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Investigation into the metal contamination of three rivers in the Western Cape and the subsequent application of a bioreactor system as remediation technology

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INVESTIGATION INTO THE METAL CONTAMINATION OF THREE RIVERS IN THE WESTERN CAPE AND THE SUBSEQUENT APPLICATION OF A BIOREACTOR SYSTEM AS REMEDIATION TECHNOLOGY

by

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Supervisor: AProf. Wesaal Khan
Co-supervisor: Dr. James Odendaal

Bellville

DECLARATION

I, Vanessa Angela Jackson, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

River systems can become contaminated with micro-organisms and metals and the routine monitoring of these rivers is essential to control the occurrence of these contaminants in water bodies. This study was aimed at investigating the metal contamination levels in the Berg-, Plankenburg- and Diep Rivers in the Western Cape, South Africa, followed by the remediation of these rivers, using bioreactor systems.

Sampling sites were identified and samples [water, sediment and biofilm (leaves, rocks and glass, etc.)] were collected along the Berg- and Plankenburg Rivers from May 2004 to May 2005 and for the Diep River, from February 2005 to November 2005. The concentrations of aluminium (Al), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) were determined using the nitric acid digestion method and analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES).

For the Berg River, the highest concentrations in water samples were recorded for Al, Mn and Fe at the agricultural area (Site A – chapter 2). In the sediment and biofilm samples, the highest metal concentrations were once again recorded for Al and Fe. The concentrations of Al and Fe were significantly higher ($p < 0.05$) than than Cu, Zn, Pb, Ni and Mn in water, sediment and biofilm samples, and were mostly higher than the quality guidelines recommended by the Department of Water Affairs and Forestry (DWAF, 1996) and the Canadian Council for the Ministers of the Environment (CCME, 2001). Possible sources of contamination in the Berg River could be due to the leaching or improper discarding of household waste from the informal- and established residential areas, as well as the improper discarding of pesticides at the agricultural area.

For both the Plankenburg and Diep Rivers the Al and Fe concentrations were higher than all the other metals analysed for in sediment and water samples. The highest concentrations recorded in the Plankenburg River was 13.6 $mg.l^{-1}$ (water - Week 18, Site B) and 15 018 $mg.kg^{-1}$ (sediment - Week 1, Site C) for Al and 48 $mg.l^{-1}$ (water - Week 43, Site A) and 14 363.8 $mg.kg^{-1}$ (sediment - Week 1, Site A) for Fe. The highest concentrations recorded in the Diep River was 4 $mg.l^{-1}$ (water - Week 1, Site A) and 19 179 $mg.kg^{-1}$ (sediment - Week 1, Site C) for Al and 513 $mg.l^{-1}$ (water - Week 27, Site A) and 106 379.5 $mg.kg^{-1}$ (sediment - Week 9, Site C) for Fe. For most of the metals analysed the concentrations were higher than the recommended water quality

guidelines as stipulated by the Department of Water Affairs and Forestry (DWAF, 1996b), the Canadian Council for the Ministers of the Environment (CCME, 2001) and the 'World average' (Martin and Windom, 1991). Point sources of pollution could not conclusively be identified, but the industrial and residential areas could have influenced the increased concentrations. Metal concentrations should be routinely monitored and the guidelines should be updated and revised based on the current state of the rivers and pollution influences.

Micro-organisms isolated from flow cells after exposure to varying metal concentrations were investigated for possible metal-tolerance. A site where high metal concentrations were recorded along the Plankenburg River was investigated. The micro-organisms isolated from the flow cells were cultured and identified using the Polymerase Chain Reaction (PCR) technique, in conjunction with universal 16SrRNA primers. The phylogeny of the representative organisms in GenBank, were analysed using the Neighbour-joining algorithm in Clustal X. After exposure, the channels were stained with the LIVE/DEAD BacLight^{TM} viability probe and visualised using Epifluorescence Microscopy. The results revealed that when exposed to the highest concentrations of Al (900 mg.l^{-1}), Fe (1000 mg.l^{-1}), Cu (10 mg.l^{-1}) and Mn (80 mg.l^{-1}), the percentage of dead cells increased, and when exposed to the lowest concentrations of Al (10 mg.l^{-1}), Cu (0.5 mg.l^{-1}), Mn (1.5 mg.l^{-1}) and Zn (0.5 mg.l^{-1}), no significant differences could be distinguished between live an dead cells. When exposed to the highest concentrations of Zn (40 mg.l^{-1}) and Ni (20 mg.l^{-1}), no significant differences between the live and dead cell percentages, were observed. The phylogenetic tree showed that a diverse group of organisms were isolated from the flow cells and that some of the isolates exhibited multiple metal resistance (*Stenotrophomonas maltophilia* strain 776, *Bacillus* sp. ZH6, *Staphylococcus* sp. MOLA:313, *Pseudomonas* sp. and *Delftia tsuruhatensis* strain A90 exhibited tolerance to Zn, Ni, Cu, Al, Fe), while other isolates were resistant to specific metals (*Comamonas testosteroni* WDL7, *Microbacterium* sp. PAO-12 and *Sphingomonas* sp. 8b-1 exhibited tolerance to Cu, Ni and Zn, respectively, while *Kocuria kristinae* strain 6J-5b and *Micrococcus* sp. TPR14 exhibited tolerance to Mn).

The efficiency of two laboratory-scale and one on-site bioreactor system was evaluated to determine their ability to reduce metal concentrations in river water samples. The laboratory-scale bioreactors were run for a two-week and a three-week

period and the on-site bioreactor for a period of ten weeks. Water (all three bioreactors) and bioballs (bioreactor two and on-site bioreactor) were collected, digested with 55% nitric acid and analysed using ICP-AES. The final concentrations for Al, Ni and Zn (bioreactor one) and Mn (bioreactor two), decreased to below their recommended concentrations in water samples. In the on-site, six-tank bioreactor system, the concentrations for Fe, Cu, Mn and Ni decreased, but still exceeded the recommended concentrations. The concentrations recorded in the biofilm suspensions removed from the bioballs collected from bioreactor two and the on-site bioreactor, revealed concentrations higher than those recorded in the corresponding water samples for all the metals analysed, except Fe. The bioballs were shown to be efficient for biofilm attachment and subsequent metal accumulation. The species diversity of the organisms isolated from the bioreactor (bioreactor two) experiment after three days (initial) differed from the organisms isolated after 15 days (final). *Hydrogenophaga* sp., *Ochrobactrum* sp, *Corynebacterium* sp., *Chelatobater* sp. and *Brevundimonas* sp. were present only at the start of the bioreactor experiment. The surviving populations present both in the beginning and at the end of the bioreactor experiment belonged predominantly to the genera, *Pseudomonas* and *Bacillus*. Metal-tolerant organisms, such as *Bacillus*, *Pseudomonas*, *Micrococcus and Stenotrophomonas*, amongst others, could possibly be utilised to increase the efficiency of the bioreactors. The bioreactor system should however, be optimised further to improve its efficacy.

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BIOGRAPHICAL SKETCH

Vanessa Angela Jackson was born in Cape Town, South Africa, on the 6th April 1976. She attended Ridgeville Primary School and matriculated at Cedar High School in 1993. She enrolled at the University of the Western Cape in 1994 and obtained a B.Sc. degree in Microbiology and Biochemistry in 1997. In 1999 she completed a B.Sc. (Hons.) degree in Microbiology at the same university. Vanessa enrolled at the University of Stellenbosch in 2000 and obtained her M.Sc. degree in Microbiology in 2004. She is presently employed in a contract position at the Cape Peninsula University of Technology in the capacity of lecturer.

DEDICATION

This thesis is dedicated to my family for their unwavering support, love and understanding during trying times

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obtained from bioballs during the course of the bioreactor run. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. The conserved regions are indicated with Roman numerals. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.

GLOSSARY

ASBR	Anaerobic Sequencing Batch Reactor
Al	Aluminium
AAS	Atomic Absorption Spectrometry
AES	Atomic Emission Spectrometry
BOD	biochemical oxygen demand
CLSM	confocal laser scanning microscopy
CDSC	Campylobacter Sentinel Surveillance Scheme
COD	Chemical Oxygen Demand
Cu	Copper
RDX	Cyclotrimethylenetrinitramine
HMX	cyclotetramethylene-tetranitramine
DGT	diffuse gradients in thin films
DO	dissolved oxygen
DDT	dichlorodiphenyl-trichloroethane
DGGE	denaturing gradient gel electrophoresis
EPS	extracellular polymeric substances
EPEC	Enteropathogenic *Escherichia coli's*
EC	Electrical Conductivity
EFM	epifluorescence microscopy
FAAS	Flame atomic absorption spectrometry
FWS	free-water surface
GC/MS	Gas Chromatography/Mass Spectrometry
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
INAA	Instrumental Neutron Activation Analysis
Fe	Iron
kg COD/m3	Kilogram chemical oxygen demand per cubic metre
kg COD $m^{-3}kg^{-1}$	Kilogram chemical oxygen demand per cubic metre per kilogram

Pb	Lead
Mn	Manganese
MMT	methylcyclopentadienyl manganese tricarbonyl
$mg.l^{-1}$	Milligrams per litre
$mg.kg^{-1}$	Milligrams per kilogram
$m^3 kg^{-1}$ COD d^{-1}	Kilogram per cubic metre chemical oxygen demand per day
$ng.g^{-1}$	Nannograms per gram
nM	nannometres
NRR	neutral red retention time assay
Ni	Nickel
Ni-DOC	Nickel-Dissolved Organic Carbon
$ng.m^{-3}$	Nannograms per cubic metre
NTA	nitrilotriacetic acid
OSHA	Occupational Safety and Health Administration
SIRAN	open-pore sintered glass beads
OCPs	organochlorine pesticides
Ppb	Parts per billion
ppm	Parts per million
PICT	pollution-induced community tolerance concept
PCB's	polychlorinated biphenyls
PCR	Polymerase Chain Reaction
PCR-DGGE	Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis
PAHs	Polycyclic Aromatic Hydrocarbons
PFGE	Pulse Field Gel Electrophoresis
RBC	rotating biological contactor
SSF	subsurface flow
TDS	Total Dissolved Solids
TNT	trinitrotoluene
TXRF	Total Reflection X-ray Fluorescence Spectrometric
TPE-LSM	Two Photon Excitation Laser Scanning Microscopy

2,4-DCP	2,4-dichlorophenol
µg/decilitre	Micrograms per decilitre
$\mu g.g^{-1}$	Micrograms per gram
$ug.l^{-1}$	Micrograms per litre
µm	micrometre
UAFF	Upflow Anaerobic Fixed Film
UASB	Upflow Anaerobic Sludge Blanket
USFF	Upflow Stationary Fixed Film
VBNC	Viable but Non Culturable
XRF	X-Ray Fluorescence Spectrometry
Zn	Zinc

LIST OF REFERENCE MAPS

LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Water Distribution and the Water Cycle

Life on earth is significantly linked to the quality and distribution of the essential element, water. This collective mass of water covers 71% of the planet, which is essentially divided into saltwater, namely oceans, and freshwater, namely rivers, lakes, groundwater and glaciers, amongst others (**Figure 1.1**). Only 3% of the water content is composed of fresh water or water suitable for drinking purposes, while the remaining 97% is made up of saltwater. Glaciers, ice caps and snow makes up the majority of freshwater on earth (68.7%), with groundwater making up 31.3% of the available freshwater (3%) (US Department of the Interior, 2006).

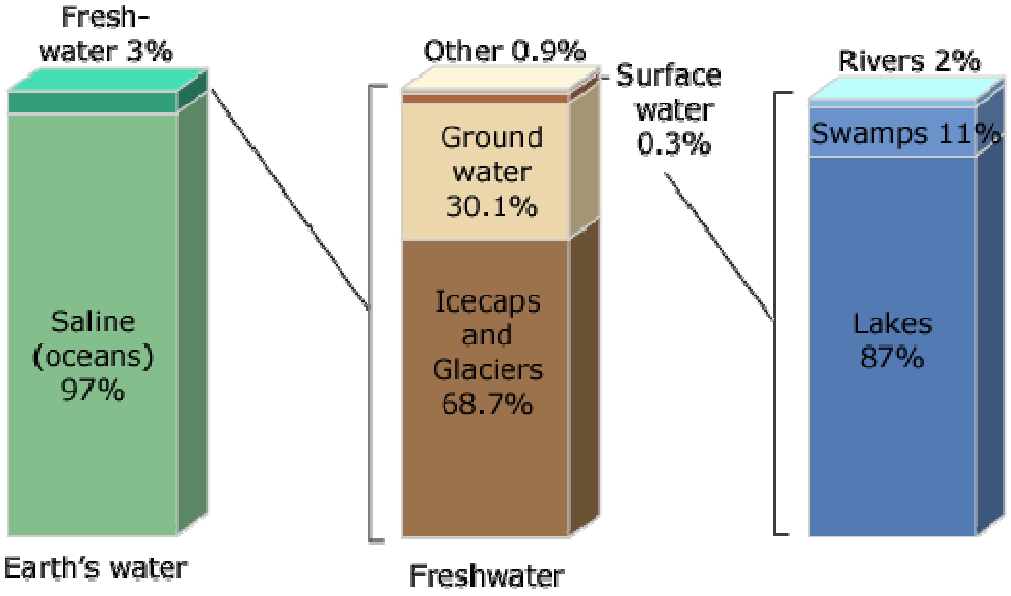

Figure 1.1. Water distribution on earth (US Department of the Interior, 2006).

The majority of available water is temporarily stored in oceans, lakes, ice caps and underground aquifers, rather than in motion in the water cycle, which is a key process in the earth's hydrosphere (**Figure 1.2**). The water cycle is generally defined as the movement of water on, in and above the earth's atmosphere, that is from the ocean to the atmosphere, back to the land and then subsequently back to the ocean (United States Geological Report, 2000; Richardson *et al.*, 2001). It includes the water in rocks (lithosphere), in plants and animals

(biosphere), in the atmosphere (precipitation, water vapour, clouds), as well as water covering the earth's surface and that beneath it.

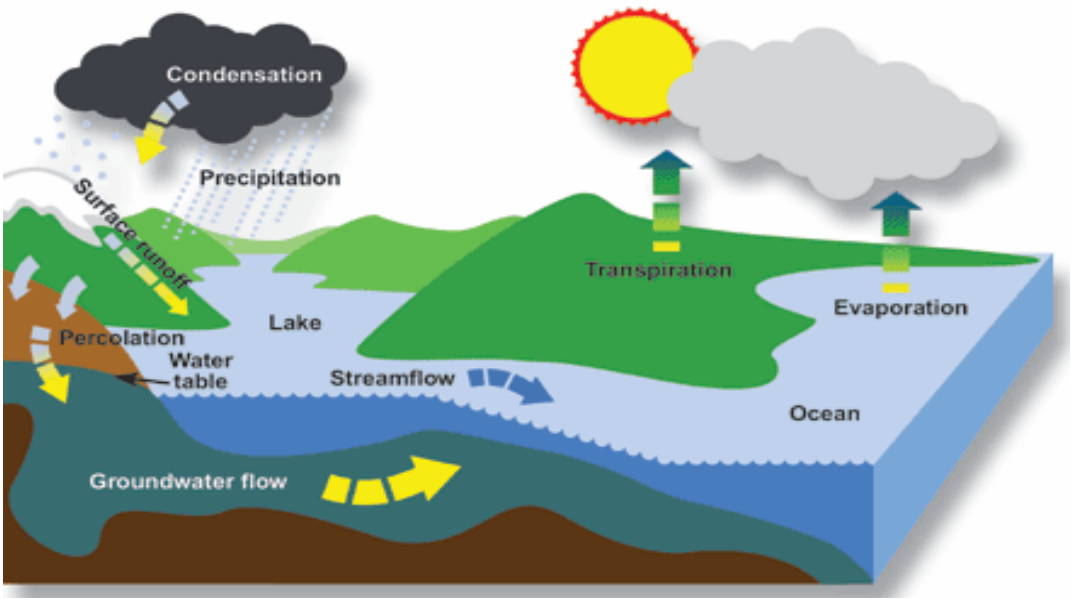

Figure 1.2. Water cycle (The Green Lane™, 2004).

The sun heats the water in the oceans, where some of the water, along with the water in the soil, and that which transpires from plants, evaporates back into the atmosphere. As the vapour rises into the air, the cooler temperatures causes it to condense into clouds. The water in the clouds precipitates, with a portion falling to the earth as snow, and accumulating as ice caps and glaciers. Most of the precipitation however, falls into the oceans or onto land, in the form of rain, where, due to gravity, it flows over the surface of the earth (United States Geological Report, 2000; Richardson *et al.*, 2001).

Surface- and groundwater serve as the two main freshwater sources, with most of the water in rivers directly resulting in runoff from the land surface. This surface runoff or the percentage of runoff entering rivers, lakes, oceans, etc. is dependent on various factors, such as land topography, human activities and meteorology (e.g. rainfall activities). Approximately, a third of the runoff enters rivers and streams, which eventually flows into the ocean, while the remaining two thirds evaporates, transpires and seeps into the ground. Groundwater is found in aquifers, where all the pores, cracks and spaces between the rocks and particles are saturated with water. It is generally situated a few kilometres below the earth's surface, which is referred to as the zone of saturation (Hoyle, 2005).

South Africa, which is located at the Southern-most tip of the African continent, generally collects it's water resources in dams or water abstraction schemes, in order to supply industry, agriculture and domestic users. Uneven distribution of rainfall and available water per capita in

South Africa (**Figure 1.3**) exists between the western and eastern parts of the country. These higher evaporation rates and lower conversion of rainfall to runoff in the eastern regions could result in water shortages in this area (Webster, 2001). The country's urban and industrialised areas (Cape Town, Port Elizabeth, East London, Pietermaritzburg, Bloemfontein, Pietersburg, and Gauteng) are the most water stressed, and will become more so as the population and the demand for water in the urban and domestic sectors increases (Department of Environmental Affairs and Tourism, 1999a).

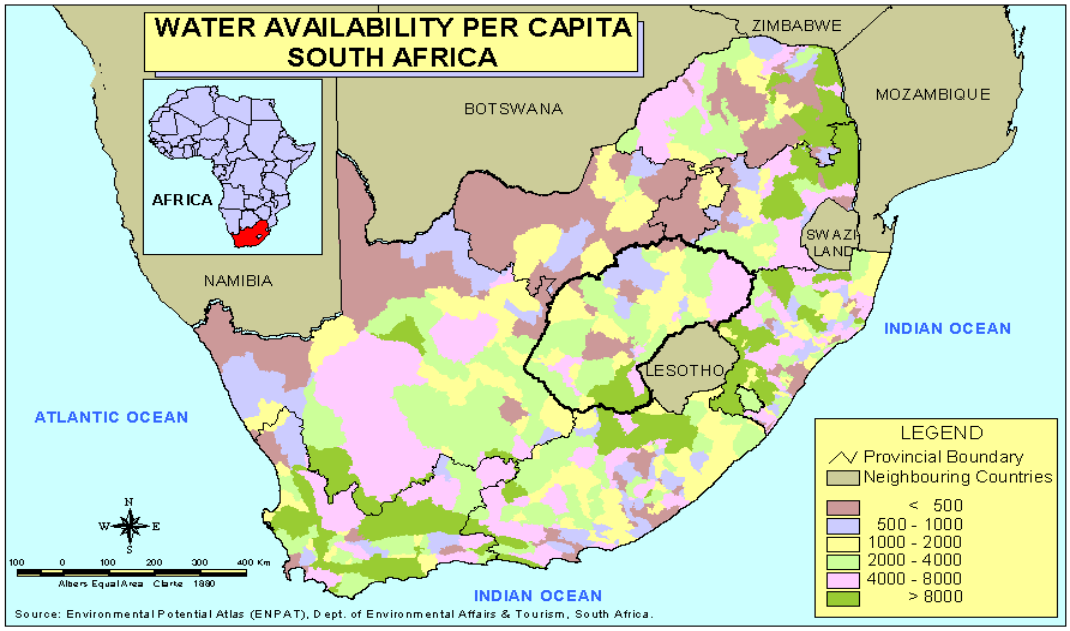

Figure 1.3. Water availability per capita in South Africa (Department of Environmental Affairs and Tourism, 1999a).

