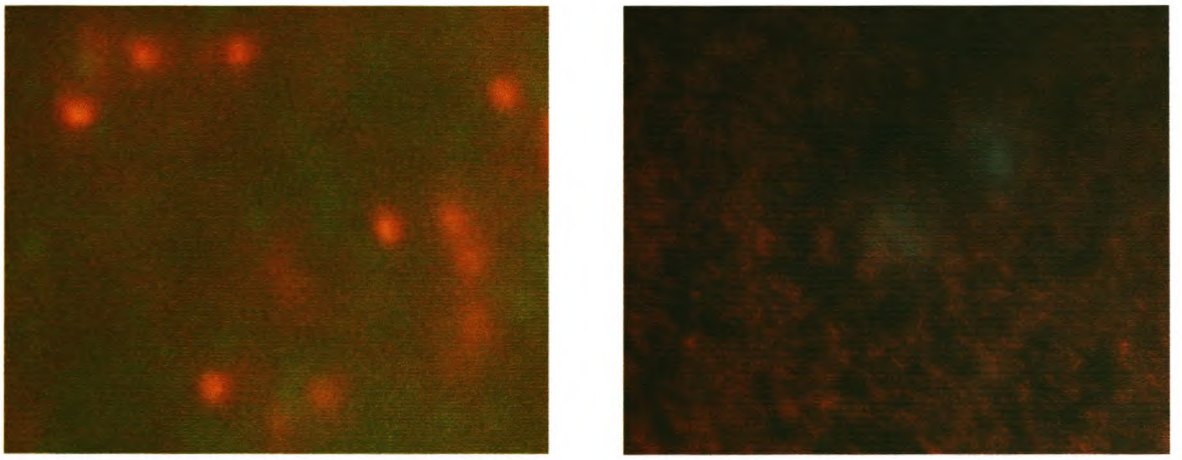
Figure 2 (continued) (D), (E), and **(F)** the introduction of the GFP labelled organism into an existing mixed species biofilm, after 6, 8 and 12 days, respectively

COPPER

(A) GFP Biofilm coverage after 4 days of inoculation: Stain Baclight™



(B) GFP Biofilm coverage after mixed species inoculation: 2 days



(C) GFP Biofilm coverage after mixed species inoculation: 4 days

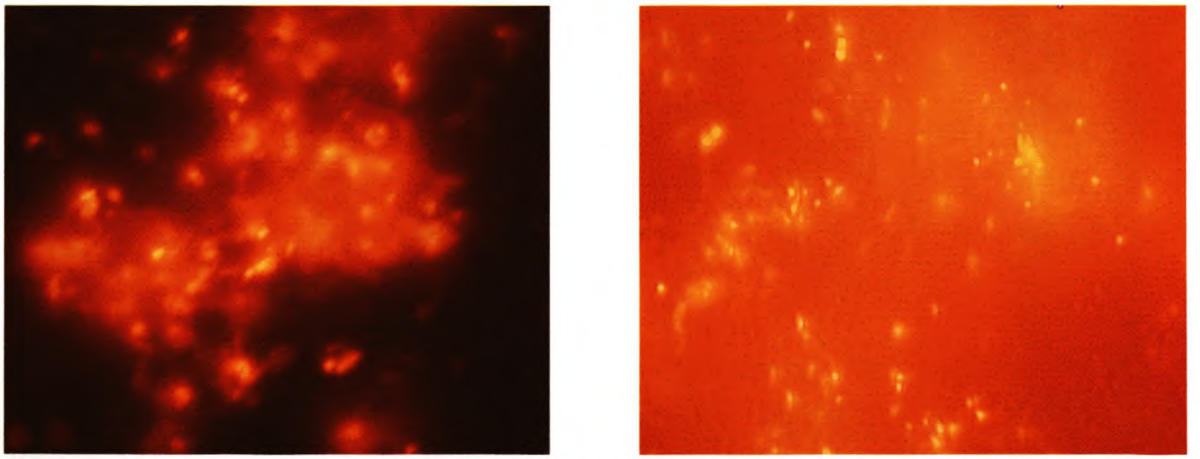
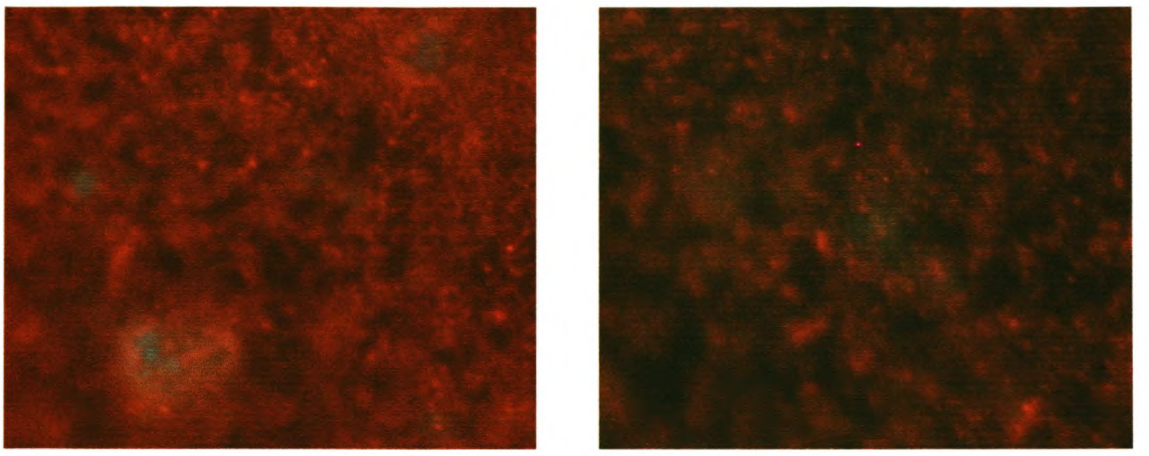
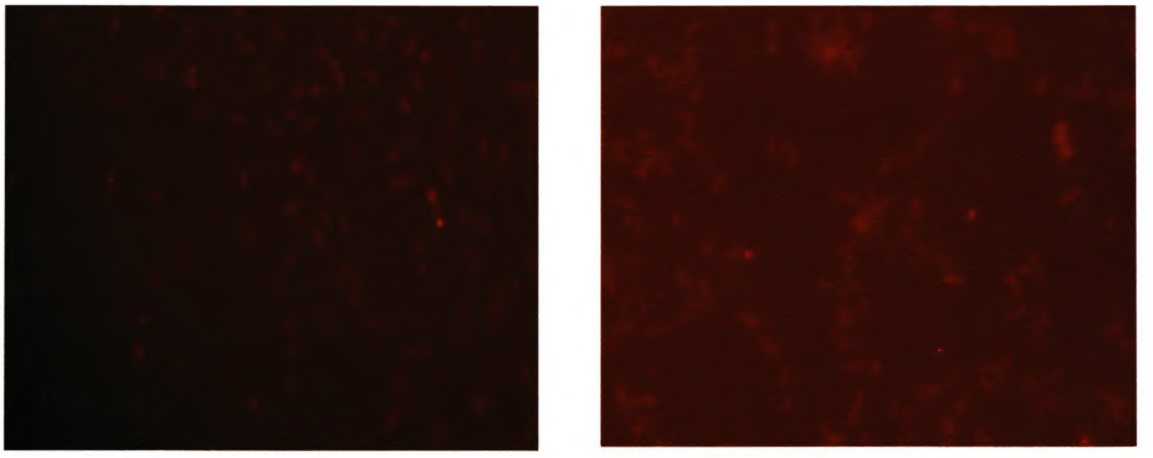


Figure 3 (A) Biofilm coverage on Copper after 4 days of inoculation (stain: Live/Dead Baclight™) **(B)** and **(C)** the introduction of the GFP labelled organism first to form a biofilm, followed by the introduction of the mixed community, after 2 and 4 days, respectively

(D) GFP Biofilm coverage after mixed species inoculation: 6 days



(E) GFP Biofilm coverage after mixed species inoculation: 8 days



(F) GFP Biofilm coverage after mixed species inoculation: 12 days

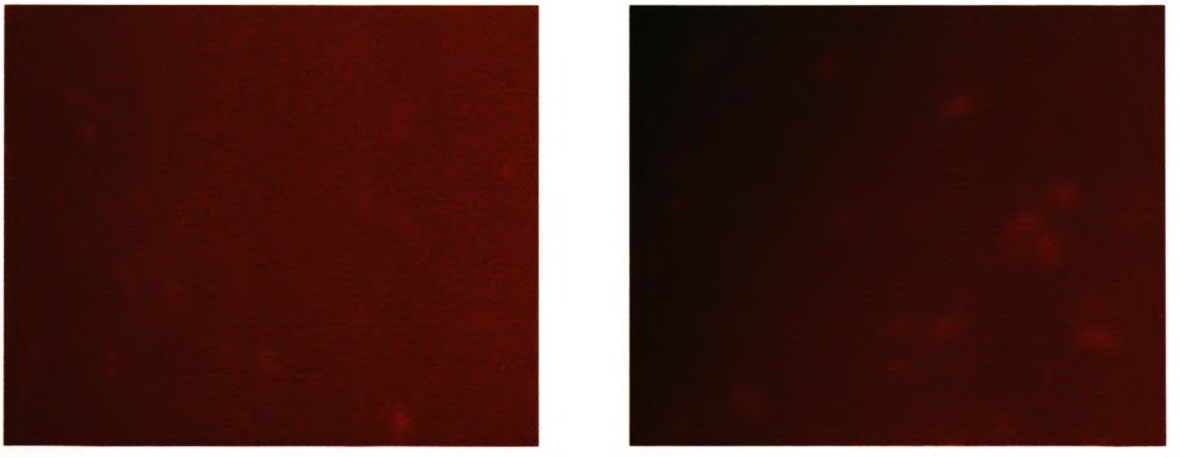


Figure 3 (continued) (D), (E), and **(F)** the introduction of the GFP labelled organism first to form a biofilm, followed by the introduction of the mixed community, after 6, 8 and 12 days, respectively

The flow was maintained at 0.2 $mm.s^{-1}$ throughout experimentation, and thus should have had little impact on the results. The study conducted by Gilbert *et al.* (1997) provides a possible explanation for the displacement of the original inoculum in the two scenarios investigated. The data obtained suggested that in natural environments, self-sacrifice on the part of the biofilm would allow it to promote the growth and development of planktonic cells, and allow the biofilms to obtain genetic information from a wider pool.

No distinct differences in the persistence of the GFP tagged organism was observed in the results obtained for the different test materials analysed, irrespective of scenario investigated. When the GFP tagged organism was introduced into an existing mixed species biofilm it could generally be detected until the 8^{th} day of sampling on copper and zinc-coated steel and until the end of the sampling period, on PE and VA 1.4301. However, detailed analysis of the copper and zinc-coated steel material surfaces was hindered by the corrosion taking place on these two materials. When the GFP tagged organism was introduced first to form a biofilm, it could be detected until the 6^{th} day of sampling on VA 1.4301 and copper and until the 8^{th} day of sampling on PE and zinc-coated steel.

Results obtained in **Section 3.2** and **Section 3.3** typically showed that various parameters such as material choice, flow conditions (laminar of turbulent water flow) and water quality, greatly influence bacterial attachment. The migration and level of protection offered by the biofilm to the tagged organism was investigated on PE, VA 1.4301, copper and zinc-coated steel.