The spatial variability of water resources and the scarcity of water throughout the country implies that in many catchment schemes the demand exceeds the supply of water (**Figure 1.4**). In 1996 the water requirements in the Vaal, Lower Orange, Sundays, Great Fish, Olifants (Mpumalanga) and Crocodile/Limpopo Rivers, exceeded the amount of available water. By 2030, it is expected that the Breede/Berg basin will be added to the list of water-scarce catchments as discrepancies exist between water requirements and water availability (Department of Environmental Affairs and Tourism, 1999a).

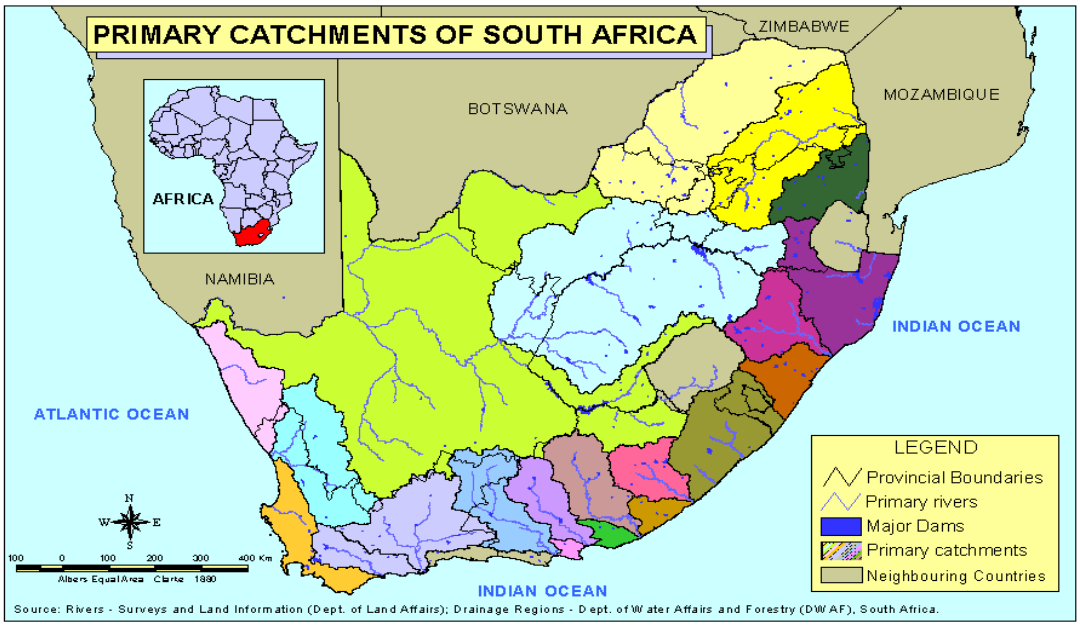

Figure 1.4. Primary water storage catchments of South Africa (Environment and Tourism, 2007).

Surface water serves as the main water resource in South Africa (Webster, 2001), and is primarily used for agricultural activities (52%), industry, mining and power generation (12.5%) and domestic and municipal uses (12%), with a further 15% needed to maintain estuaries and rivers (Schutte & Pretorius, 1997; Holtzhausen, 2002; Mack *et al.*, 2004) (**Figure 1.5**). In the North-West province of South Africa, more than 80% of rural communities depend on groundwater as their sole source, where according to the Department of Water Affairs and Forestry (DWAF, 2001), 7% of the groundwater resource was used for domestic purposes and 78% for irrigational purposes.

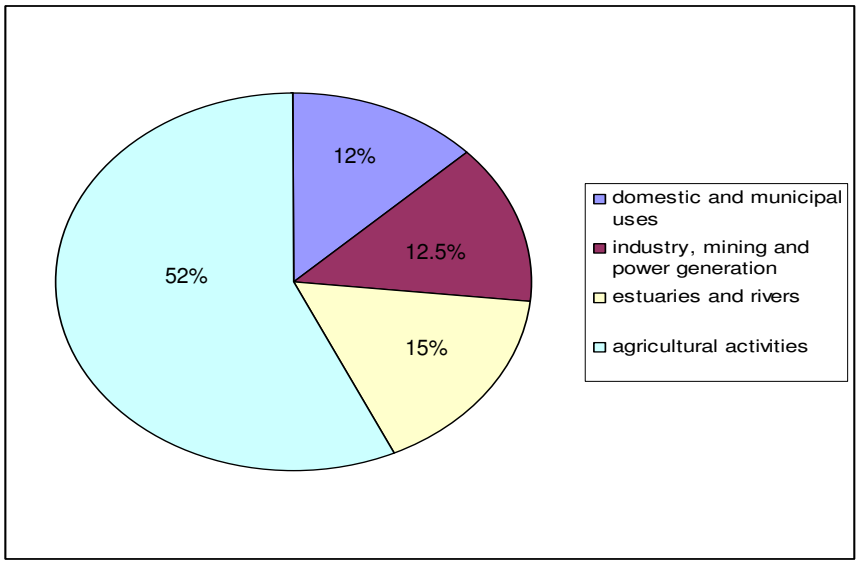

Figure 1.5. Water distribution in South Africa (Mack *et al*., 2004).

Population increases in South Africa are expected to lead to an increase in agricultural development, which will in turn lead to an increased demand for irrigation water. Agriculture is also a major contributor to the economy of the country, where the net income of the farming sector increased from 6.5 billion rand in 2000 to 9.6 billion rand in 2001 (South Africa Online, 2007). A major factor limiting agricultural activity is however, the availability of water resources. Water scarcity in South Africa, is further exacerbated by the pollution of surface- and groundwater resources. Typical pollutants of freshwater aquatic environments include industrial effluents, domestic and commercial sewage, acid mine drainage, agricultural runoff and litter. High concentrations of metals, oils, and other toxic substances, could also contribute to the overall decrease in water quality (Pegram *et al*., 1999). Of concern to water resource managers is the diffuse sources of pollution which are difficult to quantify. These sources can be clustered into two major groups, e.g. point source pollution (such as microbiological contamination and domestic sewage discharges) and nonpoint source pollution (runoff from herbicides and pesticides) (Hills *et al*., 1998; Ho *et al*., 2003).

In agriculture, groundwater pollution could result from fertiliser application, pesticide use and groundwater over-abstraction. In the coal and gold mining industries, however, the potential sources of pollution are stockpiling, slimes disposal and underground, or opencast mining areas. In the urban sector, pollution is caused by sewage effluent, leaking sewers and the lack of proper on-site sanitation at informal housing schemes. Industrial pollution arises from industrial effluent, bulk storage of chemicals, waste irrigation and air pollution. However, the increase in informal settlements, with inadequate sanitation and waste removal facilities, may become one

of the greatest localised pressures on water quality in South Africa (Department of Environmental Affairs and Tourism, 1996).

1.2 SOURCES OF CONTAMINATION

1.2.1 Microbial Contamination and Waterborne Diseases

Micro-organisms are ubiquitous in the environment, where their diverse characteristics allow them to proliferate and survive in a vast number of habitats. They have been studied for centuries and have primarily been described on a morphological, physiological, biochemical and molecular level (Singh *et al*., 2006). This opportunistic group of organisms are capable of flourishing under adverse conditions, allowing them to contaminate a broad range of fundamental resources such as water and food. Epidemics linked to waterborne pathogens occur frequently throughout the world, leading to an increase in the incidence of illnesses and even death.

As early as the 19th century, the contamination of water with *Vibrio cholerae (V. cholerae)* was investigated by John Snow. This infamous 'Broad Street Pump' cholera outbreak was the first disease occurrence to make known the dangers of a single source of polluted water, and linked the communicable, diarrhoeal disease to its causative agent, *V. cholerae* (Bailey *et al*., 2005; Fleming *et al*., 2006). Microbial contamination of water by organisms, such as *Campylobacter* spp., *Legionella* spp. and *V. cholerae*, amongst others, can however, be directly correlated to a lack of hygienic practices and water treatment. Drinking water can also become contaminated with bacteria, viruses and the protozoan parasites, *Giardia* and *Cryptosporidium*, when human waste is directly deposited into the water source (Karanis *et al*., 2006).

The Viable-but-Non-Culturable (VBNC) state of micro-organisms causes public concern, as these organisms have resuscitation abilities and can proliferate when the required nutrients are available (Lin *et al*., 2003). Smith *et al.* (1994) evaluated the survival, sublethal injury, and recoverability of *Escherichia coli* (*E. coli*), *Enterococcus* faecalis (*E. faecalis*), *Salmonella typhimurium* (*S. typhimurium*) and *Yersinia enterocolitica* (*Y. enterocolitica*), after exposure to polar marine environments at McMurdo Station, Antarctica. Temperatures below 1.8℃ were typically recorded at the station. The plate count, direct viable count and respiratory activity were determined for the different species. Upon exposure to the polar environment, all the bacteria displayed declining recoverability and increasing sublethal injury, but sublethal injury was more evident in the indicator organisms than in pathogens. All the tests showed increases in viable-but-non-culturable cells in *E. coli*, *S. typhimurium* and *Y. enterocolitica* after 58

exposure days, and resulted in an inability of these organisms to form colonies at 37°C. After the addition of nutrients, respiring cultures of *E. coli* and *S. typhimurium* increased significantly.

In Delmas, Mpumalanga, South Africa (2005), at least 18 people were admitted to hospitals with suspected typhoid fever. Up to 380 people may have been infected through the ingestion of contaminated water from the Delmas water-purification works. The disease was spread through the ingestion of the bacillus, *S. typhimurium*, as well as through direct contact with substances that were in contact with the water (Travel Doctor, 2005). Torpdahl *et al*. (2006) typed isolates of *S. typhimurium* using pulse-field gel electrophoresis (PFGE). In 2005, an outbreak with 26 cases of *S. typhimurium* infection was identified by multiple locus variable number of tandem repeats analysis (MLVA). The authors found that the discriminatory ability of PFGE for certain phage types was not high enough. The results showed that an isolate obtained from a pig herd, corresponded to an isolate from a human sample, located in the same region. The authors then concluded that the pig herd was the source of the human infections.

In 2003 the World Health Organisation estimated that over 1.1 billion people did not have access to a potable water source and that over 2.4 billion people had no access to basic sanitation (African Medical and Research Foundation, 2007). This includes 42% of the population in sub-Saharan Africa. This problem will only be amplified, as the world's population is expected to increase every year by 74.8 million people between 2002 and 2015. Natural disasters, such as flooding and droughts, along with inadequate sanitary facilities and the lack of potable drinking water, increases the potential risk of a wide range of potential waterborne diseases, such as cholera, malaria, bilharzias yellow fever, amongst others (African Medical and Research Foundation, 2007). In Kwazulu-Natal, South Africa, the Mhlathuze catchment area supports a rapidly growing industrial and agricultural community, where 78.5% of the population live in rural areas and where 34.8% of the population are without sanitation services (Lin *et al*., 2003). In 1996, the Department of Water Affairs and Forestry found that the bacteriological quality of the Mhlathuze River, which is utilised by the community for household and other purposes, was posing an increased risk of infectious disease transmission.

In addition, inadequate domestic and industrial wastewater treatment contributes to the lack of hygiene and sanitation. Only about 15% of collected wastewater undergoes treatment in Latin America, and in Venezuela, 97% of the country's sewage is discharged into the environment without treatment. In developing countries, water utilities, that is, supply and treatment is also grossly inadequate, due to a lack of funds, operational deficiency, the lack of staff and inadequate enforcement of environmental quality standards (World Health Organisation, 2003).

In 2003, the South African Department of Health reported a cholera outbreak in the Mpumalanga Province of South Africa. The outbreak included 27 areas bordering Swaziland

and Mozambique. The cumulative number of suspected and confirmed cholera cases was 174, with three deaths reported. In February 2004, another cholera outbreak was reported in the Nkomazi area, Mpumalanga Province, South Africa. The cumulative number of cholera cases reached 179, with five deaths recorded (World Health Organisation, 2004). In 2005, 49 cases and five deaths were reported in Niger by the Nigerian Ministry of Health, where laboratory testing led to the confirmation of the causative agent, *Vibrio cholerae* O1 (World Health Organisation, 2005). Angola reported 46758 cases of cholera in 2005, where 1896 deaths were recorded and 14 of the 18 provinces were affected. The most highly affected areas were Luanda (49%) and the Benguela provinces (17%). Although the spread of the disease in most of the provinces declined, a daily incidence of around 125 cases was still reported (World Health Organisation, 2006). In Sudan, in 2006, the Federal Ministry of Health reported a total of 2007 cholera cases, including 77 deaths as a result of acute watery diarrhoea. Of these cases, 35.3% occurred in Khartoum state, while 26% occurred in the North Kordofan state. *V. cholera* 01 Inaba was confirmed in 70 out of 139 stool samples (50%) by the national public health laboratory (World Health Organisation, 2007). South Africa also experienced major cholera outbreaks during 1980 to 1984, when over 22 000 people were infected in Kwazulu-Natal, and then again later, from August 2000 to February 2002, where 113 966 people were infected and 259 died in the same province (Cottle & Deedat, 2002).

Campylobacter jejuni (*C. jejuni*), *Campylobacter coli* (*C. coli*) and *Campylobacter enteritis* (*C. enteritis*) are major causes of acute enterocolitis and infective diarrhoea in most developed countries (Konkel *et al*., 2003). It is also a known fact that surface water that has been contaminated with faecal human waste has the potential to contain *C. jejuni* (Bates & Phillips, 2005). This is not surprising since many domestic animals and waterfowl shed this pathogen in their faeces, contributing to the microbiological degradation of recreational waters (Bates & Phillips, 2005). The Campylobacter Sentinel Surveillance Scheme (CDSC) of England and Wales reported 7360 laboratory confirmed cases of campylobacteriosis in the year that it was established in May 2000. Of the 7360 confirmed cases, 3% directly consumed untreated river-, stream- or spring water. Affected individuals were ill for a total of 11 days, while 732 patients required admission to hospital for at least five days (CDSC, 2000).

The two protozoan gastrointestinal disease-causing pathogens, *Giardia* and *Cryptosporidium* have been identified as the causative agents for the vast majority of outbreaks associated with water (Karanis & Kourenti, 2004). Karanis *et al*. (2006) investigated the water supplies (surface-, tap-, bottled-, well-, spring- and wastewater) in Russia and Bulgaria for the presence of *Giardia* and *Cryptosporidium* and found both parasites present in tap-, well-, surface- and wastewater samples. *Giardia* cysts were also detected in bottled water. Hsu *et al.* (2007) used the immunofluorescence- and enzyme-linked immunosorbent assay to detect

Giardia cysts. Out of the 107 collected samples, *Giardia* was present in eight samples, with these results confirmed in six of the samples by immunofluorescent microscopic examination, and four of the samples by the polymerase chain reaction method.

1.2.2 Xenobiotics

Xenobiotics are large-moleculed, man-made compounds believed to be resistant to environmental degradation (Chong, 2005). Human exposure to these compounds occurs via the ingestion of contaminated food and water, which leads to their bioaccumulation in food webs, resulting in the increased risk of environmental contamination and deterioration in human health (Belgiorno *et al*., 2007). Examples of these contaminants include polycyclic aromatic hydrocarbons (PAHs), alkylphenols (APs), organotins (OTs) and brominated flame retardants (Stasinakis *et al*., 2005). Xenobiotics have also been linked to alterations in endocrine functions and multiple hormonal systems in experimental animals, humans and wildlife (Desantis *et al*., 2005). Sources include hydrocarbons, insecticides, polychlorinated biphenyls (Otitoloju, 2003), herbicides (Dorigo *et al*., 2004), agricultural fertilisers and pesticides, heavy metals and organic synthetic compounds from industry and shipping (Quintaneiro *et al*., 2006).

Research has shown that herbicides and pesticides negatively affect the quality of the natural environment they leach into. Dorigo *et al*. (2004) investigated the impact of atrazine and isoproturon on periphyton and phytoplankton samples in the river Ozanne (France) and its tributaries. The sampling sites were reported to be contaminated with varying levels of atrazine and isoproturon. Microalgal communities inhabiting the contaminated area as well as those inhabiting pristine stations were investigated. A greater tolerance to atrazine and isoproturon was observed in the microalgal community inhabiting the contaminated area. In addition, the phytoplankton and the periphyton communities both complied with the pollution-induced community tolerance concept (PICT) as described by Blanck *et al.* (1988). The PICT concept establishes a cause-effect relationship between a toxicant and microbial communities (Schmitt *et al*., 2005). The phytoplankton and periphyton communities shifted their composition towards diatom-domination due to the continuous presence of atrazine and isoproturon. *Achnanthes lanceolata* and *Nitzschia palea* were nearly always present at the sampling sites. This result was corroborated by a previous study by Kasai (1999), where he proved that these algae were highly resistant to atrazine. The phytoplankton and periphyton communities displayed increased tolerance to the PICT studies. In PICT studies the increased tolerance of the total community to the introduced toxicant is investigated making them an effective tool in ecotoxicology due to their high sensitivity and specificity to the effects of toxic substances (Boivin *et al*., 2002).

Weston *et al*. (2001) and Kolpin *et al*. (2002) identified the occurrence of multiple pharmaceuticals, such as fluoxetine in surface waters, while other studies recorded the occurrence of estrogenicity steroid therapeutics in municipal effluents (Foran *et al*., 2003; Huggett *et al*., 2003). Brooks *et al*. (2003) showed that fluoxetine reduced the growth of the green algae, *Pseudokirchneriella subcapitata* (*P. subcapitata*). Fluoxetine was added to three flasks containing *P. subcapitata* at concentrations of 0 (control), 43.6, 87.3 and 174.4 nM. Algal growth was evaluated by enumeration, using a haemocytometer and a compound microscope, and turbidity measurements were done by absorbance readings at 750 nm. Cell deformities were observed and cell sizes appeared smaller at 87.3 and 174.4 nM treatment levels, where cells appeared shrivelled and on occasion were not crescent shaped, which is a normal characteristic of *P. subcapitata*. Although cell deformities and biovolumes were not quantified in this study, this effect of fluoxetine on algal cells warrants further investigation. Richards *et al*. (2004) also evaluated the effects of fluoxetine, ibuprofen and ciprofloxacin mixtures on a bacterial community in aquatic microcosms. The three compounds were used in combination to provide a high exposure scenario for future risk assessment. The sampled zooplankton and phytoplankton communities showed decreases in the number of certain organisms, while other organisms flourished. *Lemna gibba* and *Myriophyllum spp*. showed increased mortality when exposed to high concentrations of the mixture, whereas the bacterial community did not change at all.