Control channels to which no GFP-labelled organism was added, were included for each test material. These control coupons were then analysed for live/dead ratio as well as surface coverage analysis using a combination of Epifluorescent microscopy (Nikon Eclipse E400) and the Baclight™ Live/Dead stain by collecting a minimum of 10 points for each control material in the flow-cell system. This was followed by analyses with ScionImage™ to obtain a comparison of the surface coverage and cell activity over time. Analysis of the control channels (**Figure 4**) four days after inoculation, revealed a similar trend as observed in **Sections 3.2** and **3.3**. Extensive biofilm formation occurred on polyethylene, while surface coverage percentages were the lowest for stainless steel VA 1.4301. After four days both the zinc-coated steel and copper coupons were starting to corrode. Results from **Section 3.2** also revealed that corrosion of copper and zinc-coated steel complicated measurements of surface coverage and total cell count analysis,

as results for ATP analysis and viable colony count on R2A agar for these two materials were constantly considerably higher than all the other materials, indicating that copper and zinc-coated steel were indeed favourable for biofilm formation, although not easily measurable due to the accumulation of corrosion products.

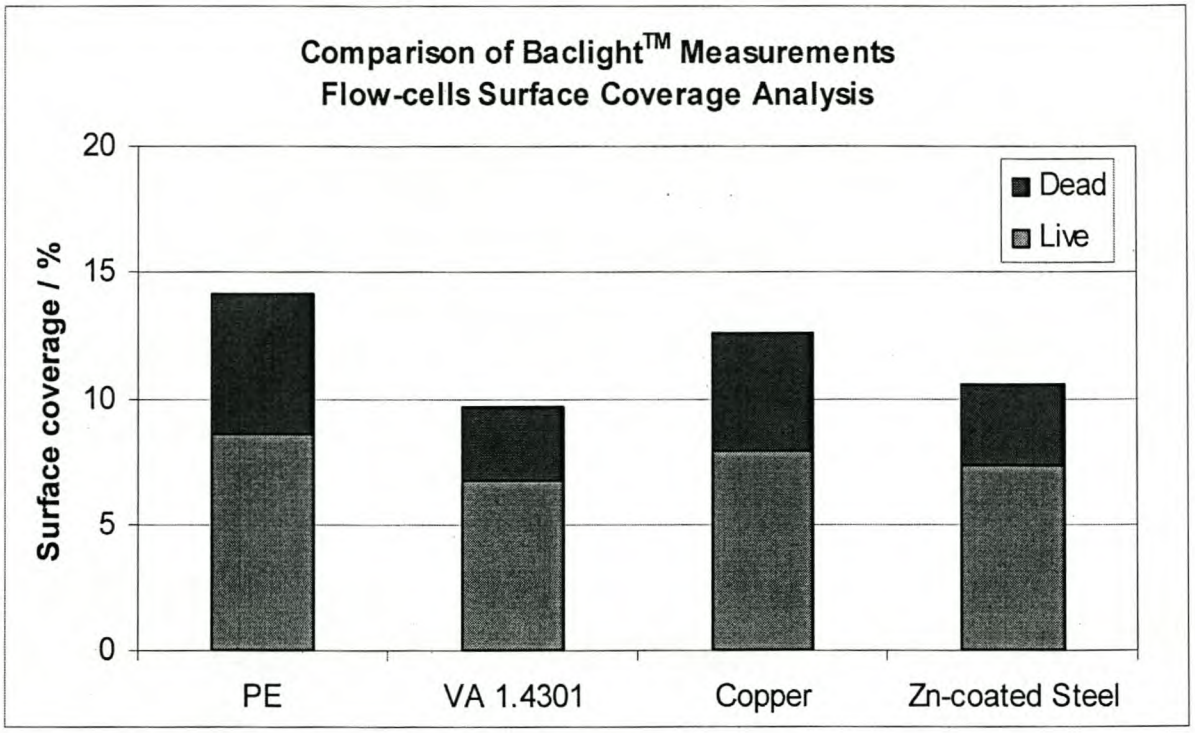


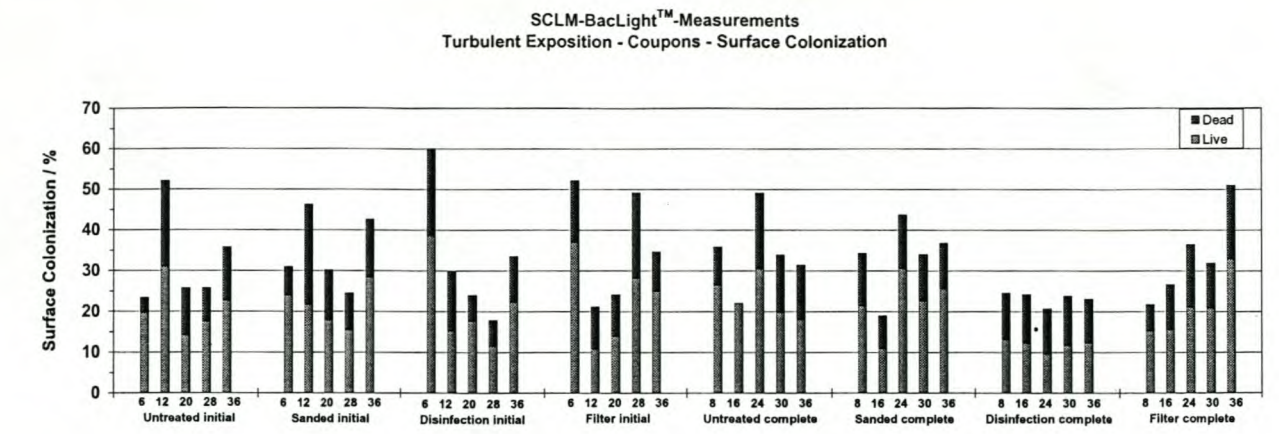
Figure 4 Comparison of the Live/Dead ratio and surface coverage analysis on polyethylene, VA 1.4301, copper and zinc-coated steel.

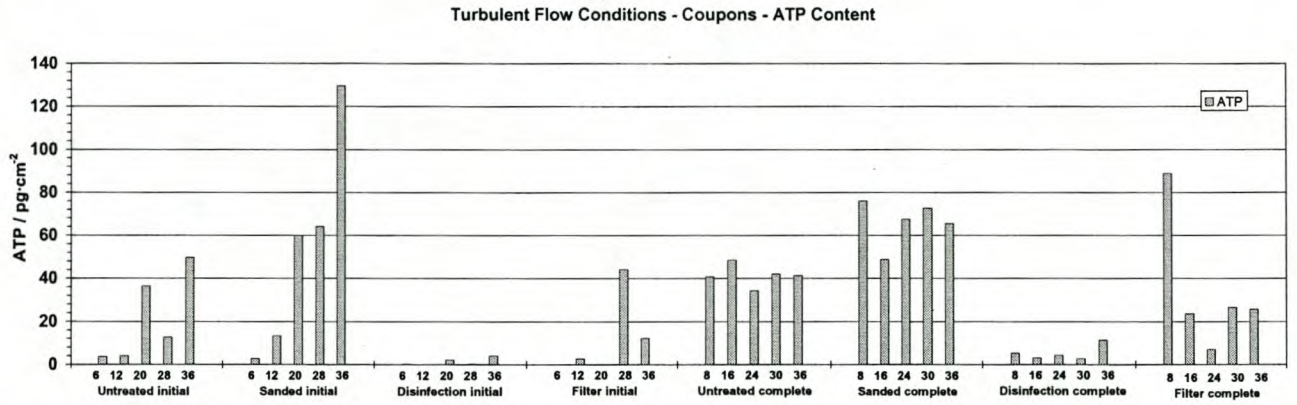
General Conclusions

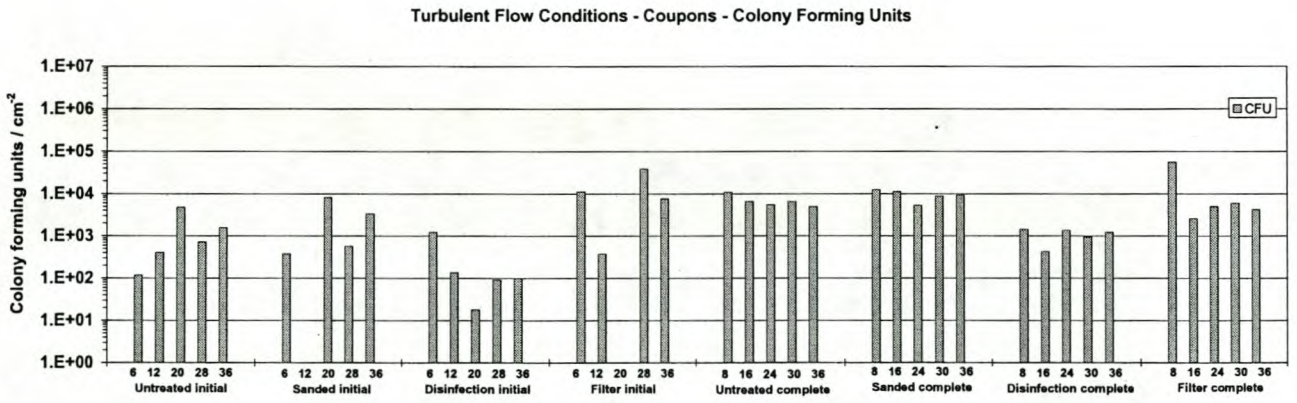
Results for the first scenario, which investigated the introduction of the GFP labelled organism into an existing mixed species biofilm on PE, VA 1.4301, copper and zinc-coated steel, showed that after the initial incorporation of the tagged organism into the mixed community, it could be detected until the 8th day of sampling on copper and zinc-coated steel and until the end of the sampling period (14 days) on PE and VA 1.4301. Even though it cannot be concluded that other organisms will behave similarly, these results suggest that an organism or pathogen entering the water distribution system after treatment may persist and be offered a habitat by the biofilm community, possibly forming part of a micro-environment for other waterborne organisms, which may enter the system.

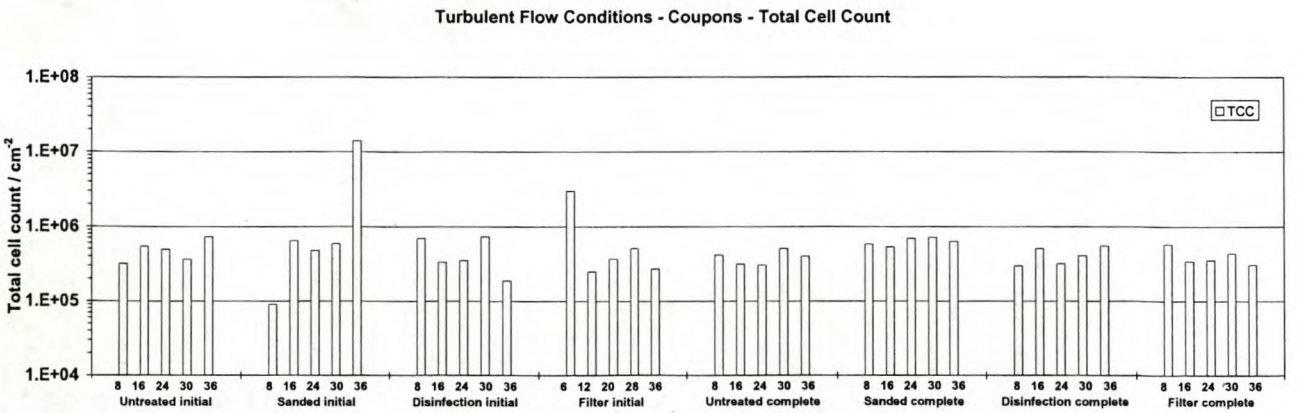
Results for the second scenario investigated, the introduction of the GFP labelled organism first to form a biofilm, followed by the introduction of the mixed

community, revealed that the tagged organism could typically only be detected until the 6th day of sampling on VA 1.4301 and copper and until the 8th day of sampling on PE and zinc-coated steel. Irrespective of scenario investigated, results obtained suggest that an organism entering the distribution system after treatment will persist and either be taken up by an established biofilm community or may even become a dominant organism in the biofilm community.

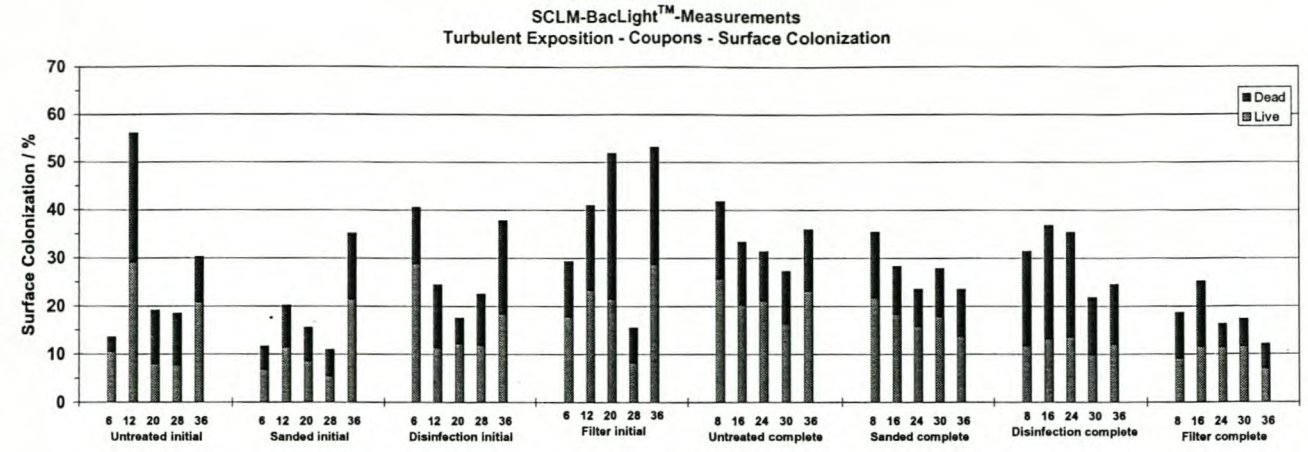


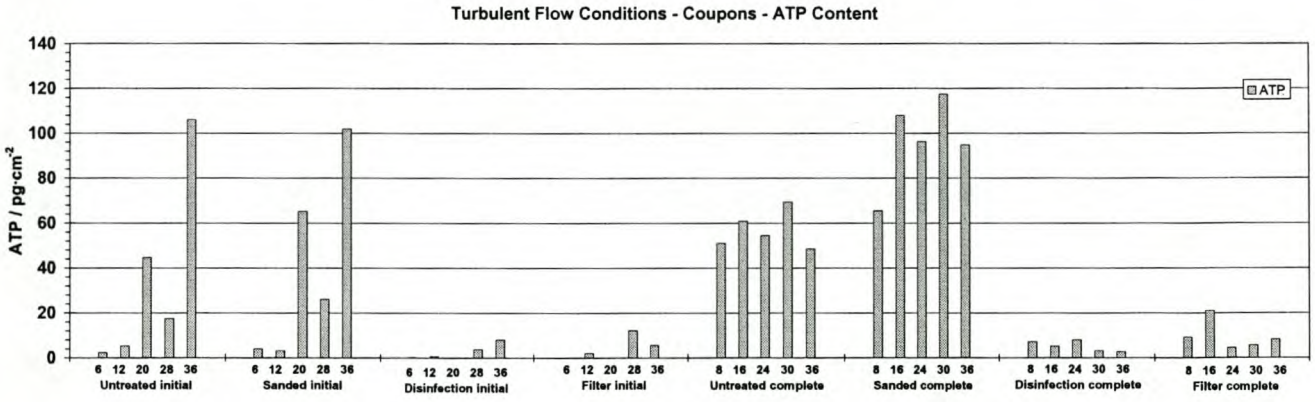


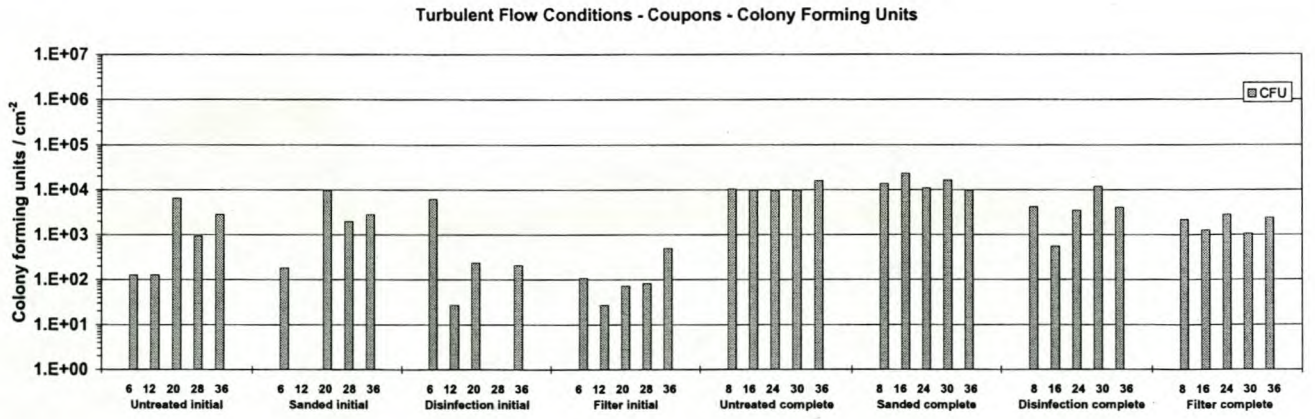


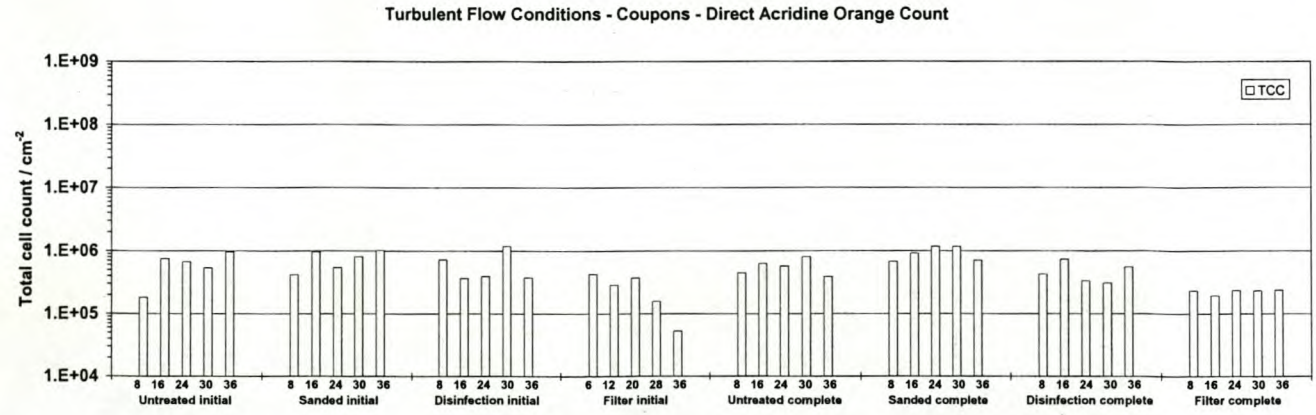


Addendum (a) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for POLYETHYLENE (PE)