1.2.3 Polycyclic Aromatic Hydrocarbons (PAHs)

Contamination of aquatic environments with polycyclic aromatic hydrocarbons (PAHs), a ubiquitous group of organic pollutants, is a matter of great environmental concern. Polycyclic Aromatic Hydrocarbons (PAHs) enter the environment via the atmosphere and adsorb onto particulate matter. Their affinity for organic fractions in sediment, soil and biota is high and PAHs accummulate in organisms, water, sediments and in their nutrient source. They enter the organisms by absorption, through the pulmonary tract, the skin and the gastrointestinal tract (Coman *et al*., 2006).

Polycyclic Aromatic Hydrocarbons have carcinogenic and mutagenic properties (Watson *et al*., 2004) and are used as intermediates in the production of polyvinylchloride and plasticisers (naphthalene), pigments (acenaphthalene, pyrene), dyes (anthracene, fluoranthene) and pesticides (phenanthrene) (National Pollutant Inventory Substance Profile, 2004). They have been known to leach from electrochemical industries, such as aluminium, iron and steel production implants and foundries (Wiles, 2004). Forest fires also contribute to the release of PAHs into the atmosphere, while the majority of the contamination originates from the discharge

of effluent from settling ponds (Watson *et al*., 2004). Other sources of PAH contamination include fossil fuel-burning, oil refineries, motor vehicle emissions, waste incineration, coke and asphalt production industries and aluminium production plants, amongst others (Srogi, 2007). Polycyclic Aromatic Hydrocarbons also result from cigarette smoke and some of these compounds are highly carcinogenic or mutagenic (Sakai *et al*., 2002).

The relationship between sediment contamination and toxicity in San Francisco Bay, USA, a highly industrialised and urbanised estuary composed of many connected bays, was evaluated by Thompson *et al*. (1999). Collected sediment samples were digested using aqua regia (Flegal *et al*., 1994) and analysed with atomic absorption spectrometry, with PAHs and chlorinated hydrocarbons evaluated using gas chromatography. The results indicated that toxicity was widespread in the Bay and that it fluctuated over time in the areas located close to harbours, closed military bases and superfund sites, containing highly toxic sediments. The results obtained correlated with a study performed by Swartz *et al*. (1994) in which highly toxic sediment samples were also evaluated for metal contamination. Sediment samples evaluated at the respective sites consisted of varying combinations of contaminants. The sediment elutriate bioassay and the bulk sediment assay, were performed to determine the toxic effects of the sediment. Results showed that sediment samples at the Redwood Creek site, proved to be most toxic.

1.2.4 Metal Contamination

Metal contamination occurs due to the accumulation of herbicides, pesticides, petroleum by-products (Dorigo *et al*., 2004; Mowat & Bundy, 2001) and urban- and industrial runoff (Ohe *et al*., 2004) in the environment. The overall natural occurrence of certain metals in the soil, atmospheric deposits (Radenac *et al.,* 2001) and corrosion of building materials (Maanan *et. al.,* 2004) also contributes to the increase of metals in the environment.

Domestic and household sources of contamination occur as a result of corrosion of metal plumbing fittings, galvanised roofs and wire fences [(zinc (Zn), cadmium (Cd)] and healthcare products, such as Zn- or selenium (Se) containing shampoos and Zn-containing baby creams (Alloway, 1995b). Silver paint containing aluminum (Al), Al-coated roofs, saucepans and other household utensils containing metals are also possible sources of contamination (Friberg *et al.,* 1986).

The metals most commonly associated with river water are lead (Pb), copper (Cu), iron (Fe), Cd, Al, mercury (Hg), arsenic (As) and manganese (Mn). Many aquatic organisms live in water bodies and increased concentrations of these pollutants could be detrimental to the aquatic environment, thereby directly contaminating the food web, and also higher animals, such

as humans. Metal pollutants also have a detrimental effect on human health, where exposure is mainly due to the ingestion of contaminated food and water (Wright & Welbourne, 2002). The leaching of metals into groundwater can result in the further contamination of drinking water and can, due to this toxicity, be detrimental to human health (Piver, 1992). **Table 1.1** represents the recommended safe metal concentrations in aquatic ecosystems as stipulated by the Department of Water Affairs and Forestry (DWAF, 1996) and the Canadian Council of Ministers of the Environment Quality Guidelines (CCME, 2001). It is important to adhere to the stipulated guidelines for metal concentrations in aquatic samples in order to determine whether the water is safe for utilisation.

Table 1.1 Recommended safe metal concentrations as stipulated by the Department of Water Affairs and Forestry (1996) and the Canadian Council of Ministers of the Environment Quality Guidelines (2001) in aquatic samples.

Metals	Recommended safe concentrations as stipulated by DWAF (1996) ($mg.l^{-1}$)	Environmental quality guidelines as stipulated by CCME (2001) ($mg.l^{-1}$)
Al	0.1 – 0.15	0.005 – 0.1
Cu	0.002 – 0.012	0.002 – 0.004
Fe	N/A	0.3
Mn	1.3	N/A
Ni	N/A	0.025 – 0.15
Pb	N/A	0.001 – 0.007
Zn	0.036	0.03

1.2.5 Metal Accumulation in Sediment and Water

The deposition of solid particles on the bed or bottom of a body of water or other liquid is known as sedimentation. This suspended material (sediment) is essentially fragmented organic or inorganic material derived from the weathering of soil, alluvial and rock materials. It is removed by erosion and can be transported by fluid flow to settle on different locations at the bottom of the river (Ebner *et al*., 1999). In aquatic environments, high concentrations of metals are usually integrated in surface sediments (Prange & Dennison, 2000; Marchand *et al*., 2006). The highest metal concentration is stored between the sediment-water interface (Maanan *et al*., 2004), as according to Peijnenburg *et al*. (2005), sediment acts as sinks for suspended material in surface water.

Increased levels of heavy metals were reported in the Mooi River sediment in South Africa (Wade *et al*., 2000). The release of mine water into a tributary of the Mooi River from a nearby goldmine apparently resulted in the increased levels of potentially toxic metals in both the water and sediment. Mzimela *et al.* (2003) conducted a study on sediment, water and fish samples collected on a quarterly basis from the Mhlathuze Estuary, South Africa, to investigate seasonal bioaccumulation patterns of selected metals. The highest metal concentrations (Al, Fe

and Mn) in water and sediment were recorded in December, coinciding with extremely high freshwater inflow from the Mhlathuze River. Metal concentrations were generally lower during April, which coincides with the reduced riverine runoff from the catchment of the estuary. Iron was found in the greatest concentrations in the fish tissue, followed by Al, Zn, Mn, chromium (Cr), Cu and lastly Pb.

The primary sources of heavy metals in the environment are waste discharge, stack emissions from industrial sources and coal power production (McComb & Gesser, 1999). Common environmental pollutants such as Zn, Cd and Hg are accumulated in aquatic environments through the weathering of minerals and soils (Merian, 1991; Kļaviņš *et al*., 2000), coal combustion, refuse incineration and Fe metal industries (Merian, 1991). This in turn, results in the contamination of freshwater sources and the alarming increase in metal accumulation in aquatic systems. The subsequent poisoning of the human food chain then increases the need for further studies to monitor metals in all water bodies (McComb & Gesser, 1999).

The Arges River (Romania) was analysed by Stoica (1999), for the presence of various metals using Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). The River was chosen because it flows past treated industrial-, and domestic wastewater and other waste material. Using these techniques, it was found that concentrations of Cu and Pb did not exceed the recommended Romanian standards in natural waters, while the concentrations of Cd and Zn did exceed the recommended concentrations for metals in the river water. The authors however, concluded that the river was relatively free of metal pollutants.

Macklin *et al.* (2003) monitored the cyanide and metal contamination into tributaries of the Tisa River, which is a major tributary of the Danube River, to record the effects of a release of 200 000 m^3 of contaminated water and 40 000 tonnes of tailings into the tributaries. Sixty-five water, 65 river- and 45 floodplain sediment samples were collected and analysed for Pb, Zn, Cu and Cd. The concentrations decreased downstream as water flowed away from the mines and tailings ponds, and generally fell below European Community (EC) concentrations. In contrast, Zn, Cu and Cd concentrations in river sediments approached or exceeded recommended concentrations close to the Romanian border.

The concentrations of Cd, Hg and Zn in the Umtata, Buffalo, Keiskamma, and Tyume Rivers and in the Sandile and Umtata Dams were determined by Fatoki & Awofolu (2003). These catchments support rapidly growing populations and concerns arose regarding the quality of the surface waters. Cadmium levels in the Umtata River and the Umtata Dam were normal but in the Keiskamma-, Buffalo- and Tyume Rivers elevated Cd levels were recorded. These elevated Cd levels may affect the health of the rural communities who use the river water before

the water is treated. The levels for Hg and Zn appeared to be normal in the river and did not exceed the recommended concentrations of metals in water as stipulated by DWAF (1996).

1.2.6 Bioindicators

Microbial organisms growing in a biofilm community are capable of adapting to and surviving in nature, due to the protection offered by the surrounding matrix, especially during stressful situations (Decho, 2000). The close and beneficial relationships among organisms within the biofilm, accelerates xenobiotic usage as well as the subsequent immobilisation and degradation of pollutants (Singh *et al*., 2006).

A biofilm can be defined as a collection of microbial cells organised within extracellular matrices, which are mostly associated with flowing systems, but can also occur as aggregates, occurring at interfaces such as solid/air-, inert solid/liquid-, and solid nutrient/liquid (Gilbert & Allison, 1993). The development of the biofilm can broadly be divided into a reversible and irreversible stage. Lawrence & Caldwell (1987) and Marshall (1988) proved that cells initially become attached to a surface, using a portion of their flagella during the reversible attachment stage, after which they either may become detached or irreversibly attached. Their research showed that cells chemically gauge their compatibility with potential binding sites before the irreversible attachment stage. Once irreversibly attached, cells will proliferate into a mature biofilm. In 1989, Lawrence *et al.* stated that irreversibly attached micro-organisms could, however, slough off because of adverse environmental factors, and could then reattach at another section of the material surface (**Figure 1.6**).

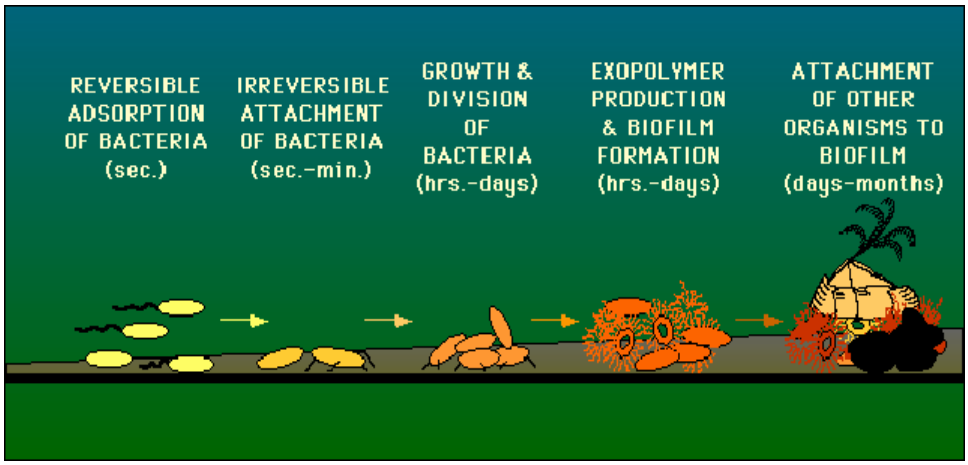

Figure 1.6. Biofilm formation (Biofilms: Online Manual – American Society for Microbiology, 2007).

Bacteria produce extracellular polymeric substances (EPS), within the biofilm matrix, which assist in improving their survival rate. This EPS assists in biofilm development by

providing a substrate for other micro-organisms and toxic molecules to adhere to (Costerton *et al*., 1978). It also has a high metal absorption capacity (Marín-Guirao *et al.,* 2005). It is due to this binding capacity of EPS that microbial biofilms are amongst the most common treatment for the removal of metals from metal-contaminated waters (Roane & Pepper, 2000).

Resistance of micro-organisms to metal pollution depends on the developmental stage of the organism as well as the taxonomic group to which the organism belongs (Wright & Welbourne, 2002). The concentrations of metals in aquatic systems are also dependent on fluctuations in environmental conditions as well as the interactions between environmental factors such as dissolved oxygen (DO) levels, water hardness, conductivity, temperature and pH. Variations in these conditions then also contribute to the release of metals back into the flowing water. Due to a resistance build-up, certain microbial species are also more effective in the removal of particular metals from the system, e.g. *Citrobacter* spp. biofilms are used for the removal of uranium, *Arthrobacter* spp. biofilms are used in the recovery of Pb, Cu, Cr, Zn and Cd, while *Bacillus* spp. effectively binds Cd, Cu, Hg, Cr and Ni (Roane & Pepper, 2000). The important biofilm-producing organisms in domestic wastewater treatment include, *Klebsiella*, *Zoogloea* and *Pseudomonas* spp. (Roane & Pepper, 2000). *Pseudomonas aeruginosa* biofilms were shown to cause the precipitation of lanthanum (Langley & Beveridge, 1999), while mercury-reducing *Pseudomonas putida* biofilms showed elemental mercury accumulation on the exterior of the biofilms (Wagner-Döbler *et al*., 2000). *Burkholderia cepacia* biofilms were found to sequester Pb^{2+} at concentrations higher than 1 µm (Templeton *et al*., 2001), while a study by Suh *et al.* (1999) found that the EPS was responsible for more than 90% of Pb^{2+} accumulation of the total Pb dissolved. Kröpfl *et al.* (2003) studied Pb and nickel (Ni) contaminated biofilms by evaluating their accumulation and their effect on biomass production. Results revealed that the total biomass production decreased by 14%, in comparison to the control, in the presence of increased Ni concentrations. This indicated that the bacteria exhibited a lower tolerance to Ni than to Pb.

Metal-resistant organisms such as *Rhizopus arrhizus* are generally used in continuous systems over an extended time period, due to their capacity to self-replicate and their ability to proliferate in the presence of metals (Donmez *et al*., 1999). Prat *et al.* (1999) analysed metal accumulation in biofilms from nine different stations in the Guidamar River, Spain. Metal accumulation was 15 times higher in the stations affected by metal discharge than in the unaffected stations, especially in the case of Zn, Pb, As and thallium (Tl). As mentioned above, the binding capacity of EPS depends on the state of the polymeric layer (EPS) but as was shown in the above-mentioned study, also on the pH of the water (Kröpfl *et al*., 2003).

Research has shown that the behaviour of a particular organism or animal could serve as an indication of chemically induced stress and would make the particular animal beneficial as a biomarker (Petrauskiené, 2003). Ecotoxicological biomarkers based on molecular and cellular level responses of an organism to adverse conditions represent the earliest signals of environmental disturbances (Lowe *et al*., 1995).

Petrauskiené (2003) assessed the behavioural responses of medicinal leeches to the effect of exposure to water from the Drukshiai Lake, to sediments of the Nemunas River, and to a solution of heavy metals. The mobility, avoidance response, changes in body shape and the feeding activity of the medicinal leeches were investigated. Avoidance response, which is defined as the amount of individuals escaping the tested water or sediment as well as mobility change, were recorded during the first hour of exposure to the tested samples and could be used as a marker for water and sediment pollution. After one to three weeks their feeding activity was reduced, indicating their usefulness in assessing the chronic toxicity of pollution, and making the medicinal leech effective as a biomarker in ecotoxicological studies.

Nigro *et al.* (2006) investigated genotoxicity and lysosomal alterations in the Mediterranean mussel (*Mytilus galloprovincialis*) from an estuary of the Cecina River. This river is subject to chemical impact mainly associated with industrial activities and untreated urban wastewater discharge. Native and transplanted mussels, which were transferred to the affected area and left for four weeks, were used for this study to compare metal accumulation and the degree of pollution. Metal concentrations in the digestive glands of the transplanted mussels were similar to those of the native mussels. Transplanted mussels could therefore be implemented as a model biomonitoring tool (Regoli & Orlando, 1994) as they rapidly equilibrate their tissue metal levels according to the metal bioavailabilty of the surrounding environment.

The snail, *Helix aspersa*, was used as a biomarker to determine whether copper oxychloride exposure induced stress (Snyman *et al*., 2002). Snails collected from vineyards treated with copper oxychloride were compared to those from an untreated vineyard. Using the neutral red retention (NRR) time assay, it was found that after only one week of exposure to copper oxychloride, the snails from the test site showed significantly shorter NRR times and significantly higher whole body copper concentrations. These results indicated that the NRR time assay is effective as an indication of copper oxychloride-induced stress.

1.2.7. Metal Contaminants

The metal contaminants most commonly associated with water include Al, Cu, Fe, Mn, Ni, Pb and Zn (Wright and Welbourne, 2002). **Table 1.2.** summarises the uses and health effects of the different metal contaminants.

Table 1.2 The uses and health effects of the metal contaminants most commonly associated with water.