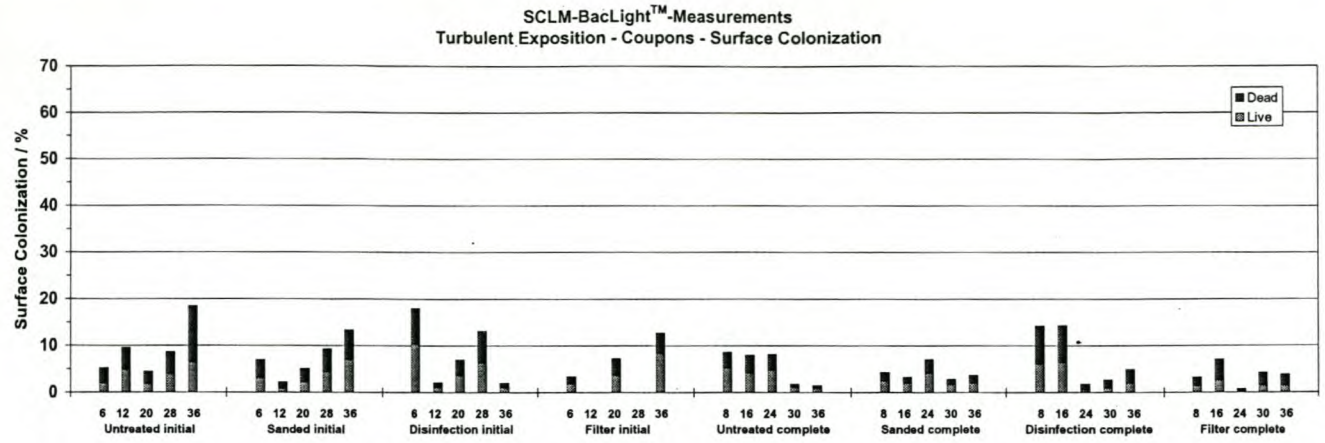


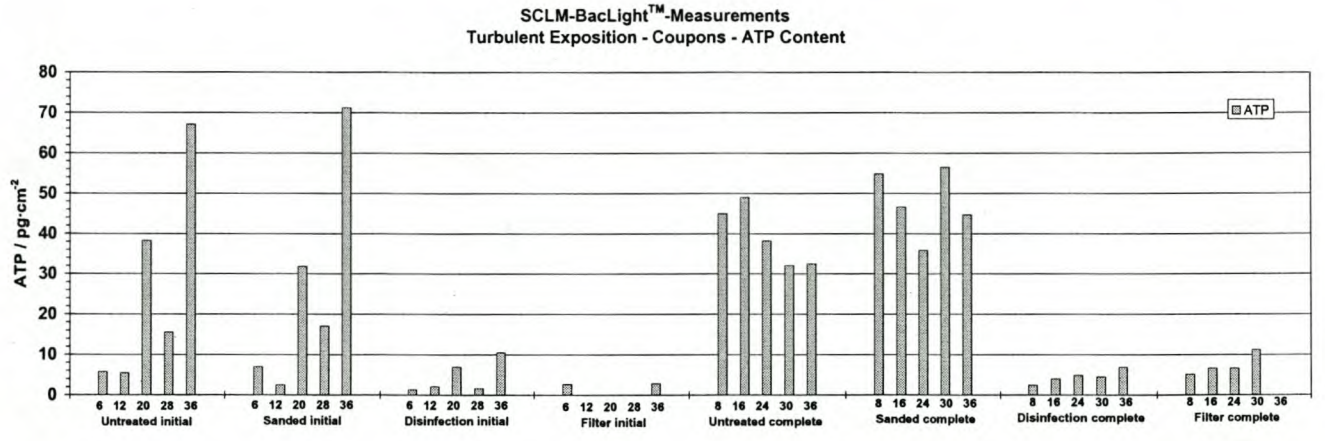


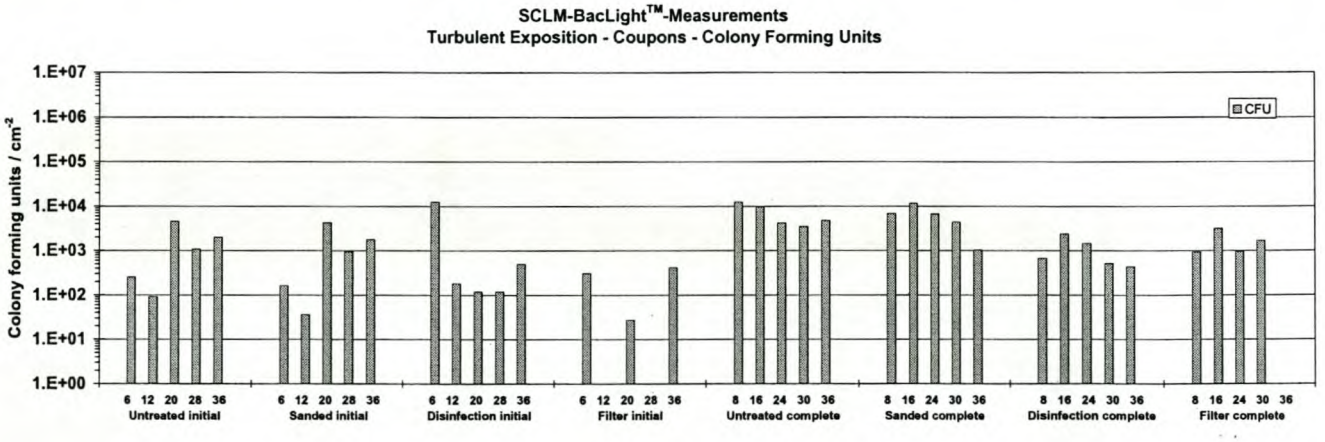


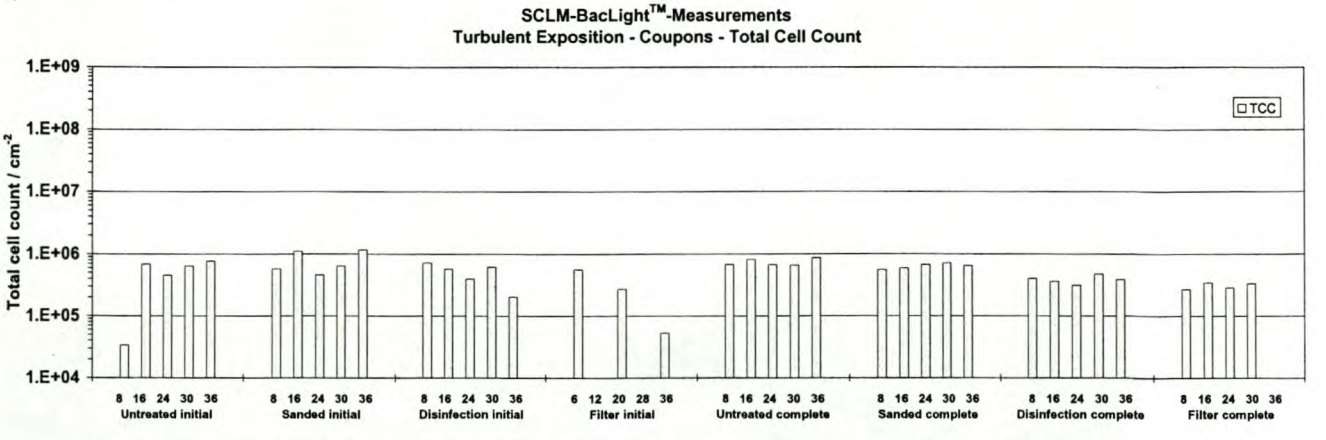


Addendum (b) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for POLYVINYLCHLORIDE (PVC)

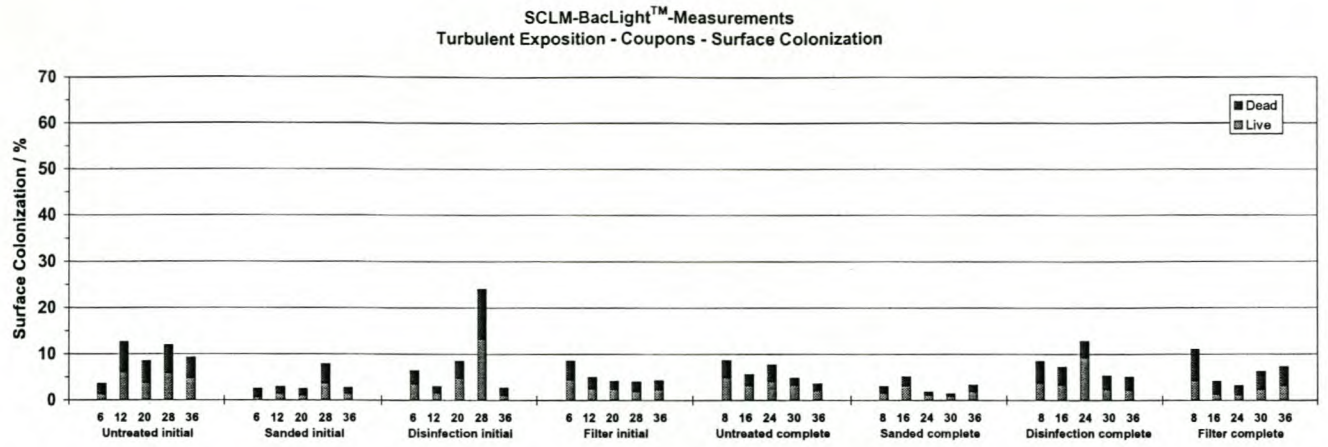


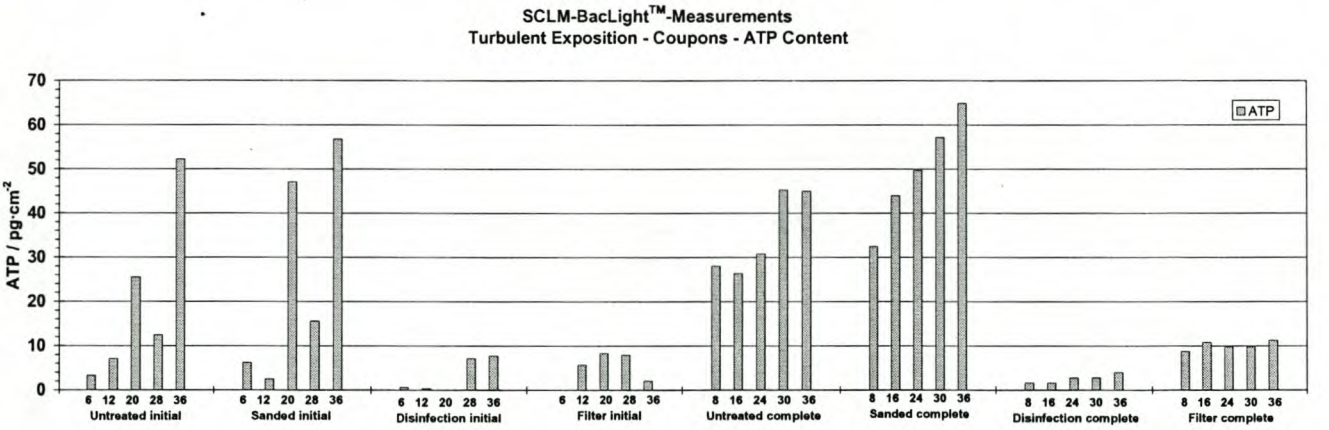


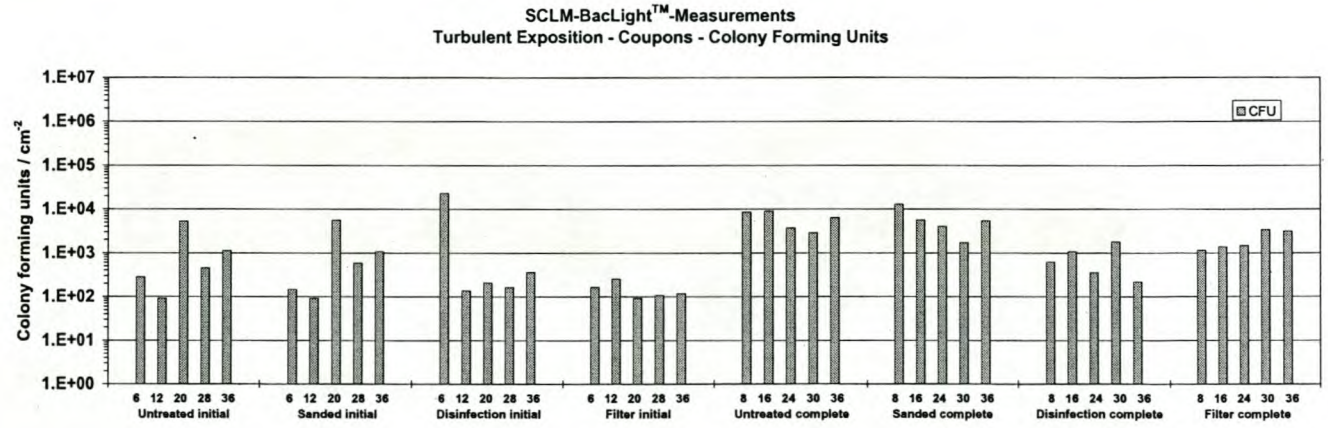


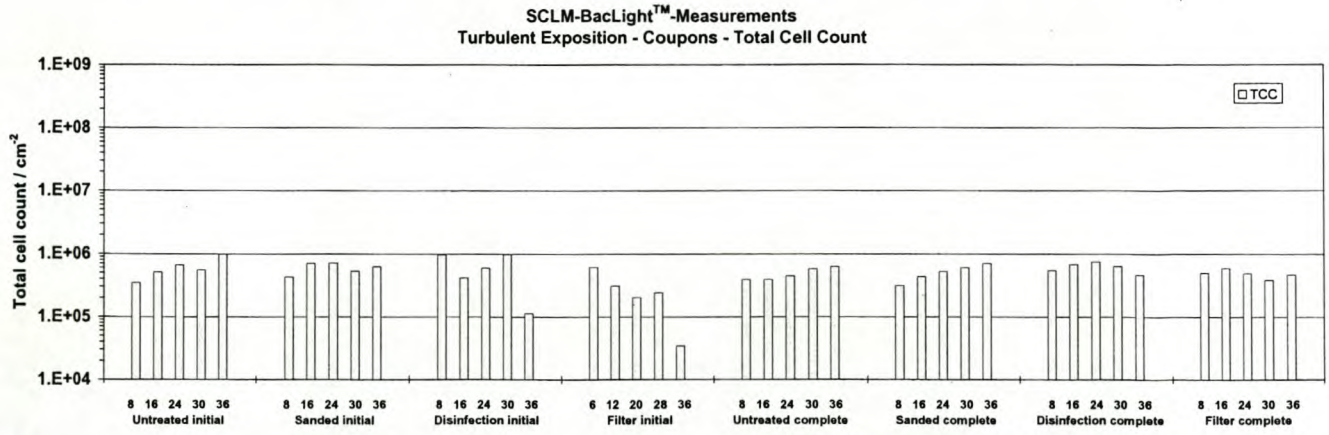


Addendum (c) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for VA 1.4301 (SS 304)

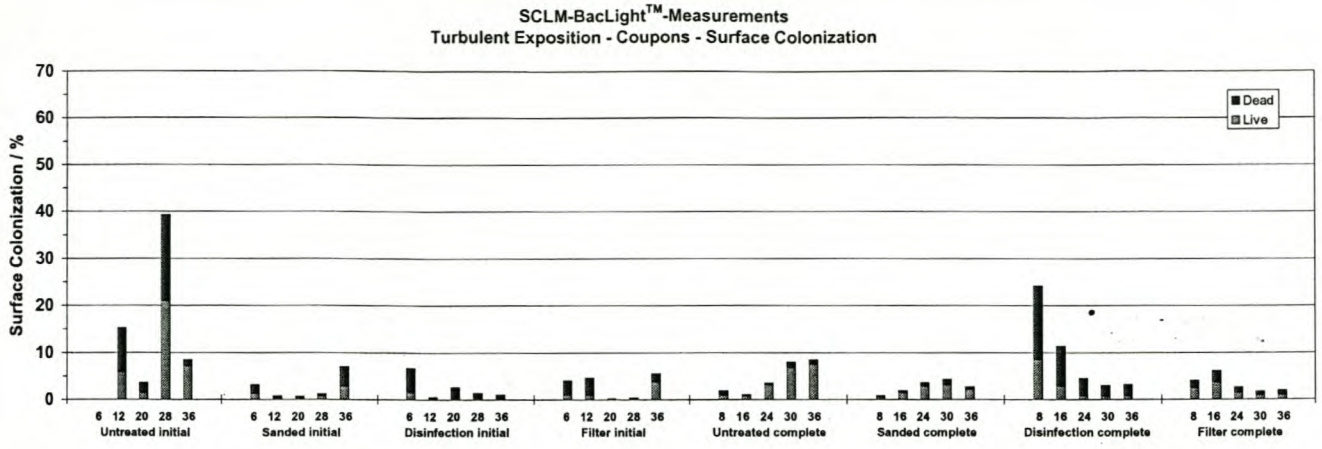


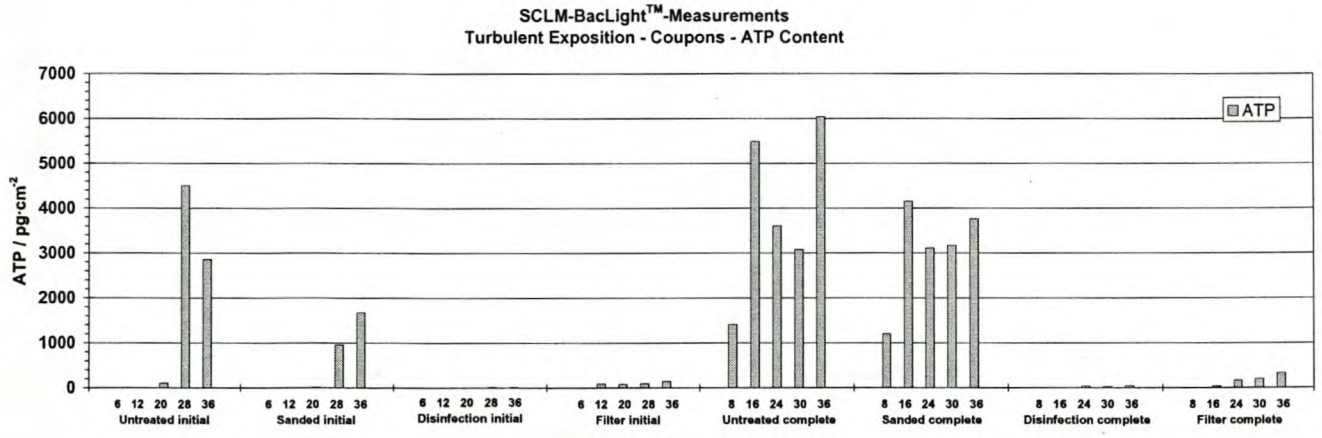


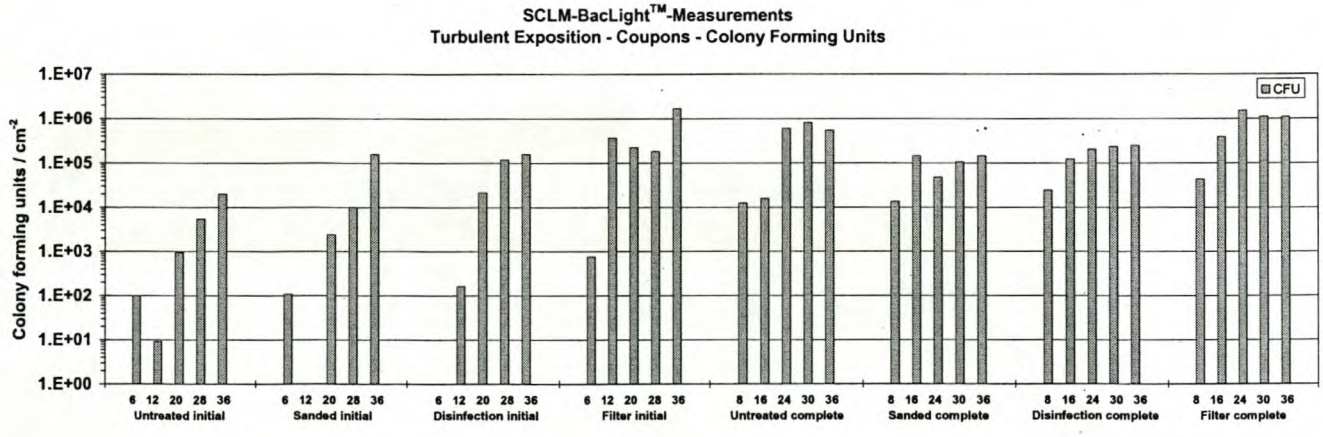


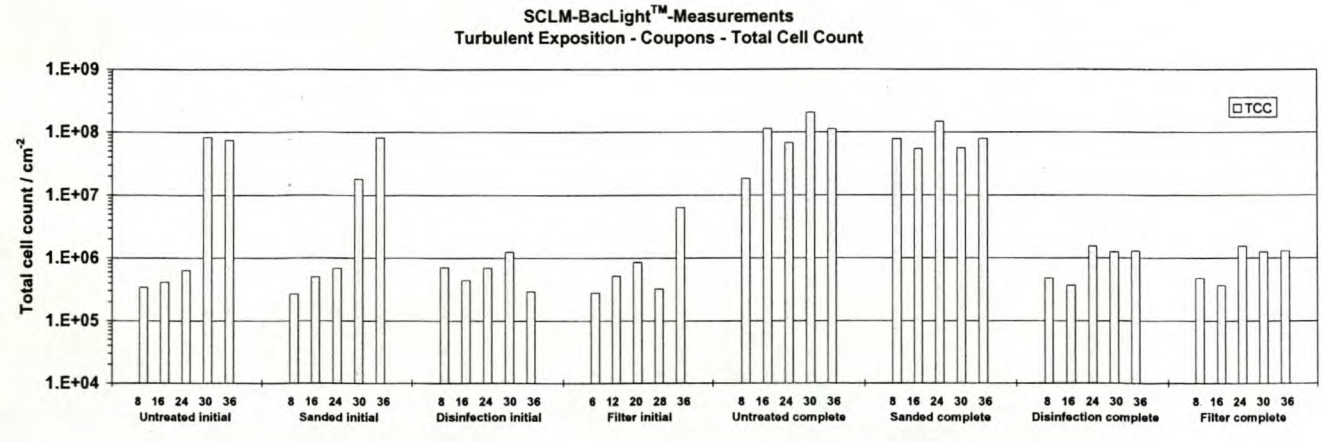


Addendum (d) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for VA 1.4571 (SS 316)