Metals	Uses	Health Effects	References
Al	Pressed into sheets or foil[a]. Pesticides and algicides[b].	Poison neurotoxin[c]. Overexposure has been linked Alzheimer's disease[d] and could be associated with contaminated drinking water[e,f,g,h] Memory loss, depression and long-term muscular weakness[i]. Damage to all tissue types[j].	History of Aluminium, 2006[a] Seachem, 2006[b] Miu *et al.*, 2004[c] Kawahara, 2005[d] Gardner & Gunn, 1991[e] Doll, 1993[f] Werbach, 2003[g] Werbach, 2007[h] Sears, 2008[i] Exley, 1996[j]
Cu	Building industry[k]. Animal feeds and fertilisers[l].	Micronutrient for all aerobic life forms. Development and performance of the human nervous- and cardiovascular systems, as well as the skin, bone, immune and reproductive systems. Inhibits the growth of certain microbes. Low Cu - heart and circulatory problems, bone abnormalities and complications in the immune system[m]. Increased concentrations - gastrointestinal distress, as well as kidney- or liver damage[n] Decrease in biological diversity[o,p].	Copper Facts, 2006[k] Mineral and Information Institute, 2006[l]. Copper and Human Health, 2006[m]. Saleh *et al.*, 2001[n] Medina *et al.*, 2005[o] Stauber *et al.*, 2005[p]
Fe	Alloys[q] Component of haemoglobin[r] Transportation,	Associated with several chronic diseases, such as heart disease, cancer and diabetes[t,u,v]	Webelements - Fe Periodic Table, 2006[q]. Powell *et al.*, 1994[r]. Minerals Education -

	construction, machinery manufacture, cans and containers and in the oil and gas industries[s]		Fe, 2008[s]. Stevens *et al.*, 1988[t] Tuomainen *et al.*, 1997[u] Klipstein-Grobusch *et al.*, 1999[v]
Mn	Pesticides and oil additives[w,x].	Strong yet flexible bones Aids the body's absorption of Vitamin B1 Enzyme activator Excess of Mn in the water supply - Parkinson's disease[y,z] Shortages - obesity, glucose intolerance, blood clotting, skin problems, lowered cholesterol levels, skeletal disorders, birth defects and neurological symptoms[1]. Swelling of cell walls, withering of leaves, and brown spots on leaves[1].	Acrobat®, 2005[w]. Vermeulen *et al.*, 2001[x]. ToxFaqs for Manganese, 2006[y] Wright & Welbourne, 2002[z] Lenntech - Mn, 2006[1]
Ni	Fair conductor of heat and electricity[2]. Stainless steel, jewellery, coins and items such as valves and heat exchangers. In certain batteries, to colour ceramics and for nickel plating[3].	Skin effects or allergic reactions. Asthma attacks, chronic bronchitis and lung infections. Stomach cramps and damage to the kidneys[3]. Increased concentrations - cancer[4]. Spontaneous abortions and structural malformations, especially cardiovascular and musculoskeletal defects in newborn babies[5]. High concentrations - inhibit algal growth[6].	Webelements Periodic Table - Ni, 2006[2]. ToxFaqs for Nickel, 2006[3]. Agency for Toxic Substances and Disease Registry, 2005[4]. Chaschschin *et al.*, 1994[5]. Mandal *et al.*, 2002[6].
Pb	Plumbing materials and water services, Pb paint chips[7].	Decrease in intelligence scores, concentration spans, reading and language, anaemia, hearing loss, and abnormal development of tissues and organs, such as the kidneys, heart and brain. Extremely high levels - ataxia, cerebral oedema, paralysis, coma and death may	Lead – Safewater, 2006[7]. Goyer, 1993[8]. Wright & Welbourne, 2002[9] Bogden *et al.*, 1997[10] National Lead Information Centre,

		result[8]. Hyperactivity, poor attention span, IQ defects[9] and palsy, or wrist drop[10] Pb-contaminated drinking water - increase in blood pressure, kidney problems. Difficulties during pregnancy, reproductive, digestive and memory problems, nervous disorders[8]. Pb smelting plants - elevated blood-lead levels[11].	2006[11].
Zn	Conductor of electricity. Galvanising[12]. Deodorants, wood preservative, suntan lotion, topical cream to prevent nappy rash[13].	Essential element in plant and animal growth[14]. Deficiency - hair loss, skin lesions, diarrhoea, wasting of body tissues and eventually death, brain development is stunted *in utero* and in infancy[13], malfunctions in cerebral activity, as well as negatively affecting eyesight, taste, smell and memory[13]. Elevated concentrations - suppress Cu and Fe absorption.	Webelements Periodic Table - Zn, 2006[12]. Zinc, 2006[13]. Zinc and the Environment, 2007[14].

1.3 BIOREMEDIATION

Bioremediation systems are gaining increased interest, as they are more economically viable and require less maintenance than more traditional treatment systems. The principle of bioremediation is to utilise microbial degradation processes in technical and controlled treatment systems (Langwaldt & Puhakka, 2000). Eccles (1999) stated that biological systems were gaining increased interest due to the fact that they are as effective as other more physical techniques, are cost-effective and environmentally friendly, which is why they are referred to as green technologies (Mack *et al.,* 2004).

Bioremediation is used to reduce or eliminate contaminants by encouraging bacterial growth through the addition of nutrients to the contaminated area. The organisms then degrade organic matter into simpler compounds such as water, methane, inorganic salts and carbon dioxide (Farhadian *et al.*, 2008). Specific bacteria can also be introduced into a system to

metabolise a particular contaminant in a process known as engineered bioremediation, where the bioremediation process is artificially enhanced (Scow & Hicks, 2005). Ideally, indigenous microbes will degrade organic contaminants (Röling & Verseveld, 2002) if a supply of nutrients is available for their metabolism (Parales & Haddock, 2004; Scow & Hicks, 2005). Bioremediation can therefore be defined as treatment technology which uses living organisms, i.e. biofilms (Singh *et al.*, 2006), to reduce the concentration or toxicity of contaminants in soil (Law & Aitken, 2003), water (White, 1995) and wastewater (Kargi & Eker, 2005). The two most important processes involved in metal removal from contaminated sites are biosorption and bioaccumulation. In these processes the metals are removed from the system by binding to specific functional groups on the outer surface of the biomass (Volesky, 1990).

The objective when choosing a bioremediation system is to find the most efficient, as well as the most cost-effective system for treatment (Liu *et al.*, 2001). The process of bioremediation can thus contribute to cost-efficiency (Adriaens *et al.*, 2006) by treating contamination in place, meaning that nutrients can be delivered to contaminated soil, without removal-disposal costs, using natural microbial processes to break down pollutants, including metals (Park *et al.*, 2008), and by reducing environmental stress, by minimising site disturbances. Bioremediation systems can be divided into natural bioremediation systems such as wetlands, and artificial systems such as bioreactors.

1.3.1 Wetland Systems

The use of wetlands is an emerging, reliable technology used primarily for the tertiary treatment of contaminated effluents. They are defined as terrestrial and aquatic systems which have the water table at, near, or above the land surface. They are also widely distributed throughout the world where low lying lands meet water. Water moves very slowly through the wetland areas and the wetland soils remain water-logged (soil that contains so much water that there is no room for oxygen). Marshes, swamps, bogs, wet meadows, sloughs, potholes, river overflow lands, and tide flats are all examples of wetlands. Wetlands consist of waterlogged, or water loving plants, known as hydrophytes, which can grow without much oxygen from hydric (saturated, flooded, or ponded) soils, which supports hydrophytic vegetation (Sheoran & Sheoran, 2006). The soil can be either organic or mineral, where organic soils are characterised by a continuously accumulating deep layer of decaying plant matter at the soil surface. The properties of hydric soils especially the lack of oxygen, retards the decomposition of the dead plants. In the water table, which is the point below the land surface where the earth is saturated with water, the mineral layer can sometimes be either wet or dry, depending on seasonal changes, which causes elements like Fe and Mn to be reduced

(Woulds & Ngwenya, 2004). Small inland wetlands, usually less than 20 hectares, are used by farmers in Southern and Eastern Africa, to grow vegetables and other crops vital to household nutrition and incomes. The wetland allows the resource-poor farmers to farm throughout the entire year, because of the moisture-rich wetland environment. This allows for a year-round food supply and source of income (International Water Management Institute, 2007).

Constructed wetlands are as functional as natural wetlands, and are also comprised of plant-, microbial-, soil-, and animal components, and have been successfully employed in the removal of pollutants from river water (Hammer, 1989). The utilisation of wetlands as successful remediation systems is based on the microbial adsorption of metals, metal bioaccumulation in plants, bacterial metal oxidation, and sulphate reduction (Roane & Pepper, 2000). Metal sulphide precipitation has also been found to be useful in the operation of natural and constructed wetlands (White *et al.*, 1997). Collins *et al.* (2004) and Murray-Gulde *et al.* (2005) showed that sulphide precipitation was the dominant process for the removal of metals from wetlands. Kadlec & Knight (1996) stated that a wetland system would be ideal for treatment of water systems, as it is environmentally safe as well as cost-effective. **Figure 1.7** outlines the distribution of wetlands in South Africa.

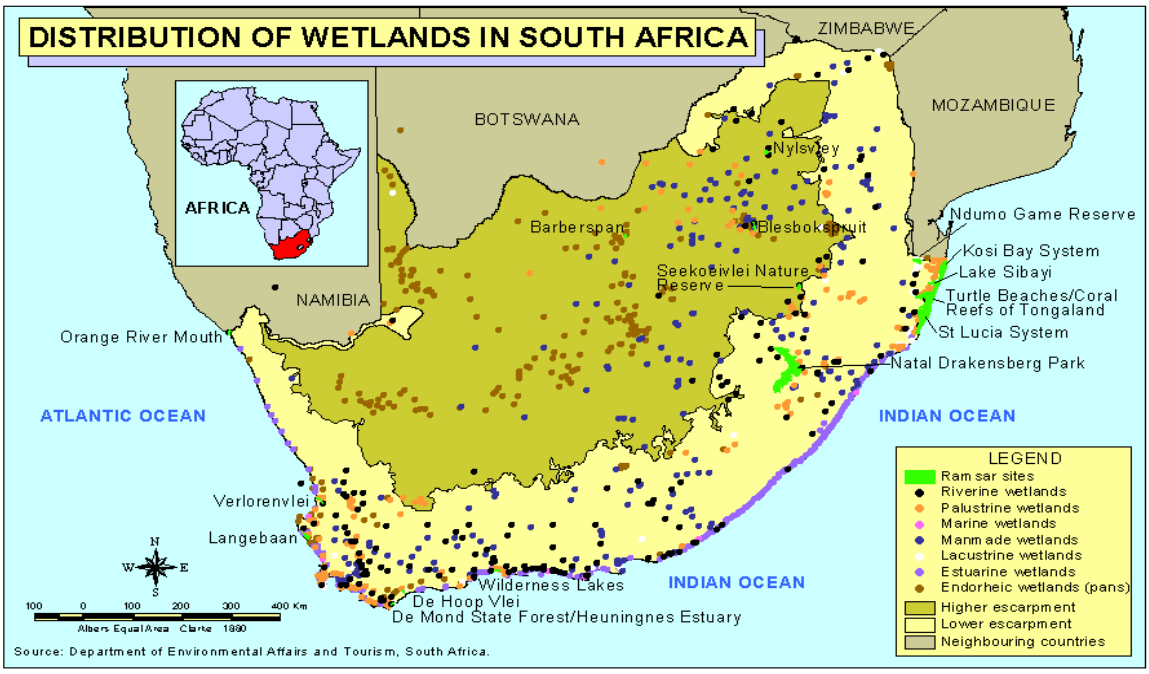

Figure 1.7. Distribution of wetlands in the nine provinces of South Africa (Department of Environmental Affairs and Tourism, 1999b).

There are many different types of wetlands which include emergent-, aquatic-, forested-, scrub/shrub-, free-water surface-, and subsurface flow wetlands, constructed for the removal of specific pollutants.

An emergent wetland is characterised by swaying grassy plants, which have parts of their soft stems and roots embedded in the wet soil. The grassy plants, cattails, bulrushes and reed canary grass are found in marshes and sedges, while grasses and willow grow on peat in fens, which have slow moving water which rinses acid from the soil. This wetland system is home to ducks, geese, migrating birds and muskrats, amongst others (Wetlands, 1999). Bulrushes and Cattails have been successfully used to reduce the total amounts of trinitrotoluene (TNT), Cyclotrimethylenetrinitramine (RDX) and cyclotetramethylene-tetranitramine (HMX) from explosives-contaminated wastewater (Qazi & Kanaras, 1999). Emergent wetlands in coastal systems, protect the shoreline from erosion, filter pollutants, enhance water quality and promote primary production (US Fish and Wildlife Service, 2007). A wetland was constructed consisting of various layers, such as a clay layer, a layer consisting of typical emergent wetland plants (*Typha latifolia*, *Typha angustifolia*, *Phragmites communis*, *Scirpus lacustris* and *Juncus* spp.), various types of algae and different heterotrophic and autotrophic micro-organisms, including different oil-degrading bacteria and fungi and manure, which provided the essential nutrients required for vegetative growth (Groudeva *et al*., 2001). Results revealed that the highest concentrations of pollutants, such as PAHs and heavy metals (Cd, Cu, Pb and, to a lesser extent, Fe) were found in the roots of the plants, as well as adsorbed to some of the algal and bacterial species.

In an aquatic wetland, not all of the plants are submerged and grow underwater. Some of the wetland plants or their leaves float on top of the water. Waterlily, duckweed and pondweed grow in water that may be three to six feet deep. Aquatic beds are generally found near the edges of lakes or streams (Wetlands, 1999). Duckweed has been used to accumulate metals, such as Pb, Cd, Cu and Zn (McIntyre, 2003). *Lemna perpusilla* or Duckweed plants were collected from a heavy ash settling basin to determine its ability to accumulate Cd, Cu, Fe, Mn, Zn, Cr, Pb and Ni, which are metals that are expected with heavy ash. The Duckweed plants accumulated higher concentrations of the metals analysed for than was recorded in the water or coal ash sediment samples collected (Clark *et al*., 1981).

Bog or Forested wetlands have very acidic soil, which allows the growth of only acid-resistant plants, such as both evergreen and deciduous trees. Due to the acid-rich and oxygen-deprived soil, dead plants do not decay and become a mat of rotting plants. Deer, raccoons, rabbits, hawks and owls make this wetland system their home (Wetlands, 1999). The deciduous plant, White birch, has been used to remove methyl bromide from the atmosphere (Jeffers & Liddy, 2003).

Swamp or Shrub wetlands are characterised by small trees and bushes. The water is close to the surface and next to rivers, lakes and streams. Willows, Spirea and common rush are well suited to this wetland type as these plants have more than one flexible stem. The willow

(*Salix* spp.) has been used to phytoextract Cd, Zn, Hg, Cr, Se and Cu from contaminated water (McCutcheon & Schnoor, 2003). The open water of a shrub wetland is used by wood ducks and song birds, while Herons, muskrats and deer are also at home in these wetlands (Wetlands, 1999). Dos Santos Utmazian *et al.* (2007) reported that willows accumulated high amounts of Zn and Cd from a contaminated site in Arnoldstein (Austria), and De Dousa *et al*. (1999) showed that metal uptake could be enhanced as a result of the presence of certain bacteria. Kuffner *et al.* (2008) evaluated the metal uptake of willows (*Salix* sp.) at a lead mining area. Four pot experiments with the soil collected from the polluted area (Zn/Cd/Pb polluted) were inoculated with Zn resistant bacteria isolated from the mined area after the bacteria had been grown up overnight in media amended with 1 mM $ZnSO_4$. After exposure to the contaminated soil, the predominant strain isolated was *Streptomyces* AR17, which was thought to be responsible for enhancing Zn and Cd uptake by the *Salix* sp.

Free-water surface wetlands are wetlands that have the water surface above the wetland bed or substrate. They are also referred to as surface flow, free surface or open water surface wetlands (Merz, 2000). Subsurface flow wetlands are designed so that the flow moves through a soil or gravel matrix which is planted with macrophytes, large macroscopic plants which are able to be seen with the naked eye (Merz, 2000). Subsurface-flow wetlands move effluent (agricultural or mining runoff, tannery or meat processing wastes, wastewater from sewage drains), through gravel or sand on which plants are rooted. Surface-flow moves effluent above the soil in a planted marsh or swamp (Kadlec & Knight, 1996). They have also been used to remove Cu, Pb and Zn from wastewater and stormwater (Nelson *et al*., 2004).

Pilot wetland units were set up in the United States in 2001, to upgrade an existing facility. The aim was to remove petroleum hydrocarbons and salts from the Naval Petroleum Reserve. Wetland pilot units, the free-water surface (FWS) and subsurface flow (SSF), were set up outdoors and followed up for three months. Dissolved Oxygen (DO), pH, Electrical Conductivity (EC), Total Dissolved Solids (TDS), Chemical Oxygen Demand (COD) and temperature were measured. The biological activity of certain types of bacteria was also determined for the soil and water samples collected. The temperatures averaged between 37°C to 39°C, where the warmer water supported good plant growth in each wetland. The dissolved oxygen (DO), ranged from 1.2 to 7.2 $mg.l^{-1}$, which was a bit low for the pilot wetlands, and increased the microbial community's demand for DO. Both pilot wetlands were well buffered with pH levels from 6.5 to 8.5, supporting microbial activity and plant growth. The water and sediment columns evaluated for microbial activity, showed variations in microbial species, capable of metabolising organic and inorganic compounds throughout the FWS pilot wetland system, with the most abundant bacteria, being heterotrophic and sulphur-reducing bacteria. The presence of heterotrophic bacteria indicated that the wetland soils and water contained

sufficient energy for the aerobic degradation of organic compounds. It was ultimately found that both pilot wetland systems were efficient and capable of improving the overall water quality (Jackson & Myers, 2002).

Metal retention by wetlands have been used to reduce the levels of Zn, Cu, Ni, Pb, and other metals in runoff and drainage from mining regions (Mays & Edwards, 2001). Nelson *et al*. (2004) studied the efficiency of a constructed wetland system to remove metals from the Savannah River Site, which receives wastewater discharges and stormwater runoff. The wetland consisted of four pairs of one acre wetland cells with water flowing from one cell to the next and then on to the discharge point. The soils in the wetland were modified by adding organic matter, fertiliser and gypsum as well as giant bulrushes (*Schoenoplectus californicus*). This provided a continuous source of organic material to the sediment, allowing for the decomposition of plants by bacteria and fungi. The anoxic conditions were also maintained in the hydric soil, thereby allowing for the capture and immobilisation of metals in the soil. A total of 11 water samples were collected monthly and analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and ion chromatography. Results for the samples collected during the fourth year of running the wetland system, validated the efficiency of the system, in that copper and mercury removal efficiencies were still very high (in excess of 80% removal from water after passage through the wetland). Lead removal from the water was 83%, Zn removal was 60% and Ni concentrations were unaffected. Dissolved organic carbon in the water column increased as a result of the anaerobic conditions in the wetland system, which then in turn reduced the toxicity of the effluent.

Wetland efficiency depends on the type of plants utilised and the potential loss of the plant community due to a lack of nutrients. It takes a longer time to remediate, as evidenced by the study performed by Jackson & Myers (2002), where their pilot study was performed over a three year period. Over the remediation period, long-term performance problems, like plugging of the porous media, maintenance of plant communities and the release of nutrients and accumulated metals during warm periods (McCutcheon & Schnoor, 2003), were identified as potential problems associated with the use of wetlands.

1.3.2 Bioreactor Systems

Wastewater treatment generally involves physical, chemical and biological processes, to remove contaminants from the affected systems. These impurities must be collected, handled and disposed of in a manner which is not detrimental to the surrounding environment or human health.

A bioreactor can broadly be defined as a tank in which cells, cell extracts or enzymes carry out a biological reaction. The efficiency of bioreactors is based on the ability of bacteria to grow at interfaces, making microbial biofilms the most effective mechanism in the removal or uptake of contaminants from the environment. This is collaborated by research, which shows that biofilms are the most common treatment for the removal of metals from metal-contaminated waters (Roane & Pepper, 2000).

Furthermore, bioreactors are advantageous as they can function under both aerobic and anaerobic conditions (Langwaldt & Puhakka, 2000), which increases their efficacy. They are easy to use and to maintain (Evangelho *et al*., 2001), and require very little energy (Laopaiboon *et al*., 2006). Fluidised bed reactors also require low retention time to generate high biomass (Sokół & Korpal, 2004). Biofilms grow on submerged inert packing, such as granular activated carbon, which allows for maximum microbial growth, and improves the bioreactors contaminant removal potential (Bouwer & McCarty, 1982).

1.3.2.1 Aerobic Bioreactors

Aerobic bioreactors fall into the category of secondary treatment. Secondary treatment utilises aerobic micro-organisms in biological reactors to remove or reduce organic matter, and are designed in a way which substantially degrades human and food waste, soaps and detergents (United Nations, 2003). These reactors require oxygen for the degradation of organic material, such as sugars, fats, organic short-chain carbon molecules, etc. by microbial organisms (Mack *et al*., 2004).

The efficiency of the bioreactor relies heavily on the substrate utilised for microbial growth and attachment. The ideal substratum material should be highly durable, have a low cost, should not clog easily and should have a high surface area for maximum microbial adsorption (Metcalf & Eddy, 1991). In Thailand, Polyvinyl Chloride (PVC) was used as an attachment surface to cultivate micro-organisms from recycled sludge from a pulp and paper company (Laopaiboon *et al.,* 2006).

The trickling filter bioreactor evolved from the early bioreactor experiments conducted by Alexander Mueller in 1865. It is one of the most commonly used fixed-film bioreactors, and is aerated from the bottom by natural draughts, or air blowers (Langwaldt & Puhakka, 2000). In this bioreactor, micro-organisms attach to a solid substratum, where their concentrations can flourish. This system has been shown to be suitable for treating wastewaters, as it is simple to use, easy to operate and has low energy requirements (Evangelho *et al*., 2001). Evangelho *et a*l. (2001) used a trickling filter bioreactor, to determine the influence of recirculation ratio (which is the fraction of water that is returned through the pumping station and

media filters) vs. the fraction that goes on to other treatment and the role and characteristics of a biomass in cyanide removal. The authors varied the recirculation ratio from between 0, 0.24, 0.43 and 0.75. Two reactors, one with (biotic) and one without biomass (abiotic), were set up, using polypropylene Pall rings as the support media. The reactors were fed with a mixture of synthetic gold milling effluent and sewage, with the treatment efficiency evaluated by monitoring chemical oxygen demand (COD). Free cyanide, thiocyanate, Cu, Zn and Fe concentrations, were also analysed using $HNO_3/HClO_4$ digestion and atomic absorption analysis. The results showed the removal of more than 90% of the free cyanide, thiocyanate, Cu and Zn, at the start of the experiment, without recirculation (0). Recirculation decreased pH and lowered the efficiency in the removal of Zn. It was found that thiocyanate degradation and Cu removal could be attributed to microbial activity. In the reactor without the biomass, cyanide removal was low and decreased further with recirculation. These results confirm the importance of biomass in pollutant degradation.

The rotating biological contactor (RBC) was patented in 1900 by Weigand and has been used in Germany since the 1920's. The RBC is similar to a trickling filter reactor, as they are both fixed-film reactors. Unlike a trickling filter, the media is supported horizontally across a tank of wastewater. Rotating biological contactor bioreactors consume very little energy, are simple and inexpensive to design. They are therefore recommended for wastewater treatment (Laopaiboon *et al*., 2006). The reactor is made up of discs, which allow for biofilm development as they are partially in contact with the air and approximately 40% of the discs are in contact with the surrounding water (Langwaldt & Puhakka, 2000). The micro-organisms in the biofilm then break down and stabilise organic pollutants within the reactor. Protozoan and metazoan species are primarily involved in this process, as they reduce dissolved organic matter and the majority of dispersed bacteria in wastewater (Martín-Cereceda *et al*., 2001).

Whitlock (1990) used the rotating biological contactor to treat gold milling effluents at the Homestake Treatment Plant (Dakota, United States of America). The process was carried out using 48 RBCs, allowing for maximum biomass immobilisation, chemical adsorption and precipitation. The results yielded a reduction of 95 to 98% cyanide, thiocyanate and heavy metal concentrations in the gold milling effluent.

The resistance of micro-organisms to a biocide and their effects on biocide degradation when it is used as a sole carbon source were evaluated in a RBC bioreactor by Laopaiboon *et al.* (2006). An inoculum of recycled sludge from the phoenix pulp and paper company in Thailand was used to cultivate microbial biofilms in a three unit bioreactor, using PVC as its growth surface. The biofilms were exposed to 0 to 180 ppm of the biocide (gluteraldehyde) and biofilm formation on the RBC was observed. Upon the re-establishment of the biofilm, the chemical oxygen demand (COD) and gluteraldehyde concentrations, as well as

the enumeration of viable populations of biofilm and planktonic bacteria, and population changes of higher organisms, were determined. At the end of the experiment, single colonies of bacterial cells in the biofilm were isolated, plated and tentatively identified, via the API 20 strip test. These results showed that the biofilms became resistant to gluteraldehyde and could eventually degrade it. The higher the biocide concentration, the longer it took for the micro-organisms to develop resistance to gluteraldehyde. The resistant species were tentatively identified as *Burkholderia cepacia*, *Aeromonas hydrophila* and *Aeromonas salmonicida*. The bacterial cells in the biofilm were also less susceptible to gluteraldehyde than their planktonic counterparts.

Fluidised-bed reactors are useful in groundwater remediation (Massol-Deyá *et al.,* 1997), as they are easy to set up, maintain and operate (Tsezos & Deutschmann, 1990). A fluidised bed reactor is capable of treating effluents in a lower retention time because of the high biomass concentrations achieved in the reactor (Sokół & Korpal, 2004). The low retention time is efficient because the particles in the beds are small, offering greater surface area for biofilm growth (Sokół & Korpal, 2006). They are based on the dilution of the influent to reduce the toxicity of the contaminants, making it easier for the biofilm organisms to remove or reduce pollutants in the reactor. As with the upflow-bed reactor, granular activated carbon is used as the surface for biofilm attachment (Sutton & Mishra, 1994).

Ochieng *et al*. (2002) evaluated the phase hold up, phase mixing, aspect ratio and superficial gas velocity of a three phase fluidised bed reactor in order to reduce the chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in brewery wastewater. The bioreactor was set up and a low-density support particle with an internal interstice was employed, to enable cost-efficiency at a relatively low gas superficial velocity. The results revealed that biodegradation increased when particle loading was at its maximum and then decreased thereafter.

Activated sludge bioreactors are generally not effective in the removal of contaminants from polluted groundwater, as shown by Ettala *et al.* (1992), where trace organic removal and optimisation by the reactor was low. The reactor essentially works by removing the sludge from the effluent (Langwaldt & Puhakka, 2000). The activated sludge bioreactor was used by Gonçalves *et al.* (1998) to evaluate the biological degradation of cyanides. They observed desorption of copper, and concluded that the mechanism responsible for the removal of copper was the adsorption of the anionic complexes formed between copper and cyanide within the biomass.

Uysal & Türkman (2005) evaluated the efficiency of a biosurfactant, rhamnolipid, on 2,4-dichlorophenol (2,4-DCP) biodegradation using an activated sludge bioreactor. Two reactors were set up, one containing both 2,4-DCP and rhamnolipid, with a control reactor, containing only 2,4-DCP. Rhamnolipid was added to the test reactor once the DCP concentrations ranged

between 30 and 100 mg.l^{-1}. In comparison to the control, in which no rhamnolipid was added, the test reactor only recorded a small increase in DCP biodegradation. Dichlorophenol removal for control and test reactor ranged between 97.4% to 97.7% and 99.7% to 99.8%, respectively. This verified that biosurfactants can be used to potentially degrade hydrophobic organic compounds in contaminated environments. No toxic effects on biomass, was observed with the application of rhamnolipid, and in fact, the presence of the biosurfactant stimulated bacterial growth.

An immobilised non-viable yeast biomass (*Saccharomyces cervisiae*) was used in a continuous-flow stirred bioreactor, to remove or reduce the metal concentrations of Cu, Cd, Cr, Ni and Zn from electroplating effluent (Stoll & Duncan, 1997). The authors evaluated the efficacy of two separate bioreactors; one with two tanks (dual bioreactor system) and the other with three tanks (triple bioreactor system). From the results, it was concluded that the dual bioreactor system was sufficient for the reduction of Cu, Cd and Cr from the effluent, with an average of 18% of the initial Zn and 17% of the initial Cd remaining in the effluent in tank two.

1.3.2.2. Anaerobic Bioreactors

The advantages of an anaerobic reactor includes low energy consumption, low excess sludge production, enclosure of odiferous compounds (Shink, 2002), high organic loadings and short hydraulic retention time (Najafpour *et al*., 2006). The reactor can thus be used to generate alternative fuel sources, such as methane, while assisting in waste disposal (Patel & Madamwar, 2002). As with any bioreactor, support materials are important for microbial attachment, and examples used in anaerobic bioreactors are charcoal, gravel, brick pieces, PVC and pumice stones (Patel *et al*., 1995).

Upflow fixed-film bioreactors can be both aerobic and anaerobic, with the aerobic version widely used for contaminant cleanup in groundwater (Langwaldt & Puhakka, 2000). Van der Hoek *et al.* (1989) showed that the upflow fixed-film bioreactor was efficient for the removal of PAH's and phenols. The upflow anaerobic fixed-film bioreactor has been reported by Ahring *et al*. (2002) and Seckler *et al*. (1996) to be effective in the treatment of hazardous waste with inhibitory or recalcitrant compositions. The Upflow Anaerobic Fixed Film (UAFF) reactor combines the recovery of usable energy with good process efficiency in a stable system. In addition, the fixed film reactor is capable of retaining active biomass in the system without the need for biomass recirculation.

Perez *et al.* (2006) evaluated the anaerobic biodegradation of a mixed feed composed of wine vinasses and cutting oil wastewater (COW), in a laboratory upflow anaerobic fixed-film reactor loaded with open-pore sintered glass beads (SIRAN). The experimental procedure was

designed to examine the effect of increasing the percentage of cutting oil wastewater in the feed after the first initial feed. Wine vinasses were initially fed through the bioreactor. At steady-state conditions, the chemical oxygen demand (COD) decreased by 87% and total organic carbon (TOC) decreased by 94.6% in wine vinasses after loading 22.3 kg COD/m^3 per day. The biological activity also decreased dramatically once the COW was added. Experimentally, the UAFF bioreactor, achieved more than 85.8% COD reduction and 58.1% TOC at a COD loading of 16.7 kg COD/m3 per day and hydraulic retention time (HRT) of 0.15 days. Therefore, it can be concluded that COW can be removed, if not degraded, by the anaerobic treatment in the presence of a biodegradable co-substrate, such as wine vinasses.

The anaerobic upflow fixed-film bioreactor has been used effectively to remove Pentachlorophenol (PCP), ethylene, ethane and phenol from contaminated systems (Hendriksen *et al.*, 1991; Juteau *et al.*, 1995). Treatment of contaminated groundwater has not yet been reported, as the reactor operates at high temperatures and high organic carbon supplementation (Langwaldt & Puhakka, 2000).

Different support materials, such as charcoal, gravel, brick, PVC pieces and pumice stones were evaluated to determine which material performed best at stabilising and recovering energy from cheese whey (Patel *et al.*, 1995). Twenty anaerobic upflow fixed film reactors were used in this study. Each reactor consisted of a glass column packed with one of the five materials and biofilms were allowed to develop naturally. Gas was collected and measured from the displacement of the acidified saturated salt solution and analysed using gas chromatography. The charcoal packed reactor obtained the highest total digested gas, as well as the highest methane content, presumably due to improved surface area for the attachment of methanogens and other anaerobic bacteria, and good biofilm formation. The lowest total gas and methane content was recorded for the pumice stone reactor. Chemical oxygen demand removal was also highest (76.6%) in the charcoal reactor, followed by the gravel, brick, PVC pieces and pumice stones. The anaerobic fixed film reactor with the charcoal bed was the most efficient and it was concluded that this system could be used for high-strength dairy waste management and energy recovery at relatively short hydraulic retention time.

Patel & Madamwar (2002) undertook a study to maximise methane generation from low pH petrochemical industrial wastewater, by optimising temperature and the organic loading rate in an upflow anaerobic fixed-film bioreactor (UAFF). A laboratory-scale UAFF reactor was set up with bone charcoal as a support material. Acidic petrochemical wastewater was used as a substrate and the biofilm was allowed to develop. The reactor was run at 25°C, 37°C, 45°C and 55°C, respectively. The organic loading rate for each temperature varied from 3.6 to 21.7 kg COD m^{-3} kg^{-1}. At 37°C, when 21.7 kg COD m^{-3} kg^{-1} was added to the system, COD and BOD reduction amounted to 90 to 95%, respectively, and 0.450 m^3 kg^{-1} COD d^{-1} as a methane

yield. At 55°C, the highest methane yield of 0.666 m^3 kg^{-1} COD d^{-1} was recorded which also decreased as the organic loading rate was increased. At 45°C, the highest methane yield was 0.416 m^3 kg^{-1} COD d^{-1} added, which also decreased as the organic loading rate increased. From the results obtained, it could be concluded that the best performance was observed in the reactor run at 37°C, which was the optimum temperature for methanogen growth.

The Upflow Anaerobic Sludge Blanket (UASB) reactor has become a viable technology in the treatment of industrial wastewaters (Fang *et al*., 1996). Granular sludge formation is the main distinguishing characteristics of UASB reactors compared to other anaerobic technologies. It does, however, take a while for anaerobic sludge granules to develop (Liu & Tay, 2004).

The biodegradation of tech-hexachlorocyclohexane was evaluated using an upflow anaerobic sludge blanket (UASB) reactor under a continuous mode of operation (Bhat *et al*., 2006). More than 85% removal of tech-HCH was recorded. Parawira *et al.* (2006) compared the COD reduction potential when treating potato waste leachate of a UASB to an anaerobic packed-bed reactor. The COD removal efficiencies of both reactors were greater than 90% in the leachate effluent.

Jin *et al*. (2008) compared the efficiency of the UASB, Upflow Stationary Fixed Film (USFF) and an Anaerobic Sequencing Batch Reactor (ASBR) in performing the ANAMMOX process. The ANAMMOX process is a novel and promising alternative to conventional denitrification removal of nitrogenous compounds at lower cost (Dong & Tollner, 2003). The three reactors were run in upflow mode, with the influent pumped in from below. ANAMMOX sludge was prepared by enriching nitrifying sludge. The UASB, USFF and ASBR reactors were filled with 0.8 L inoculum and the nitrifying bacteria were cultivated with synthetic wastewater containing ammonium, according to the method used by Zheng *et al*. (2004). The tolerance of the bioreactors to hydraulic and substrate concentration shock was evaluated. From the results obtained, it could be concluded that all three reactors were more tolerant to hydraulic shock than to substrate concentration shock. The UASB reactor was found to be more stable to increases in substrate concentration, than the USFF and ASBR reactors, while for flow rate shock the results showed that the ASBR reactor was the most stable, followed by the UASB.

1.4 TECHNIQUES FOR DETERMINING METAL CONCENTRATIONS IN ENVIRONMENTAL SAMPLES

An increase in pollution, industrial areas and environmental degradation has resulted in a corresponding increased concentration of heavy metals and Polycyclic Aromatic Hydrocarbons (PAHs) in water, sediment and the environment (Herbes & Schwall, 1978; Van Schooten *et al*., 1995). Research has shown that the presence of organochlorine pesticides

(OCPs), such as dichlorodiphenyl-trichloroethane (DDT), chlordane, hexachlorbenzene (Awofulu & Fatoki, 2003) and PAHs, such as naphthalene and benz anthracene (Herbes & Schwall, 1978) have increased in the environment. There has also been an increase in the occurrence of pharmaceutically active compounds, such as carbamazepine, in aquatic environments (Jos *et al.*, 2003). Contamination of riverine systems has also been shown by Prat *et al.* (1999), to be due to the continuous dumping of mining wastes, resulting in adverse effects of heavy metals on aquatic organisms at both individual and community levels (Leslie *et al.*, 1999). It is thus essential that the degree of contamination be evaluated in order to apply appropriate control or remediation strategies.

The metal concentrations in environmental samples may be determined using different analytical techniques. These techniques can be divided into single element analysis, using Atomic Absorption Spectrometry (AAS), Flame Atomic Absorption Spectrometry (FAAS) and multi-element analysis, using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and X-Ray Fluorescence Spectrometry (XRF), Atomic Emission Spectrometry (AES) and Instrumental Neutron Activation Analysis (INAA).

1.4.1 Single-element Analysis Techniques

Atomic Absorption Spectrometry (AAS) is currently widely used for the analysis of most metals, metalloids and for some non-metals, as well. The technique works by identifying the free atom of a particular element, which then absorbs light at the wavelength characteristic to that element. An AAS requires a light source, an energy source, i.e. a flame to decompose the sample into its constituent atoms, a monochromator, which would then isolate the particular wavelength, a photomultiplier detector and a readout device. The light source is usually a hollow cathode discharge lamp, composed of the element to be determined (Ure, 1995).

The AAS technique was used by Lin *et al.* (2003) to conduct a study on the water quality of the Mhlathuze River, servicing rural areas in Kwazulu-Natal, South Africa. Their results revealed high metal concentrations in the water samples from the Richard's Bay estuary and Felixton. In comparison to the other metals analysed, the Al concentration was particularly high, which probably resulted from runoff from the nearby aluminium smelters and fertiliser manufacturing factories. Kelly *et al.* (2004) also used AAS to determine the efficiency of a bioluminescent bacterium (Shk 1) in the removal of metals from an activated sludge wastewater treatment system. By using the Shk 1, the authors were able to detect the adsorption abilities of the different metals to the activated sludge. The bacterium showed greater sensitivity to Cu adsorption, followed by Cd and then Zn.

Flame atomic absorption spectrometry (FAAS), is a rapid, sample handling, measurement technique. The higher the temperature of the flame, the greater the ionisation of the elements, which will then decrease interference problems associated with incomplete dissociation. The biggest drawback in using FAAS is when chemical interference effects occur due to the low energy of the flame atomisers (Ure, 1995).

1.4.2 Multi-element Analysis Techniques

Knowledge of the extent of pollution by contaminants in environmental samples is essential in the maintenance of environmental health. It is also essential to know the concentrations of elements which are acceptable in different environments, in order to rapidly identify and prevent any pollution episodes. The techniques used to identify one or more elements/metals simultaneously, are called multi-element analysis techniques (Ure, 1995).

Atomic Emission Spectrometry (AES) consists of an exciting source and a monochromator/detector, which may be capable of wavelength scanning for rapid sequential multi-element analysis. A polychromator with a number of fixed exit slits and detectors for simultaneous multi-element analysis may also be used. Atomic Emission spectrometry differs from atomic absorption spectrometry, in that it can readily provide simultaneous or very rapid sequential, multi-elemental analysis of a sample solution.

X-ray Fluorescence Spectrometry (XRF) can detect all elements over the atomic number eight. Although XRF has successfully been employed in the detection of the major constituents of soil samples, it is less sensitive in the detection of minor and trace elements. A homogeneous sample is usually prepared for quantitative analysis by fusion with a borate flux, as particle size, composition and element form affect analysis (Ure, 1995). Kröpfl *et al*. (2003) developed a Total Reflection X-ray Fluorescence Spectrometric (TXRF) method for elemental analysis of lead and nickel contaminated natural biofilms, grown on polycarbonate substrates. When comparing the results obtained for metals in the biofilm samples with the plankton reference material (biofilms have a similar matrix to the plankton and no reference for biofilms exists), deviations from the certified concentration values were found to be less than three percent. They therefore concluded that the TXRF method is a powerful tool for studying metal accumulation in biofilms, due to its low sample demand and the multi-elemental capability of the technique.

Instrumental Neutron Activation Analysis (INAA) is a multi-element, solid sample technique for the analysis of soils, plants and biological material. It makes use of γ-irradiation, to determine the concentrations of trace elements in the sample (Ure, 1995; Freitas *et al*., 2007). Although this technique has proved successful as an analytical tool for metal content in the

analytes, access to a neutron source, usually a nuclear reactor, restricts the use of this analytical technique for routine analysis.