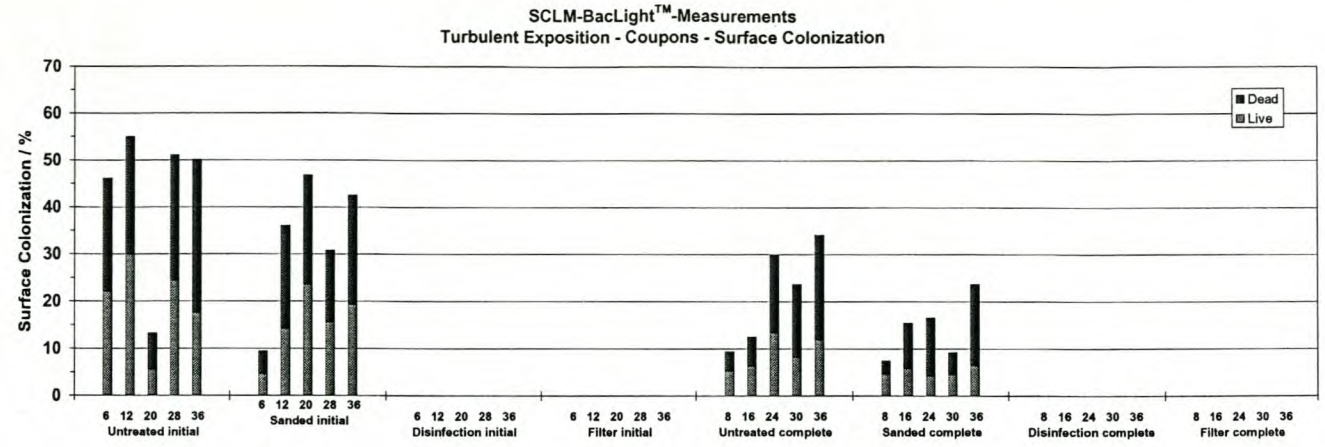


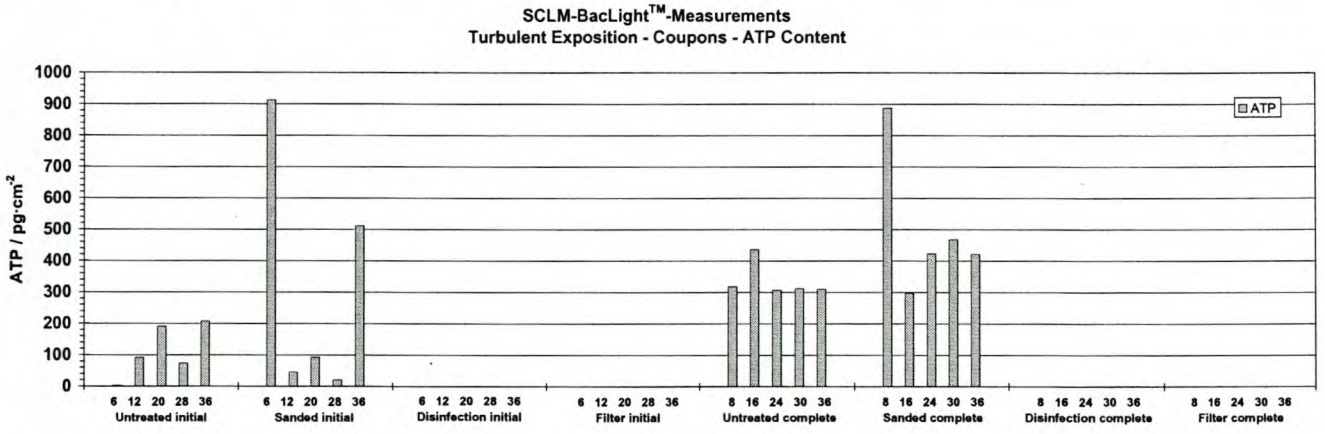


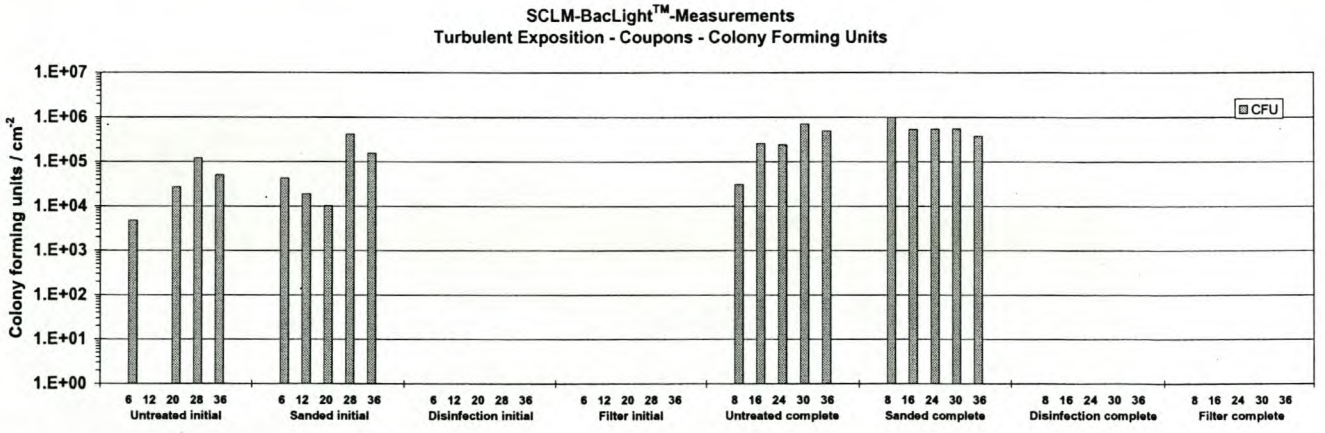


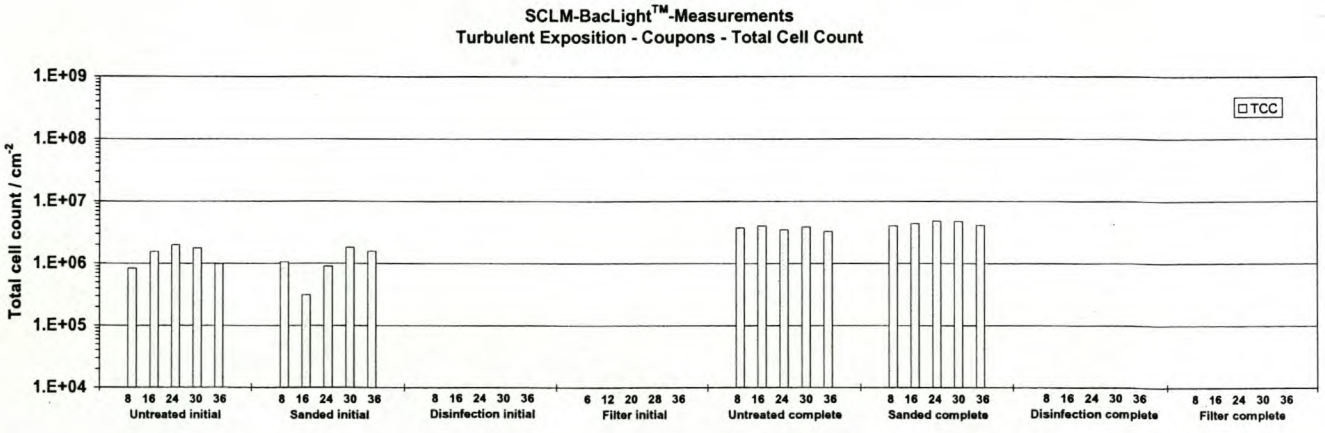


Addendum (e) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for COPPER

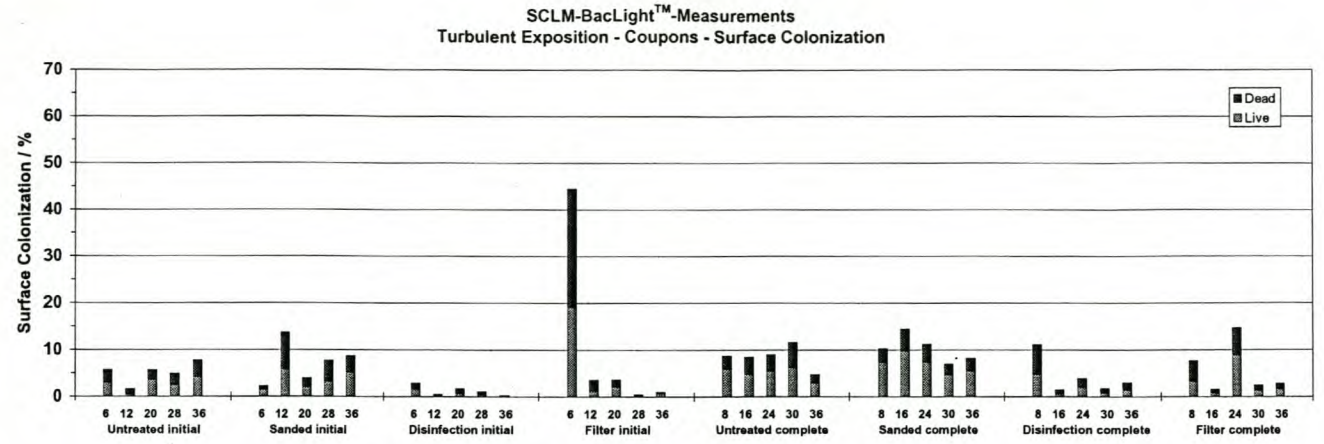


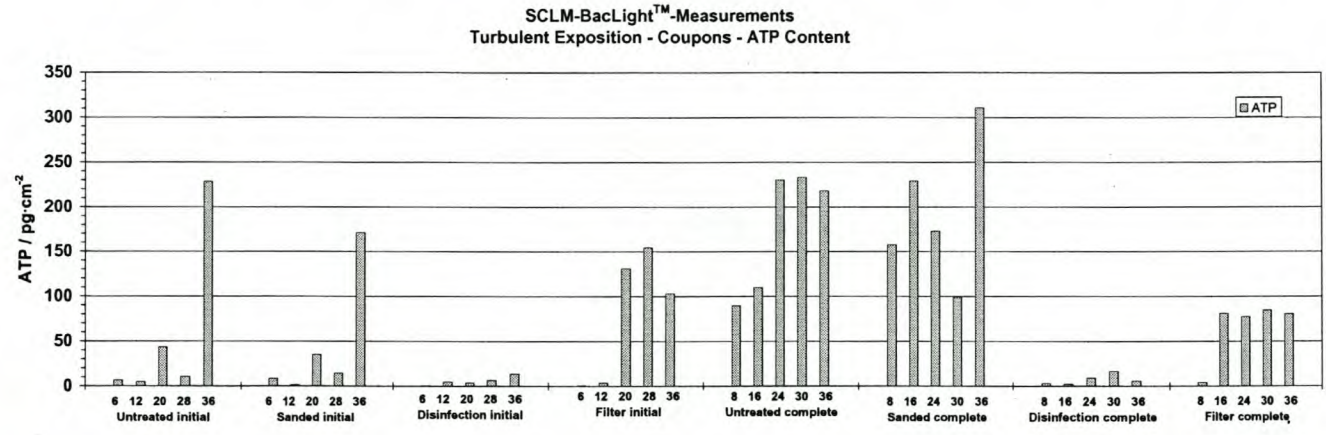


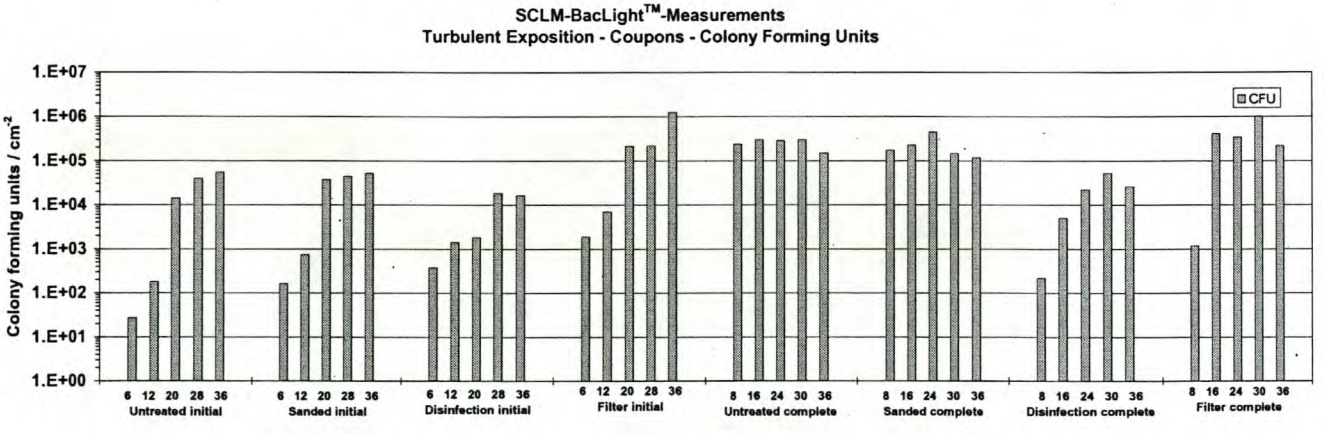