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) principally uses the emission of flame-like plasma formed on a quartz torch by coupling a radiofrequency electromagnetic field to the electrons, on ionised Argon plasma. At these high temperatures, atomisation of the sample is virtually complete for most of the elements and strong atomic and ionic line emission can occur. Two types of spectrometers, namely a single-channel scanning monochromator and a multi-channel fixed-wavelength polychromator, can be used. The monochromator is more flexible, has higher resolution, background correction can easily be made and is less expensive to operate than the polychromator. The multi-channel fixed-wavelength polychromator type is usually preferred for the routine analysis of large numbers of samples and where the range of elements is known in advance (Ure, 1995). The multi-elemental nature of ICP-AES (Saleh *et al*., 2000) has made it a powerful technique in the analysis of soil and environmental samples, as a wide range of elements as well as ion exchange resin extracts in soil can be determined simultaneously. Vermeiren *et al.* (1990) used ICP-AES to determine the presence of the metals Cd, Pb, Cu and Zn in natural waters. In order to evaluate ICP-AES as an accurate method for metal concentration determination, a portion of the samples were enriched with known concentrations of the metals, while other samples were left uninoculated and were thus designated as controls. Upon comparison of the ICP-AES analysed results for the enriched and control samples, the authors concluded that the technique accurately determined the metal concentrations. Mowat & Bundy (2001) studied the difference in metal concentrations between the highly polluted, Bayous Trepagnier (BTP) and the less polluted, Bayou St. John (BSJ), in Louisianna, USA. The authors used ICP-AES and found that in sediment samples, the metal concentrations recorded from the BTP samples were over one order of magnitude higher than in the samples from the BSJ site. The calibration of reference materials for X-ray fluorescence was done by Swagten *et al*. (2006), using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) and NAA, as no commercially available polyethylene reference material for the calibration of X-ray spectrometry existed. The reported concentrations measured by ICP-AES and NAA were accurate enough to be used as an input for the certification of the XRF reference material.

Inductively Coupled Plasma Atomic Mass Spectrometry (ICP-MS), like ICP-AES, provides comprehensive element coverage and is highly sensitive. It is however, not economically feasible for soil trace analysis. Freitas *et al*. (2007) evaluated the efficacy of INAA and ICP-MS in providing accurate concentrations of metals in *Lichen thalli*, *Parmelia caperata* (*P. caperata*) and tree bark *Petunia hybrida* (*P. hybrida*). These samples were transferred from a clean area (low pollution area) and exposed for different time periods at testing facilities in

three coastal cities in Portugal (Viana do Castelo, Lisboa and Sines). The results were compared to reference materials to assess the quality of the methods in the determination of different metals. Compared to the reference material the results obtained for Bromide (Br), Cu and Sodium (Na) using INAA were statistically lower. In contrast, the results recorded for ICP-MS were generally found to be statistically accurate when compared to the reference material.

1.5 IDENTIFICATION OF METAL TOLERANT MICRO-ORGANISMS

1.5.1 Viability Probes and Microscopy

Microscopic techniques can be used in conjunction with a singular fluorescent probe or dye to provide cell counts using images based on the relative abundance of micro-organisms (Boulos *et al*., 1999; Quéric *et al*., 2004). Fluorescent probes, allows for the distinction between live and dead cells, based on the physical integrity of the micro-organism (Ramirez *et al*., 2000). The LIVE/DEAD Baclight™ viability probe consists of two components, SYTO9, which is a green fluorescent stain, able to penetrate both the membrane and the cytoplasm of both intact and damaged cells and a red fluorescent stain, propidium iodide, which only penetrates the cell when there is a loss of membrane integrity. SYTO 9 bound to DNA, is excited at 480 nm and propidium iodide at 540 nm (Haugland, 2002). To examine the mechanisms by which biofilm organisms aggregate and respond to external stress, Epifluorescence Microscopy (EFM) and Confocal Laser Scanning Microscopy (CLSM) can be used to capture the biofilm images. Confocal Laser Scanning Microscopy (CLSM) is used to study the spatial distribution of bacteria (Hope & Wilson, 2003), microbial aggregates and activated sludge flocs and epifluorescence microscopy is used to study overall morphology and the abundance of micro-organisms (Zilles *et al*., 2002).

Membrane integrity of marine bacterioplankton collected from various sites in the western Seto Inland Sea of Japan was studied by Decamp & Rajendran (1998). The collected samples were mounted on a glass slide and viewed under an epifluorescence microscope. A higher concentration of viable cells (65.2% to 86.4%) was recorded in the winter months, where oxygen concentration was highest. In contrast, during the summer months, where temperatures of 23°C to 26°C were recorded (Decamp & Rajendran, 1998), viability decreased. The authors suspected that temperature and the concentration of the dissolved oxygen might influence viability.

Activated sludge samples (sample one to three) were stained with the Baclight™ viability probe, after collection from three different sites (Illinois, United States of America). Epifluorescence Microscopy, Confocal Laser Scanning Microscopy (CLSM) and Two Photon Excitation Laser Scanning Microscopy (TPE-LSM) were used to visualise the number of living

cells in the sludge. This was then compared to the number of nonviable cells in the sludge after an aerobic starvation period. The fresh cells contained 95% viable cells, while after biomass starvation, epifluorescence analysis showed a decrease in viability in sample 1 from 80% to 56% (activated sludge). Results showed that the selection of the most appropriate microscopic technique depended on the type of activated sludge sample. Epifluorescence Microscopy was found to be adequate for the analysis of conventional activated sludge with low-density flocs and both Epifluorescence and CLSM proved to be ineffective in visualising and thus quantifying the more dense activated sludge flocs. Two Photon Epifluorescence Laser Scanning Microscopy images revealed the internal floc structure, allowing for more accurate quantification of sludge blankets. The TPE-LSM microscopic technique is however, costly and the availability of equipment makes it difficult to use as a standard tool to monitor changes in activated sludge viability and structure (Lopez *et al*., 2005).

1.5.2 Flow Cell Cultivation

Microbial biofilms are a collection of microbial cells organised within extracellular matrices. Their growth pattern increases their chances of adaptation and survival in nature, due to protection offered by the surrounding matrix, especially during stressful situations (Decho, 2000). For effective biological treatment, degradation of organic compounds must be optimised. One way to stimulate degradation would be through increased knowledge of genetic information of the organic compounds, allowing for the optimisation of techniques, to control or remove pollutants (Christensen *et al*., 1998). The cells can also be used to identify metal-tolerant micro-organisms through exposure to varying metal concentrations.

Flow cells are used to cultivate and study microbial biofilms in vivo (Wolfaardt *et al.,* 1994; Caldwell *et al*., 2002). They are multi-channelled to allow for experimental replication and simplified handling, and are usually made of Perspex. Wolfaardt *et al.* (1994) constructed continuous-flow culture chambers, or flow cells, to observe the adaptive strategies of degradative biofilms. A microscope glass coverslip was mounted on the Plexiglass flow chambers, which measured 1 mm deep, 3 mm wide and 42 mm long. The glass slide was kept in place with an adhesive. The coverslip served as the attachment surface for biofilm development and was also the surface used to view the microbial community microscopically.

Christensen *et al.* (1998) used a biofilm community to degrade benzyl alcohol. The community consisted of three organisms, *Pseudomonas putida*, *Acinetobacter* sp. Strain C6 and an unidentified isolate, D8. Mixtures of the three strains were cultured for two days in Luria Bertani (LB) broth, followed by inoculation into two- and four chamber flow cells. Benzyl alcohol

was used as the sole carbon source for microbial growth, and after seven days, 16S ribosomal DNA fluorescent probes were used to target the three species within the biofilm. Image analysis was performed using CLSM. Analysis revealed that *P. putida* RI was the most common in the upper layers and *Acinetobacter* dominated the layers near the substratum, where most of the overall biomass was observed.

The flow cell biofilm culture technique was used by Teitzel & Parsek (2003) to visualise biofilm degradation after exposure to 1 mM Cu and 64 mM Zn over different time periods, using CLSM. Their results revealed that in the untreated control, the majority of cells were alive, compared to the Cu and Zn-treated channels, where the outer layer of cells were found to be dead while the inner layer contained viable cells.

1.5.3 Molecular Typing

Only approximately 1% of bacteria can be grown in pure cultures (Petit *et al*., 1999). Existing analytical techniques have been found to be time-consuming, laborious and generally do not provide information on bacterial viability (Yu & McFeters, 1994; Touron *et al*., 2005). Some of these techniques include plating onto selective media, followed by three to four days of incubation and then finally analysis by standard microbiological techniques. An additional difficulty with the existing analytical techniques lies in the fact that bacteria have the ability to persist in aquatic environments in a viable-but-non-culturable state (VBNC) (Touron *et al*., 2005), such as the gastroenterolitic bacteria, *Campylobacter jejuni, Yersinia enterolytica* and *Vibrio cholerae* (Alexandrino *et al*., 2003). Advances in molecular biology have been of great value in the study of microbial populations in the environment (Petit *et al*., 1999). The need for rapid detection methods for waterborne pathogens and other non-culturable micro-organisms are also essential for early detection and treatment of disease outbreaks. Molecular techniques, such as nucleic acid extraction and polymerase chain reaction analysis (PCR) can be employed to eliminate the process of prior culture isolation techniques (Ritchie *et al*., 2000).

The Polymerase Chain Reaction (PCR) technique can also be used for studying unculturable organisms. It is a rapid and relatively simple technique and can amplify minute quantities of DNA available from a particular source into billions of copies of a designated gene-sized fragment. This ensures that sufficient quantities of the starting template are available for sequencing. It is a three step process, generally repeated for 30 to 40 cycles in an automated cycler. The sequences of the forward and reverse primers do not necessarily have to match the sequences to which they anneal exactly, as some level of mismatching is expected in nature (Viljoen *et al*., 2005). Sequencing of rRNAs also provides the best descriptions of microbial diversity. Sequence divergence among the different species (5S, 16S and 23S rRNAs) assisted

in defining primary evolutionary lines, providing a framework for microbial classification (Owen, 2008). The first step in all these molecular methods is DNA extraction. Once whole genomic DNA has been extracted, the 16S rDNA region is amplified using the polymerase chain reaction (PCR) technique. This molecular approach based on 16S rDNA is useful for the detection of bacterial community composition changes, since these genes are conserved (Kozdrój & Van Elsas, 2001). The technique includes the denaturing of the double-stranded DNA region of interest (16S rDNA), the attachment of primers to each strand, followed by the synthesis of a new DNA strand behind the primers on each template strand (Rawlings, 1995). In order to amplify a particular gene, specific primers, unique to that gene, are used.

Navia *et al*. (2005) used Pulse Field Gel Electrophoresis (PFGE) to describe the genetic diversity of *Shigella* species from different intercontinental sources. Of the 7023 patients attending the Tropical Medicine Unit from 1995 to 2000, about 19% presented with travellers' dysentery, and from 9% of cases, stool samples with *Shigella* isolates were recovered. Out of a total of 124 *Shigella* spp. isolates collected from nine different geographical zones, 58 were identified as *Shigella sonnei*, 54 as *Shigella flexneri*, two as *Shigella dysenteriae* and one as *Shigella boydii*.

Petit *et al*. (1999) developed a DNA extraction protocol suitable for estuarine water, allowing for the simultaneous extraction of RNA from viruses. The detection of *Salmonella* in the estuary water was performed using both PCR and standard culture techniques. Five samples containing *Salmonella* were studied, and culture techniques detected *Salmonella* in four of them. In contrast, the PCR analysis of the same samples showed *Salmonella* in all five samples, suggesting that the bacteria were probably in a VBNC state.

An alternative 16S rDNA-based fingerprinting method is denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA fragment (Becker *et al*., 2006). It is especially useful for determining the microbial community structure, where diversity is lower in contaminated soil (Ferris *et al*., 2003). Microbial diversity in polluted soil was studied using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) (Rasmussen *et al*., 2000). The authors used the technique to detect the bioavailable fraction of mercury in polluted soil from a garden farm in Kingston (USA). Two different soil types were spiked with 2.5 $\mu g.g^{-1}$ Hg in microcosms and the bacterial diversity in response to mercury exposure, was evaluated by the number of bands visualised in DGGE gels after electrophoresis of the PCR amplified products. The initial concentration of bioavailable Hg (estimated to be 40 $ng.g^{-1}$) was recorded in agricultural soil. The technique showed that the concentration stayed the same during the first three days, coinciding with increased degrees of resistance and a decrease in diversity. Kozdrój & Van Elsas (2001) used PCR-DGGE for structural diversity determination of dominating populations in situ in heavy metal contaminated soil. Total DNA was extracted from

the soil samples and amplified using eubacterial primers, with the PCR products analysed using DGGE. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis analysis showed significant differences in microbial community structure between the soils studied and the contamination levels.

1.6 AIMS OF THE STUDY

The continued provision of safe, potable drinking water is of major international concern, due to increases in urbanisation and the growing third-world population. This results in increased pollution from anthropogenic sources, be it human or industrial, leaching into river systems and resulting in elevated concentrations of metals and xenobiotics in the environment. This current study was undertaken to determine the presence of metal contaminants in the rivers of the Western Cape, South Africa. River water could serve as a possible alternative source of potable and domestic water, but because of severe contamination due to industrial- as well as human activities, the rivers in South Africa are highly polluted with micro-organisms and metals, amongst others. The aims of the study were as follows:

1.6.1 Identifying and sampling at three points along the Plankenburg- (Stellenbosch), Berg- (Paarl) and Diep Rivers, as well as the Rietvlei (Oil Refinery) on a monthly basis from May 2004 to May 2005 (Berg- and Plankenburg Rivers). Sampling of the Diep River sites started in March 2005 and continued until November 2005.

1.6.2. Determine the presence and concentrations of metals (Al, Cu, Fe, Mn, Ni, Pb and Zn) in the river water, sediment and biofilm samples, using nitric acid digestion and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES).
Article one (chapter two - Jackson, V.A., Paulse, A.N., Van Stormbroek, T., Odendaal, J.P. & Khan, W. 2007. Investigation into metal contamination of the Berg River, Western Cape, South Africa. *Water SA.*, 33: 175-182).
Article two (chapter three - Jackson, V.A., Paulse, A.N., Odendaal, J.P. & Khan, W. 2008. Investigation into the metal contamination of the Plankenburg- and Diep Rivers, Western Cape, South Africa. Accepted for publication by Water SA).

1.6.3. Determine LIVE/DEAD ratios of micro-organisms when exposed to varying concentrations of metals, through the use of flow cells and the Baclight^{TM} viability probe, and identify metal-tolerant micro-organisms, through DNA extraction, PCR and sequencing.

Article three (chapter four - Jackson, V.A., Paulse, A.N., Khan, S., Odendaal, J.P. & Khan, W. 2008. Identification of Metal-tolerant Organisms Isolated from the Plankenburg River, Western Cape, South Africa. Submitted to the Canadian J. Microbiol.).

1.6.4. Develop and optimise a laboratory-scale bioreactor system to reduce the concentrations of metals in river water.

Appendix A (Jackson, V.A., Paulse, A.N., Bester, A.A., Neethling, J.H., Du Plessis, K.R. & Khan, W. 2007. The application of bioremediation: reduction of metal concentrations in river water and COD in distillery effluent. *Wat. Sci. Technol.*, 55: 183-186).

1.6.5. Establish an on-site large-scale bioreactor system along the Plankenburg River.

Article four (chapter five - Jackson, V.A., Paulse, A.N., Bester, A.A., Neethling, J.H., Khan, S. & Khan, W. 2008. Bioremediation of Metal Contamination in the Plankenburg River, Western Cape, South Africa. Accepted for publication by International Biodeterioration and Biodegradation).

Investigation into metal contamination of the Berg River, Western Cape, South Africa

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Abstract

A recent decline in water quality of the Berg River, Western Cape, South Africa, has lead to the investigation into the degree of metal pollution in the river system. This study was conducted over a period of one year, from May 2004 to May 2005. The nitric acid digestion technique was used to extract metals from water, sediment and biofilm samples collected at various points (Site A - agricultural area, Site B - informal settlement and Site C – Newton pumping station) along the Berg River. Metal concentrations were obtained using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). The highest mean metal concentrations recorded were as follows; water samples, 6 $mg.\ell^{-1}$ for Al, 14.6 $mg.\ell^{-1}$ for Fe and 18.8 $mg.\ell^{-1}$ for Mn; sediment samples, 17448.8 $mg.kg^{-1}$ for Al and 26473.3 $mg.kg^{-1}$ for Fe; biofilm samples, 876.8 $mg.\ell^{-1}$ for Al and 1017.5 $mg.\ell^{-1}$ for Fe. The increased availability, or noteworthy incidence of Al and Fe, could be due to the leaching of metals into the river water from waste and household products associated with the informal settlement and the subsequent settling on sediment. The highest recorded concentrations in water were recorded for site C (agricultural area). Recorded concentrations fluctuated throughout the study period for most of the metals analysed, but Al and Fe were consistently above the recommended guidelines as stipulated by the Department of Water Affairs and Forestry and the Canadian Council of Ministers of the Environment.

Keywords: metals, river systems, sediment, biofilm, inductively coupled plasma atomic emission spectrometry (ICP-AES)

Introduction

Metals occur in less than 1% of the earth's crust, with trace amounts generally found in the environment (Alloway, 1995a). When these concentrations exceed a stipulated limit (South African Bureau of Standards, 2001; World Health Organisation, 1991), they may become toxic to the surrounding environment. Sources of metal contamination include industrial and medical waste (Dorigo et al., 2004), pesticides, petroleum by-products (Mowat and Bundy, 2001), household products, as well as urban and pharmaceutical waste (Brooks et al., 2003). Domestic and household sources of metal contamination generally occur as a result of corrosion of metal plumbing fittings, galvanized roofs and wire fences [zinc (Zn), cadmium (Cd)], and healthcare products, such as Zn- or selenium (Se) containing shampoos and Zn-containing baby creams (Alloway, 1995b). Silver paint containing Aluminum (Al), Al-coated roofs, saucepans and utensils (Friberg et al., 1986), are also possible sources of contamination.

Natural watercourses can also be contaminated with microorganisms, which inhabit the natural environment in the form of planktonic organisms and sessile biofilms. Under favourable conditions, microorganisms will generally form a biofilm on any surface exposed to an aqueous environment. These biofilms can be defined as a community of attached microbial cells organized within extracellular polymer matrices (EPS). This EPS assists in the bacterial survival by providing protection against metals, predation and environmental fluctuations, and also provide increased resistance against antimicrobial agents (Decho, 1990). Biofilms are advantageous in that they encapsulate toxic molecules, such as metals (Costerton et al., 1978), by providing a substrate for them to adhere to, thereby limiting the diffusion of biocides and other toxic molecules

across the EPS (De Beer et al., 1994; Huang et al., 1995). This implies that the attached biofilm communities may then be employed in the removal of toxins from aqueous systems, as the biofilms are able to concentrate and bind ions from the passing water (Neu et al., 1992).

The presence of metals in biofilms isolated from the Elbe River in Germany was determined in a study by Friese et al. (1997). They found fractions of several elements from stones and plates incubated in the river. These elements included potassium (K), calcium (Ca), chromium (Cr), manganese (Mn), lead (Pb), nickel (Ni), copper (Cu), zinc (Zn) and iron (Fe). Factors contributing to microbial biodiversity and the tolerance of certain organisms to metals depend on the type of attachment material (e.g. glass, stones, leaves, rocks) the biofilm is isolated from, the age of the biofilm and the concentration of the EPS. In addition, research has indicated that the predominant metals present in streams and lakes are Zn, Pb, Cu, Fe and Mn (Geesey et al., 1992; Nelson et al., 1996).

Metal concentrations in environmental samples, may be determined using different analytical techniques. These techniques can be divided into single element analysis, using Atomic Absorption Spectrometry (AAS) and simultaneous multi-element analysis, using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) and X-ray Fluorescence spectrometry (XRF). Vermeiren et al. (1990) investigated ICP-AES as a method of determining the presence of the metals Cd, Pb, Cu and Zn in natural waters. Certain samples were enriched with specific concentrations of the metals, while others were not. Comparison of the results obtained for the enriched and non-enriched river water samples indicated that the technique is accurate in the determination of metal concentrations.

An increase in urbanisation has led to an upsurge in informal settlements in the Western Cape, South Africa, where the inhabitants of these settlements experience a distinct lack of resources. Inadequate sanitation behaviour and a lack of adequate sewage disposal facilities may lead to the leaching of potentially harmful substances, from waste, household products, etc. into the environment. The Berg River was specifically selected due to the increased incidence of pollution recorded within the area in recent times. This increased pollution is of great concern, as the Berg River serves as a water source to towns, cities, rural communities, farms and recreational users in the area (River Health Programme, 2004). The objective of this study was to identify the predominant metals, which occur within the river water, sediment and the biofilm samples isolated from the Berg River (Paarl) in the Western Cape, South Africa.

Materials and Methods

Site Description

Sampling commenced in May 2004 and was conducted over a period of one year until May 2005. Experimental work entailed collecting samples along the Berg River (Western Cape) after 1, 5, 9, 17, 21, 25, 33, 37, 41, 45 and 49 weeks from the commencement of the study (Figure 2.1). As indicated on Figure 2.1, the samples were collected from Site A (agricultural farming area) and Site B, known as Plot 8000 (at the informal settlement of Mbekweni). Storm water drainage pipes from the communities in the settlement enter the river at Site B. A third site, Site C (the Newton pumping

station), serves as an inlet of storm drainage water and wastewater into the river from the residential area of Newton as well as certain areas of Mbekweni.

Sampling

Water samples (200 mℓ were collected in sterile 250 mℓ narrow mouth square polypropylene bottles (Cole-Palmer Instrument Company). The sediment samples were collected in 250 mℓ plastic containers and consisted of a combination of five different subsamples (±15 cm deep) collected at different locations in a defined perimeter. The biofilm samples were obtained by collecting various materials, such as glass, leaves, stones, rocks, etc. (~100 g) along each of the different sampling points and storing them in sterile whirlpack bags. The samples were stored at 4°C during transport.

Sonication of collected biofilm samples

Bacterial suspensions were removed from rocks, leaves, glass and stones collected from each representative site, by sonication. Collected material samples (~100 g) were sonicated for 10 minutes in 30 mℓ sterile distilled water in a UMC5 sonication bath (Instrulab, Inc.). The procedure was repeated at least twice, with fresh sterile d.H_20 added after each sonication step. The sonicated samples were combined to result in a total of 60 mℓ bacterial suspension. The biofilm suspension obtained was used for further analysis.

Metal concentrations in sediment, biofilm and water samples

To determine the concentrations of Al, Zn, Cu, Fe, Pb, Ni and Mn, sediment samples (0.500 – 0.600 g; dry mass), biofilm suspensions (5 mℓ) and water samples (5 mℓ) were digested with 10 mℓ 55% Nitric acid at 40°C for 60 minutes and then at 120°C for 180 minutes, using a Grant dry-block heater. A blank (control) of 10 mℓ 55% nitric acid was analysed along with the collected samples, to check for possible contamination. The samples were cooled to room temperature, filtered with Whatman filter paper No 6 into 20 mℓ volumetric flasks, made up to a volume of 20 mℓ with distilled water and subsequently filtered for a second time using 0.45 μm cellulose nitrate ultrafiltration membrane filters (Whatman). Metal concentrations were determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) analysis according to the procedure outlined in Saleh et al. (2000).

Statistical analysis

The results presented are the averages of five repeats for each particular sampling point at the different sampling sites. For statistical analysis, the dry masses (0.500 – 0.600 g) and volumes (5 mℓ) were taken into consideration for the calculation of the final metal concentrations in a given sample. Repeated measures ANOVA (RMA) was performed on all data obtained as outlined in Dunn and Clark (1987), using Statistica™. In each RMA, the residuals were analysed to determine if they were normally distributed. In all hypothesis tests a significant level of 5% were used as standards.

Results and Discussion

Metals in Water

Comparisons of the mean metal concentrations in water are represented in Figures 2.2A and B. The highest mean metal concentrations were recorded for Mn (18.8 mg.ℓ^{-1}), Fe (14.6 mg.ℓ^{-1}) and Al (6 mg.ℓ^{-1}) during weeks 25, 45 and 49, respectively, at Site A. The concentrations of Al and Fe were significantly higher than the recommended guidelines of Al and Fe in freshwater systems as stipulated by the Department of Water Affairs and Forestry (DWAF, 1996) and the Canadian Council of Ministers of the Environment (CCME, 2001) (Table 2.1). Overall the mean concentrations; for Al ranged from 0.4 mg.ℓ^{-1} during week 1 at site B to 6 mg.ℓ^{-1} during week 49 at site A, and for Fe from 1.7 mg.ℓ^{-1} during week 1 at site C to 14.6 mg.ℓ^{-1} during week 45 at site A (Table 2.1). The particular agricultural area, situated near the Berg River in the Western Cape utilises the following pesticides and insecticides for the treatment of their crops: Acrobat® MZ, Copperflo, Cosavet, Folpan, Karathane, Mancozeb™, Phosguard™, Richter and Stroby. The increased concentrations of Al could be due to the use of Phosguard™, of which Al oxide is a component (Seachem, 2006). Phosguard™ is an algicide, which is used to control algae growth in this particular agricultural area.

Manganese concentrations fell within the recommended quality guideline range for most of the sampling period with the exception of weeks 25, 45 and 49, where increased concentrations ranging from 3.6 mg.ℓ^{-1} to 18.8 mg.ℓ^{-1} at Sites C and A (Figure 2.2), respectively, were recorded. Manganese is a major component of pesticides such as Mancozeb™ and Maneb. Mancozeb™, then in turn, makes up 60% of the fungicide,

Acrobat® MZ, which is used to manage early and late blight in potatoes (Acrobat®, 2005). Mancozeb, or manganese-zinc ethylene bis (dithiocarbamate), is a fungicide used for the treatment of plant diseases in the Western Cape. Its usage in agriculture amounts to 1343 x 10^3 kg/a (Vermeulen et al., 2001). Contamination of the water could be due to excessive use or improper discarding of these pesticides into the river (Agency for Toxic Substances and Disease Registry) (ATSDR, 2000). As previously mentioned, Site A (Figure 2.1) is situated in an agricultural area and is the site at which the highest concentrations of metals were recorded. Runoff from a nearby farm enters the river at this site, which could account for the increase in Mn concentrations.

The recorded concentrations of Cu, Ni and Zn in water samples varied throughout the study period (Table 2.1). Lead could not be detected in any of the analysed samples. The concentrations of Cu, Ni and Zn fluctuated above and below the recommended guidelines of DWAF (1996) and the CCME (2001). The mean concentrations recorded for Ni were higher than the recommended concentrations during weeks 17, 21, 25, 33, 37, 45 and 49. The recorded mean Cu and Zn concentrations fell within the recommended guidelines for week 41 only (Table 2.1). The increases in Zn could also be as a result of Mancozeb™ use, as Zn is a component of this pesticide. The increased metal concentrations could also possibly be due to recurring anthropogenic episodes associated with the Berg River during the sampling period (Barnes, 2003).

Metals in Sediment

Comparisons of the mean concentrations for Al and Fe are presented in Figure 2.3. Results for Cu, Mn, Ni, Pb, and Zn are presented in Tables 2.2 and 2.3. No

recommended sediment quality guidelines for Al, Fe, Mn, Pb, Ni, Cu and Zn were available from DWAF (1996) and guidelines for Cu and Zn only were available from the Canadian Council of Ministers of the Environment (CCME, 2001). Significant differences ($p < 0.05$) in the concentrations for Al and Fe were observed for the sediment samples. The highest mean concentration ($mg.kg^{-1}$) of 17448.8 $mg.kg^{-1}$ was recorded for Al during week 1 at Site B (Figure 2.3). In comparison to the results obtained for week 1, no significant differences in concentrations ($p > 0.05$) of Al were recorded from weeks 5 to 49, where mean concentrations ranged from 353.2 $mg.kg^{-1}$ at Site A to 2687.7 $mg.kg^{-1}$ at Site C, both during week 5. The highest mean concentrations of 21035 $mg.kg^{-1}$ and 26473.3 $mg.kg^{-1}$ were recorded for Fe in weeks 1 and 5, at Sites B and C, respectively. In comparison to the results obtained for weeks 1 and 5, no significant ($p > 0.05$) differences in concentrations of Fe were recorded for weeks 9 to 49, where the mean concentrations ranged from 700 $mg.kg^{-1}$ to 7014.9 $mg.kg^{-1}$, during weeks 33 and 45, at Sites A and C, respectively.

The Agency for Toxic Substances and Disease Registry (ATSDR, 1995) reported that Al composes 8% of the earth's crust, with dissolved Al and Fe primarily derived from soils (Neal et al., 1997). In addition, Al forms complexes with organic matter in soil, which could contribute to elevated concentrations (Tipping et al., 1991).

Effluent from a nearby stormwater drain enters the river at site B, where the highest concentration of Al was recorded in week 1. The high concentrations of Al and Fe in the sediment samples could partly be due to the leaching of metals from housing materials and household products, utilised by inhabitants of the informal settlements, into the river. In addition, the galvanised sheeting used to build informal dwellings is primarily composed of Fe. When pure iron reacts readily with oxygen and moisture in the environment, a red or brown ferric oxide coating is formed, which destructively

corrodes the galvanised sheeting. Excess Fe in aqueous environments can be the cause of chronic and acute health effects, but according to the Department of Water Affairs and Forestry, accidental Fe poisoning is rare (DWAF, 1996).

Tables 2.2 and 2.3 represent the mean concentrations for Cu, Mn, Ni, Pb and Zn. Manganese, Ni and Pb concentrations were significantly low throughout the study period with the highest mean metal concentrations for Mn, Ni and Pb recorded at: 70 $mg.kg^{-1}$ in week 5 at Site C; 44 $mg.kg^{-1}$ in week 1 at site B and 23 $mg.kg^{-1}$ in week 45 at Site B (Tables 2.2 and 2.3), respectively. No recommended sediment quality guidelines for Pb, Mn and Ni were available from DWAF (1996) and the CCME (2001).

The highest mean Cu concentration of 74 $mg.kg^{-1}$ was recorded at Site B, during the first sampling week (Table 2.2). This result for Cu was higher than the recommended environmental quality guideline of 35.7 $mg.kg^{-1}$ in freshwater sediment as specified by the CCME (2001). Thereafter, during weeks 5 to 49, concentrations for Cu decreased significantly ($p < 0.05$), to levels lower than the guideline (CCME, 2001).

The highest mean Zn concentration of 395 $mg.kg^{-1}$ was recorded at Site B, during week 1 (Table 2.3). This result was higher than the recommended Canadian sediment quality guidelines of 123 $mg.kg^{-1}$ (CCME, 2001). During the subsequent sampling weeks the mean Zn concentrations ranged from 4 to 36 $mg.kg^{-1}$ (Table 3), which falls within the accepted CCME guidelines. During week 1 of sampling the region experienced very low rainfall (6 mm). In the following weeks, the recorded rainfall ranged from 52.2 mm to 154 mm. The lower rainfall could imply that the metals were not readily available in the water column, impeding metal transportation from site to site and increasing metal accumulation in sediment.

Metals in Biofilms

No recommended biofilm quality guidelines were available from DWAF (1996) and the Canadian Council of Ministers of the Environment (CCME, 2001). Biofilms are layers of organisms, organic matter and inorganic material, organised within extracellular polymeric substances (EPS) (Decho, 1990). These organisms attach and develop on biologically active or non-active surfaces (Stickler, 1999).

Comparisons of the mean concentrations for Al and Fe are presented in Figure 2.4. Results for Cu, Mn, Ni, Pb, and Zn are represented in Tables 2.4 and 2.5. The highest metal concentrations recorded in the biofilm samples ($mg.\ell^{-1}$), were for Al and Fe, as in the case of sediment. Throughout the entire study period, Fe was consistently present in elevated concentrations in water, sediment and biofilm samples when compared to the other metals analysed for. The recorded results for Fe and Al fluctuated throughout the entire study period. The mean concentrations recorded for Al ranged from 14.1 $mg.\ell^{-1}$ during week 33 to 876.8 $mg.\ell^{-1}$, during week 37, both at Site A (Figure 2.4). In addition, the mean Fe concentrations in biofilm samples ranged from 18 $mg.\ell^{-1}$ during week 33 to 1017.5 $mg.\ell^{-1}$ during week 37, again both at Site A (Figure 2.4).

As indicated above, an increase in the concentrations of Al and Fe in the biofilm samples was observed at site A in week 37. For the same time period, a corresponding study investigating microbial contamination of the river revealed a microbial count in sessile samples of 3.9×10^7 organisms/ml. This increased number of microorganisms could have facilitated the Al and Fe accumulation in the biofilm. It is well recognised that microorganisms have developed unique means of resistance to specific metals (Roanne and Pepper, 2000), the mechanisms of which are not yet fully understood. Extracellular

Polymeric Substances contains various constituents such as polysaccharides, proteins, nucleic acids, lipids or humic substances (Mayer et al., 1999). Decho (1990) showed that the EPS assists in the bacterial survival by providing protection against predation and environmental fluctuations.

Tables 2.4 and 2.5, represents the metal concentrations recorded in biofilm samples for Cu, Mn, Ni, Pb and Zn. These metal concentrations were consistently lower than the concentrations recorded for Al and Fe. The highest mean metal concentrations for Cu, Mn, Ni, Pb and Zn were recorded at: 2 $mg.\ell^{-1}$ in weeks 1 and 37 at sites C and A; 71 $mg.\ell^{-1}$ in week 25 at Site A; 19 $mg.\ell^{-1}$ in week 17 at site A; 1.6 $mg.\ell^{-1}$ in week 5 at site C and 8.4 $mg.\ell^{-1}$ in week 25 at site A, respectively (Tables 2.4 and 2.5).

Kröpfl et al. (2006) studied metal accumulation by biofilms in the Tisza River, Hungary. The authors utilised different substrates to cultivate biofilms, with the aim of investigating the applicability of biofilms as biomonitors. The different substrates used were andesite, polished granite, Plexi-glass, granite and polycarbonate. The biofilms were cultivated for 6 weeks, after which, the samples were analysed using Total Reflection X-Ray fluorescence (TXRF) for elemental analysis. The concentrations of essential elements and heavy metal pollutants (Cu, Ni, Pb and Zn) were highest in biofilms on polished granite or granite (rocks and stones) (Kröpfl et al., 2006). In the present study, various substrates such as rocks, leaves, glass and stones were collected as representative biofilm samples form the Berg River. Biofilms are currently being studied as biomonitors in ecological research, but it is important to note that as yet no guidelines exist for biofilms in aquatic systems.

Research has shown that the EPS layer also exhibits a high metal absorption capacity. A study by Suh et al. (1999) investigated the accumulation of Pb^{2+} into the EPS layer of pure species *Aureobasidium pullulans* biofilms using Transmission Electron

Microscopy (TEM). The results of the study showed that the EPS was responsible for more than 90% of Pb^{2+} accumulation of the total Pb dissolved. The TEM microphotographs showed that the Pb^{2+} penetrated the cell wall, cell membrane and even into the inner cellular parts of the cell.

Prat et al. (1999) studied the recovery of an aquatic ecosystem after toxic mining waste was dumped into the Guadiamar River Basin, Sevilla, Spain in April 1998. Nine sites were sampled, three of which were unaffected and designated as the control or reference sites. Sampling was carried out over a period of five months, in the form of plankton and particulate material, naturally occurring biofilms and macroinvertebrates obtained from water, riffles (stones), introduced artificial substrates (large tiles), vegetation and sediment. These samples were analysed for water quality and for arsenic (As), cadmium (Cd), antimony (Sb), thallium (Tl), Cu, Pb and Zn concentrations, as these were the most abundant heavy metals in the mine spill. Compared to the reference stations, the concentrations of metals were higher in the polluted sites, with As, Cu, Pb and Zn, being the most abundant. Except for Cd, Cu and Sb, all the other metals in biofilms from the polluted sites were found to be more than 15 times higher than in the reference stations (Prat et al., 1999). In addition, the metal concentrations recorded in the biofilm samples were five to 20 times higher than the recorded values in macroinvertebrates (Prat et al., 1999). This study proved that biofilms are effective in the accumulation of metals from the environment.

Conclusions

The conclusions of this study were as follows:

- Al and Fe were recorded at consistently higher concentrations than all the other metals analysed for in water, sediment and biofilm samples.
- In both the sediment and biofilm samples, the concentrations of Al and Fe were significantly higher ($p < 0.05$) than Cu, Zn, Pb, Ni, and Mn.
- On average, the results generated for water for Al and Fe, were higher than the quality guidelines recommended by DWAF (1996) and the CCME (2001).
- The results for Cu and Zn were higher than the recommended quality guidelines in freshwater sediment (CCME, 2001). No guidelines for Al, Fe, Mn, Pb and Ni were available in sediment.
- The highest metal concentrations were obtained in the sediment and biofilm samples, yet no freshwater guidelines for metals in sediment were available from DWAF and no guidelines for metal concentrations in biofilms were available from either DWAF or the CCME.

Future research

Future research would include the setting up of parameters or guidelines for acceptable metal concentrations in rivers. Research at the Cape Peninsula University of Technology is currently being conducted on the Lourens-, the Diep-, the Kuils- and the Plankenbrug Rivers, as well as the Berg River, as discussed in this article.