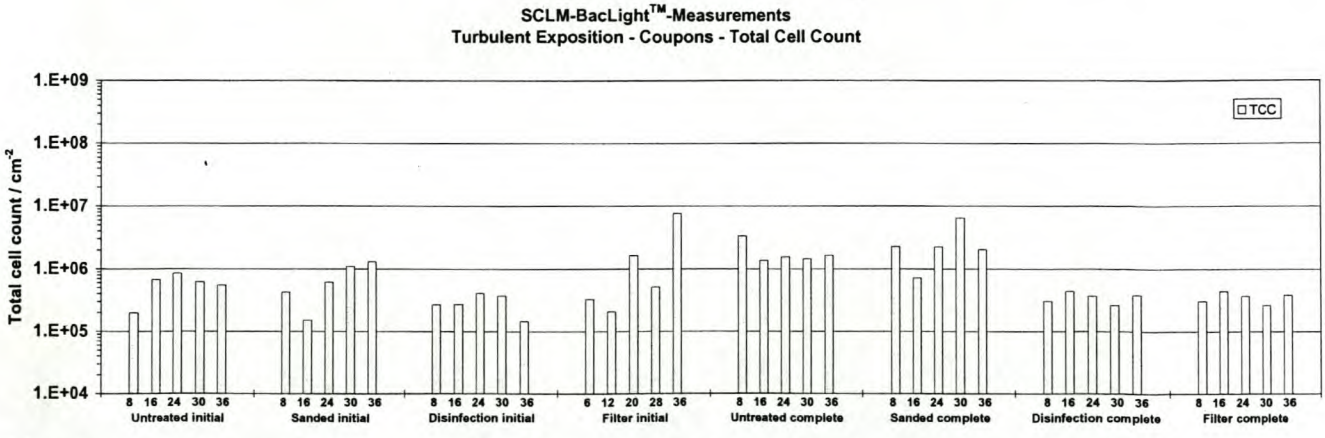


Addendum (f) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for ALUMINIUM

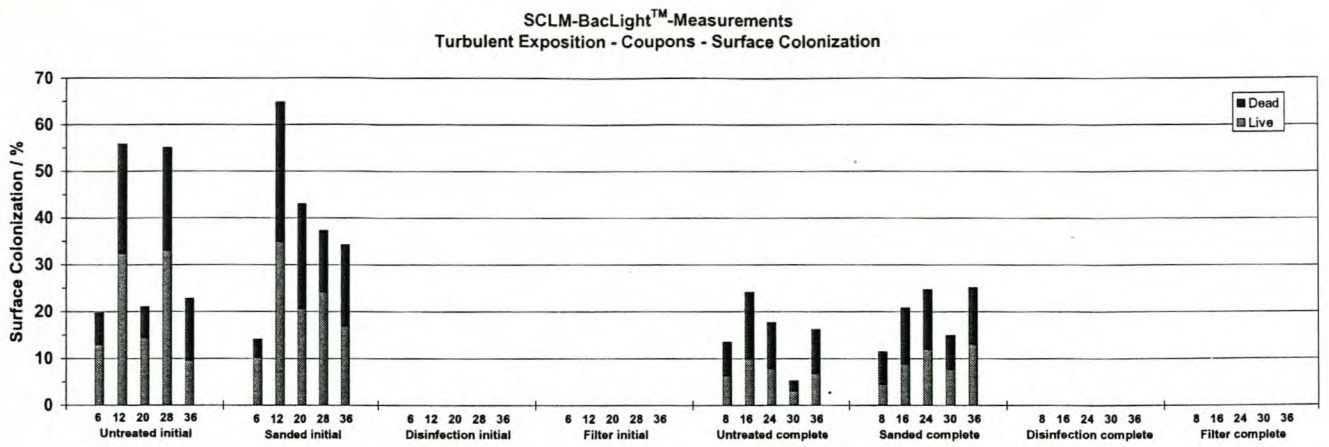


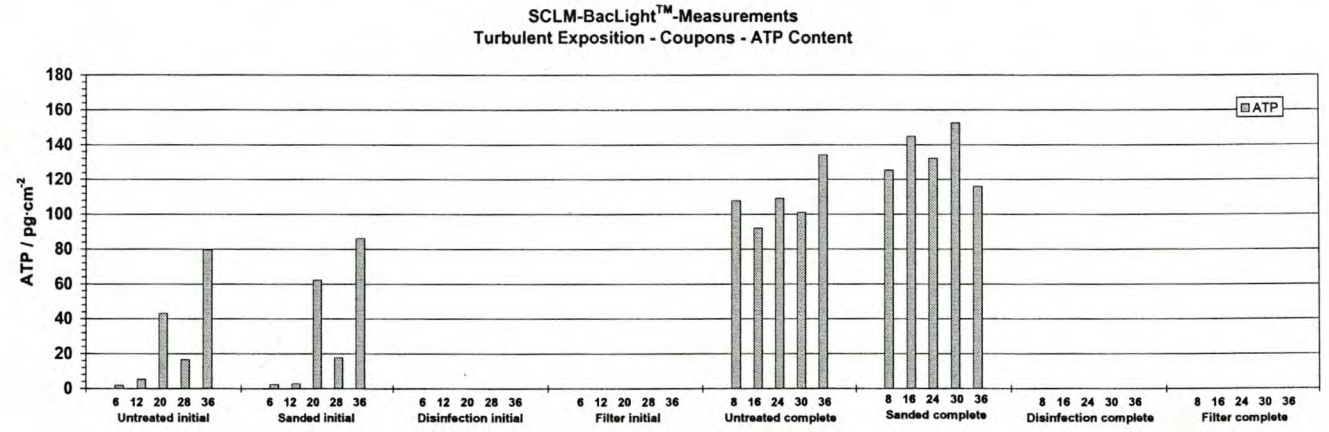


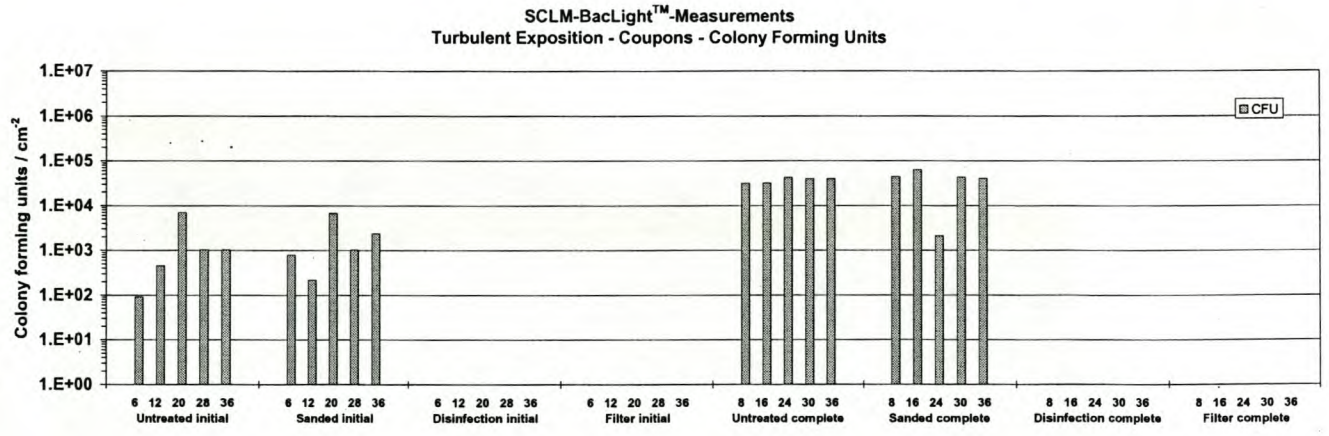


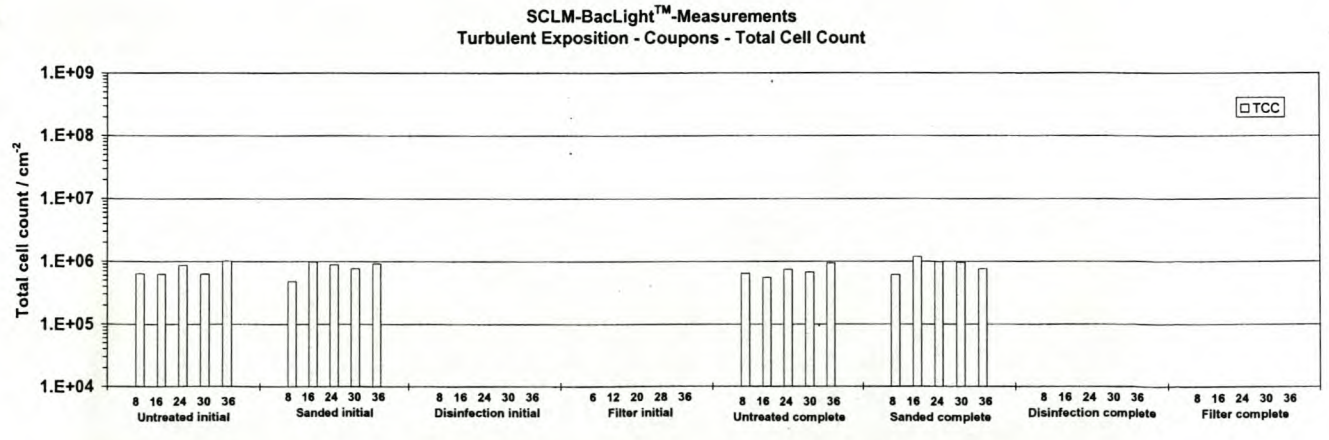


Addendum (g) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for ZINC-COATED STEEL

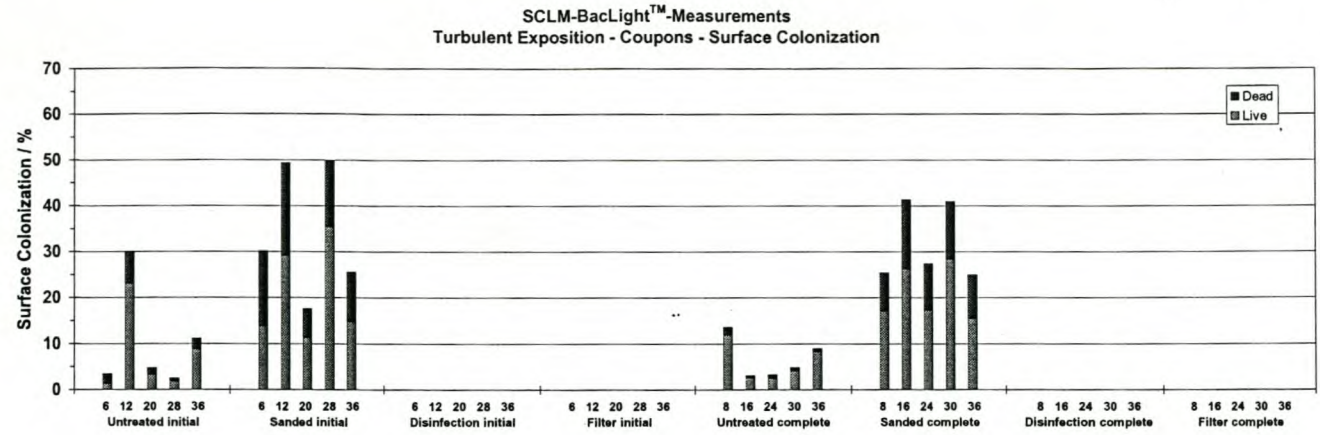


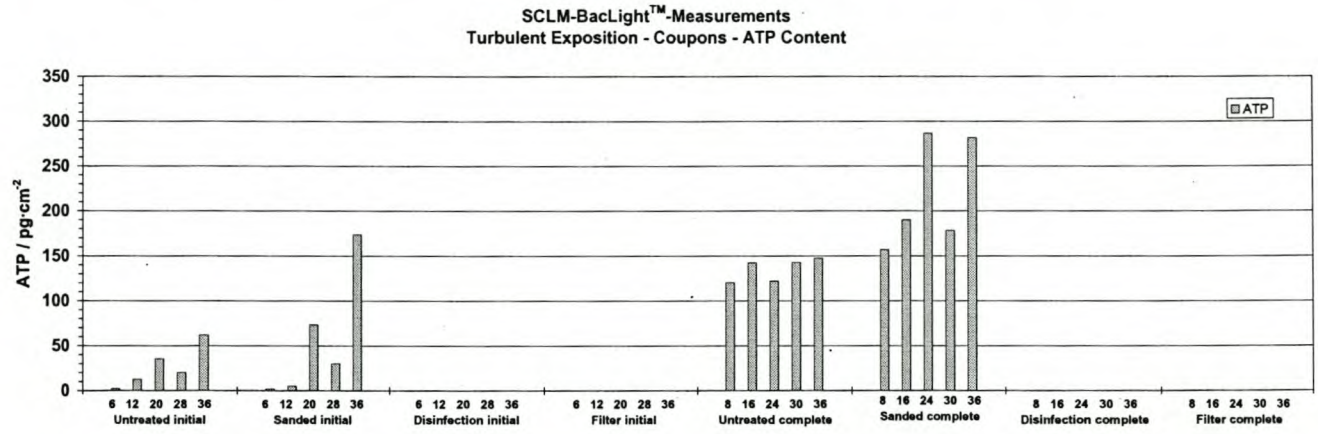


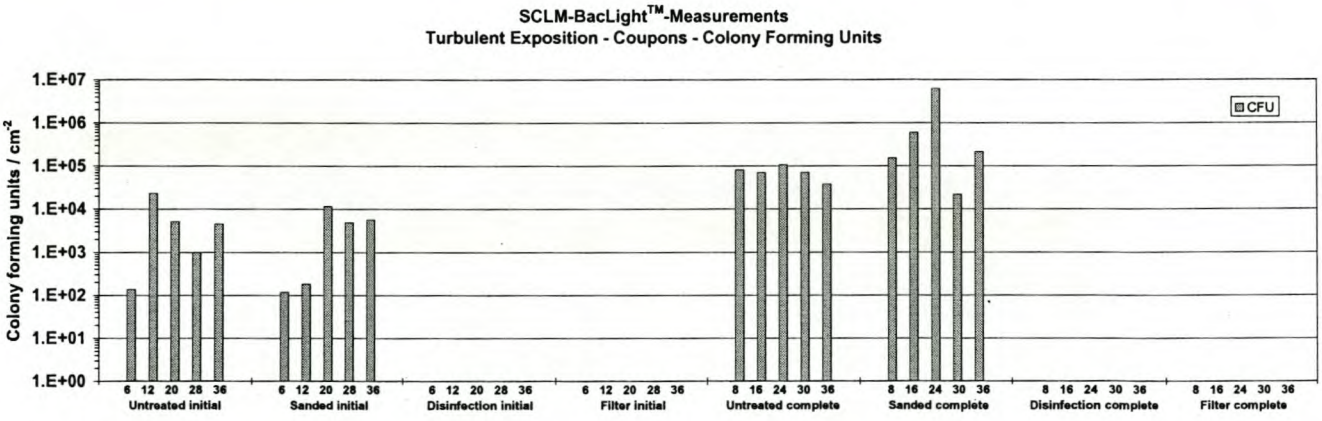


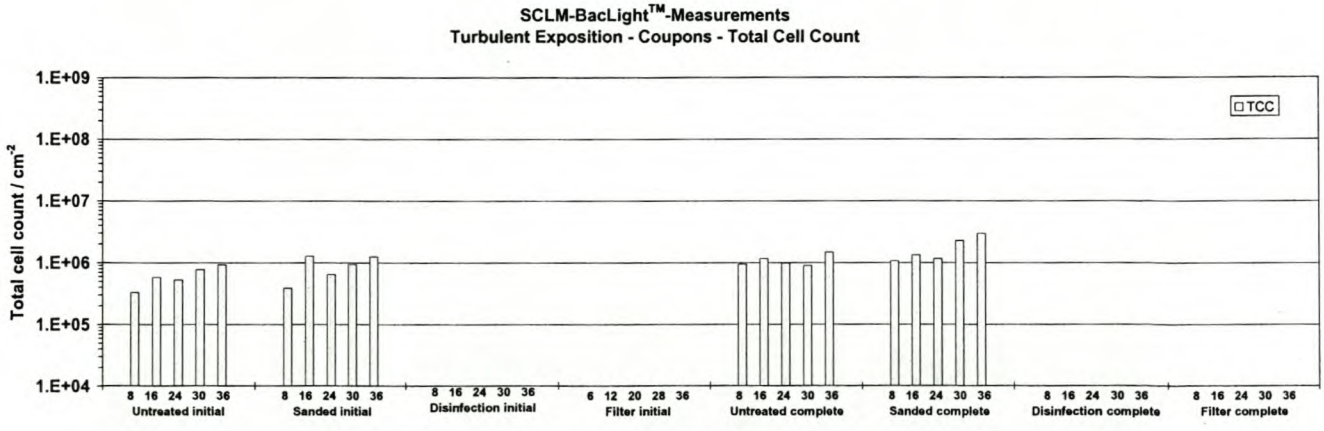


Addendum (h) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for TEFLON (PTFE)

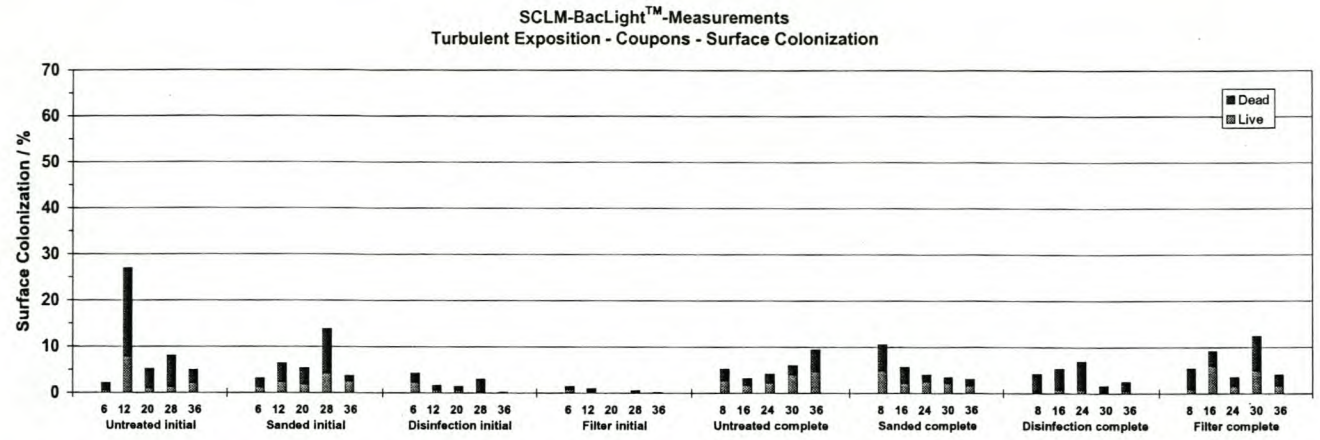


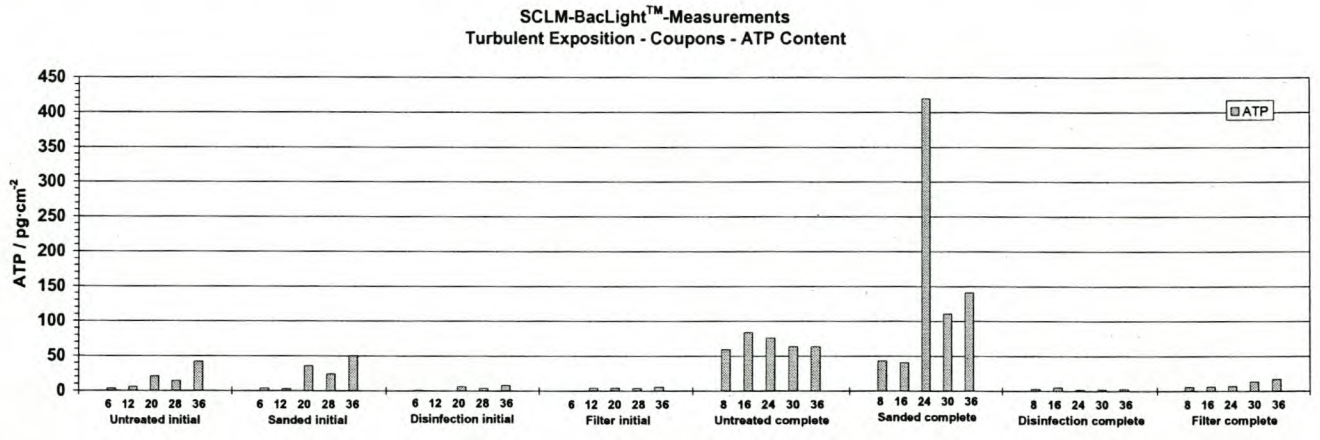


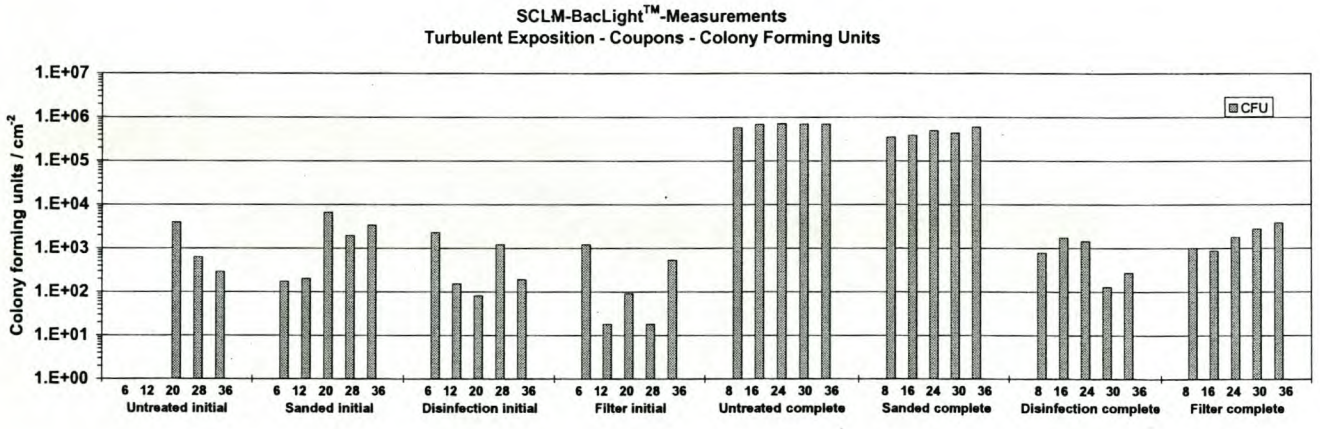


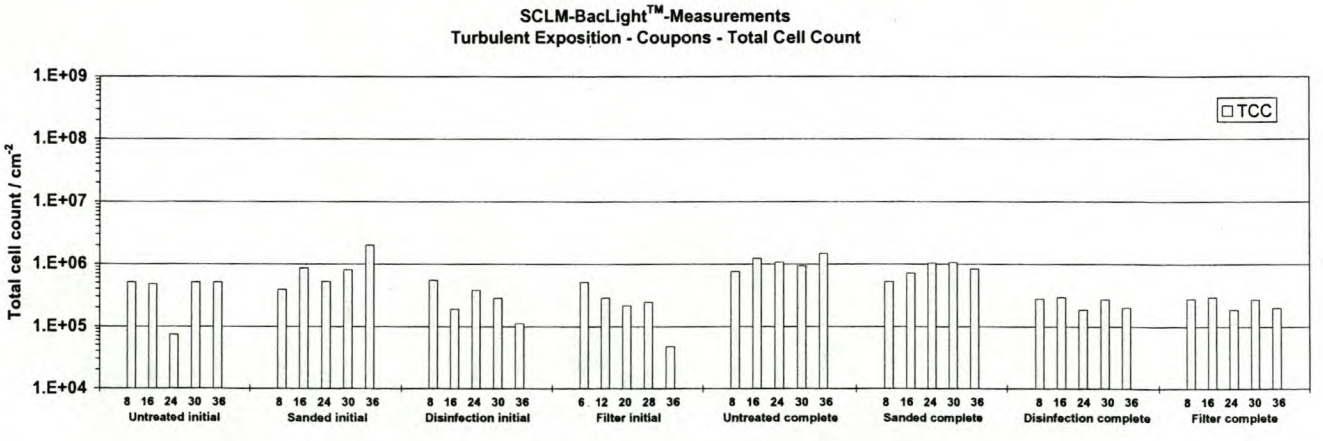


Addendum (i) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for PLEXIGLASS (PMMA)









Addendum (j) An overall summary of results obtained for surface coverage and Live/Dead ratio, direct acridine orange count, ATP concentration and viable plate count (CFU) for GLASS