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List of figures and tables

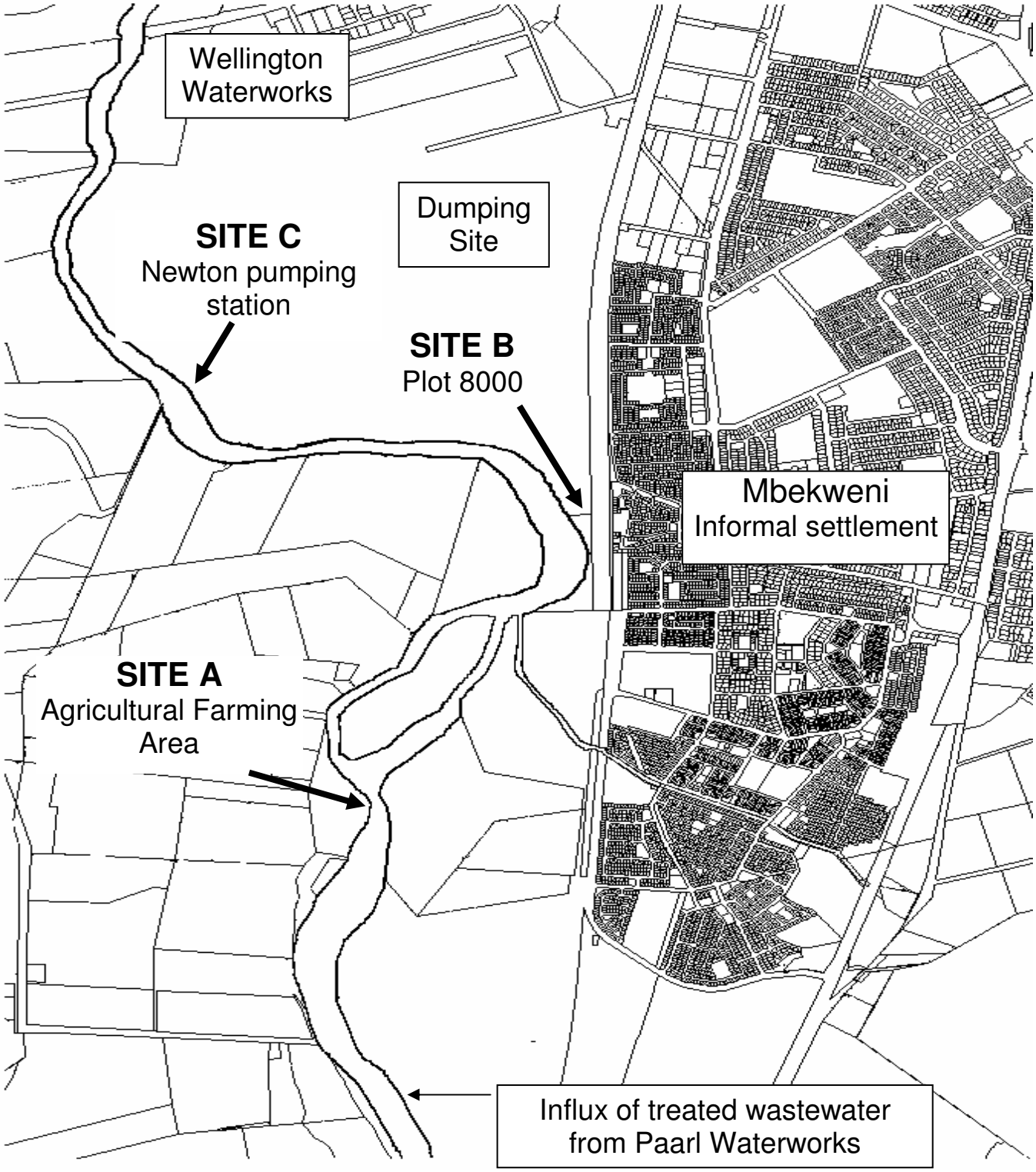

Figure 2.1

Map of the Berg River indicating the different sampling points: Site A, indicates the agricultural farming area; Site B, indicates Plot 8000, close to the informal settlement of Mbekweni; and Site C, the Newton Pumping station

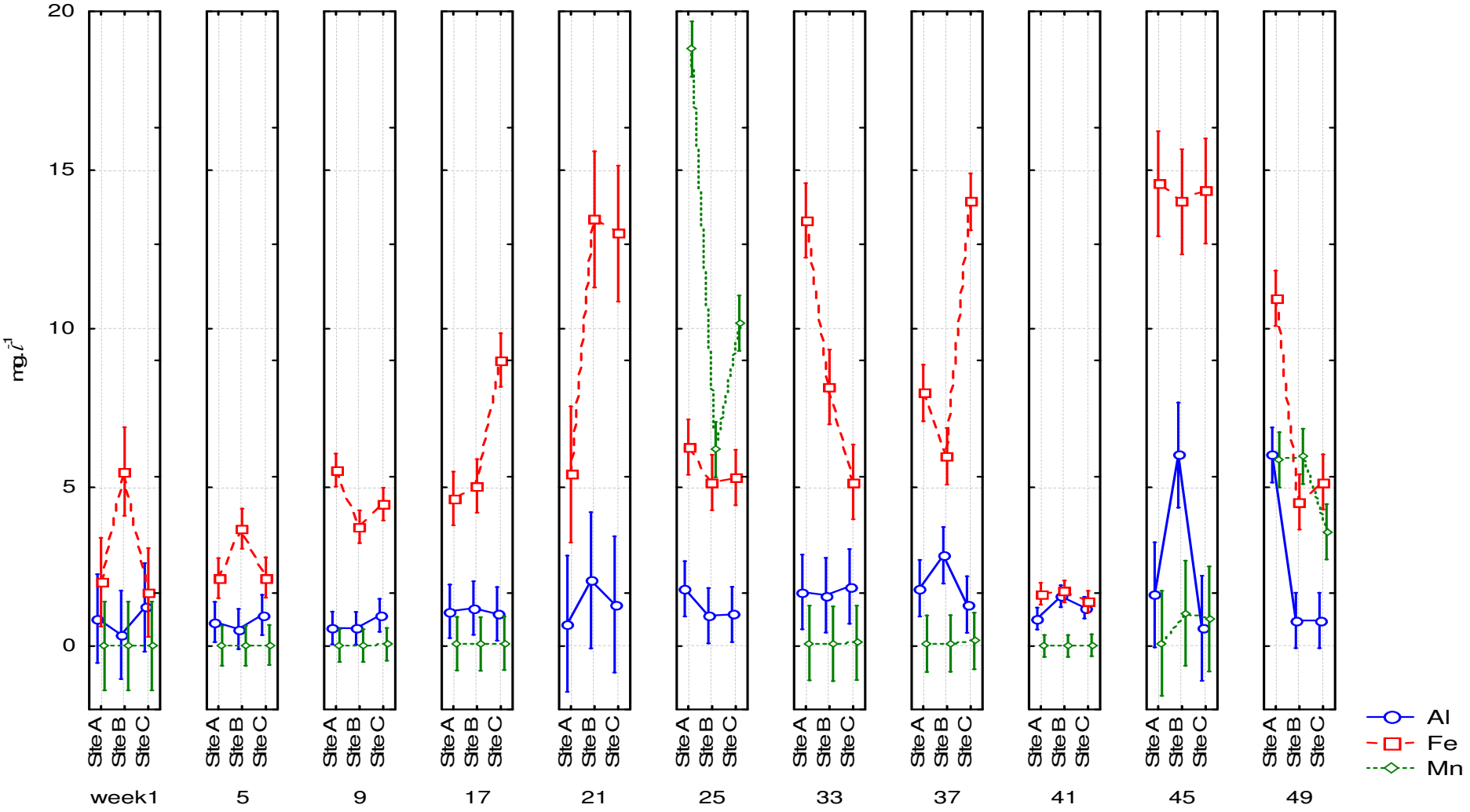

Figure 2.2A

Mean metal concentrations of Al, Fe and Mn in water samples from the Berg River

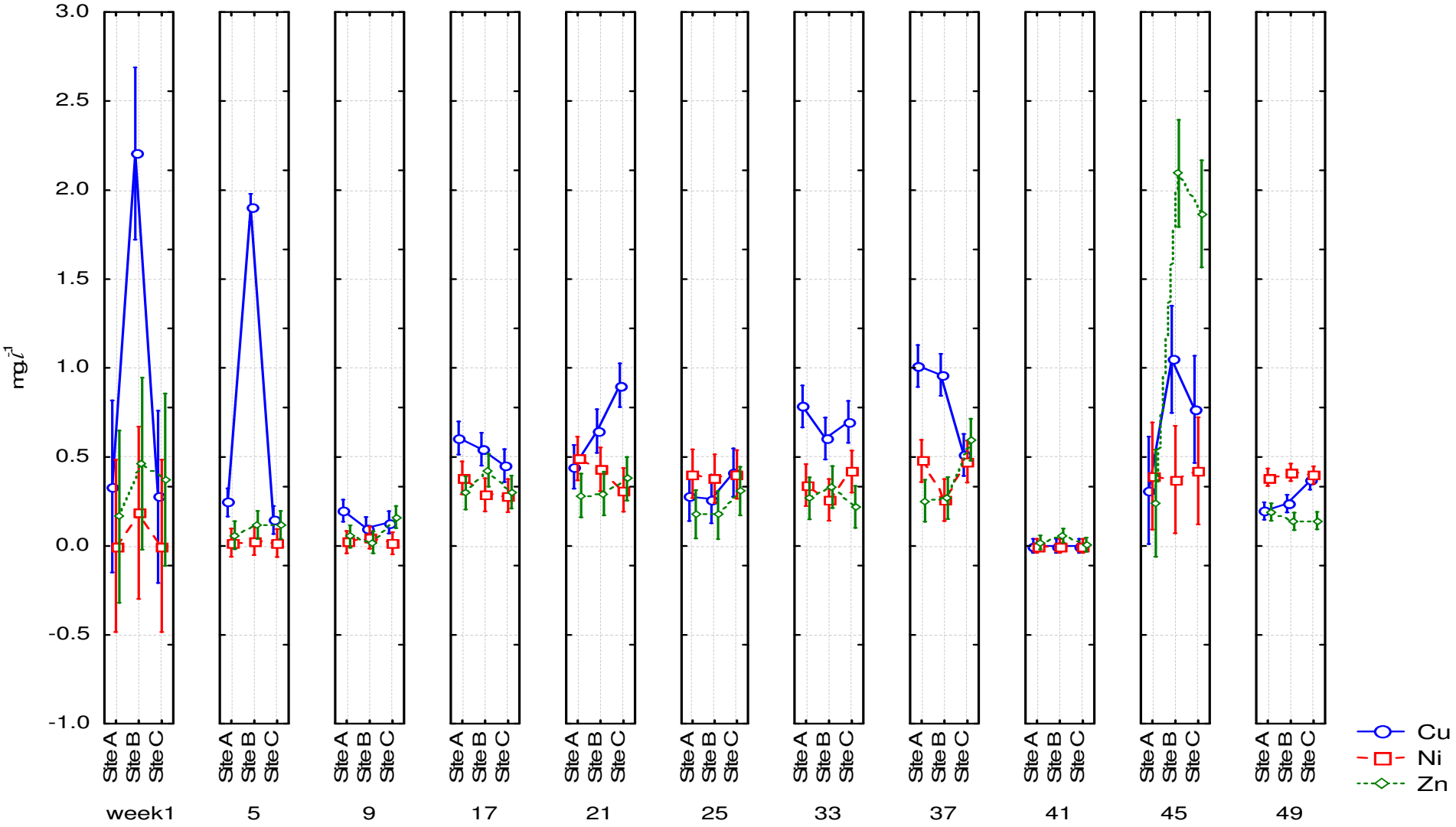

Figure 2.2B

Mean metal concentrations of Cu, Ni and Zn in water samples from the Berg River

Table 2.1. Concentrations obtained in water of the Berg River compared to recommended safe concentrations as stipulated by the Department of Water Affairs and Forestry (1996) and the Canadian Council of Ministers of the Environment Quality Guidelines (2001)

Metal	Recommended safe concentrations as stipulated by DWAF (1996) (mg.ℓ^{-1})	Environmental quality guidelines as stipulated by CCME (2001) (mg.ℓ^{-1})	Mean meal concentrations obtained in water (mg.ℓ^{-1})
Al	0.1 – 0.15	0.005 – 0.1	0.4 – 6
Cu	0.002 – 0.012	0.002 – 0.004	0– 2.2
Fe	N/A	0.3	1.4 – 14.6
Mn	1.3	N/A	0 – 18.8
Ni	N/A	0.025 – 0.15	0 – 0.5
Pb	N/A	0.001 – 0.007	0 – 0
Zn	0.036	0.03	0.01 – 2.1

N/A = Data not available

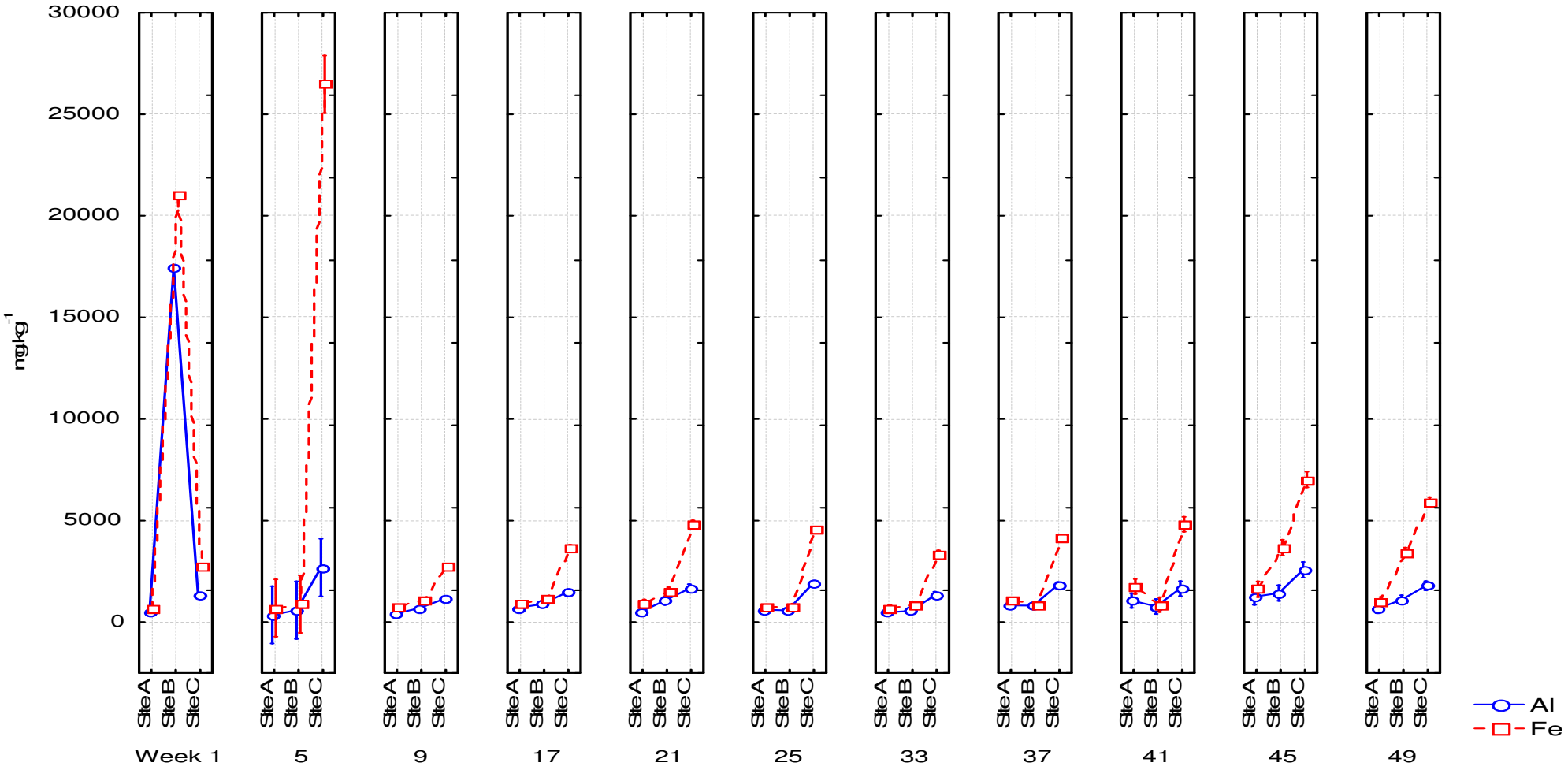

Figure 2.3

Mean Aluminium and Iron concentrations in sediment samples from the Berg River

Table 2.2. Mean metal concentrations of Cu, Mn and Ni recorded in sediment samples for the different sampling weeks

Metals (mg.kg^{-1})									
Time (weeks)	Cu			Mn			Ni		
Sites	A	B	C	A	B	C	A	B	C
1	5.0±4.5	74.0±0	8.2±2.1	3.0±0.5	11.0±0	11.0±0.9	5.0±0.2	44.0±0	5.0±0.1
5	4.0±1.6	3.8±1.8	12.0±2.9	5.0±0.6	7.5±0.8	70.0±5.8	5.0±0.3	5.0±0.4	5.0±0.1
9	2.0±0.9	3.2±1.8	4.4±2.2	3.0±0.5	8.4±1.1	12.0±3.4	4.0±0.4	5.0±0.5	8.0±0.3
17	2.0±0.1	5.9±2.5	9.9±2.1	7.0±0.6	9.4±0.6	12.0±0.7	5.0±0.1	5.0±0.2	5.0±0.4
21	3.0±0.6	3.3±0.4	2.9±0.4	5.0±1.3	8.6±0.5	13.0±2.5	0.0±0.0	0.0±0.0	6.0±0.5
25	1.0±1.7	12.0±1.9	11.0±3	8.0±0.4	3.9±0.3	13.0±0.9	0.0±0.0	0.4±0.0	0.0±0.0
33	5.0±1.3	4.9±0.9	6.7±2.2	3.0±0.2	4.1±0.4	5.9±0.4	2.0±0.3	2.0±0.4	1.0±0.3
37	7.0±0.9	6.3±0.8	7.4±1.2	7.0±0.9	5.1±0.5	15.0±2.3	2.0±0.4	2.0±0.3	3.0±0.2
41	5.0±0.8	4.9±1.3	4.5±0.2	9.0±0.7	4.4±0.4	19.0±1.4	0.0±0.0	0.0±0.0	3.0±0.3
45	7.0±0.9	6.7±1.6	6.9±0.4	11.0±0.6	16.0±3.8	43.0±7.2	4.0±0.6	5.0±0.5	1.0±0.7
49	4.0±0.7	2.9±0.7	7.8±4.9	7.0±0.2	12.0±1.4	44.0±1.4	4.0±0.4	4.0±0.3	5.0±0.4

Table 2.3. Mean metal concentrations of Pb and Zn recorded in sediment samples for the different sampling weeks

Metals (mg.kg^{-1})						
Time (weeks)	Pb			Zn		
Sites	A	B	C	A	B	C
1	0.0±0.0	2.0±0.3	2.1±0.3	7.6±1.4	395.0±0.0	11.0±0.5
5	2.0±1.3	2.0±0.6	11.0±1.7	6.5±0.7	12.0±2.3	22.0±2.3
9	1.0±0.8	2.0±1.9	3.0±0.4	7.0±1.4	11.0±2.3	11.0±1.4
17	3.0±2.2	2.0±0.2	3.0±0.4	8.5±0.5	9.5±0.5	9.0±1.4
21	1.0±0.3	3.0±0.4	4.2±0.7	4.3±0.8	9.2±0.8	7.3±0.9
25	3.0±0.4	2.0±0.2	4.2±0.3	13.0±1.4	9.2±0.5	11.0±0.9
33	2.0±0.5	2.0±0.3	2.9±0.6	4.0±2.1	3.8±0.4	6.2±0.7
37	3.0±0.6	3.0±0.5	4.9±0.3	8.6±0.5	10.0±0.7	11.0±1.3
41	4.0±0.6	3.0±0.3	4.6±2.6	13.0±1.0	12.0±0.9	14.0±2.3
45	5.0±1.5	23±34	8.9±6.8	19.0±2.5	23.0±7.9	16.0±1.3
49	2.0±0.4	6.0±2.9	5.1±0.6	12.0±1.0	27.0±6.7	36.0±41.0

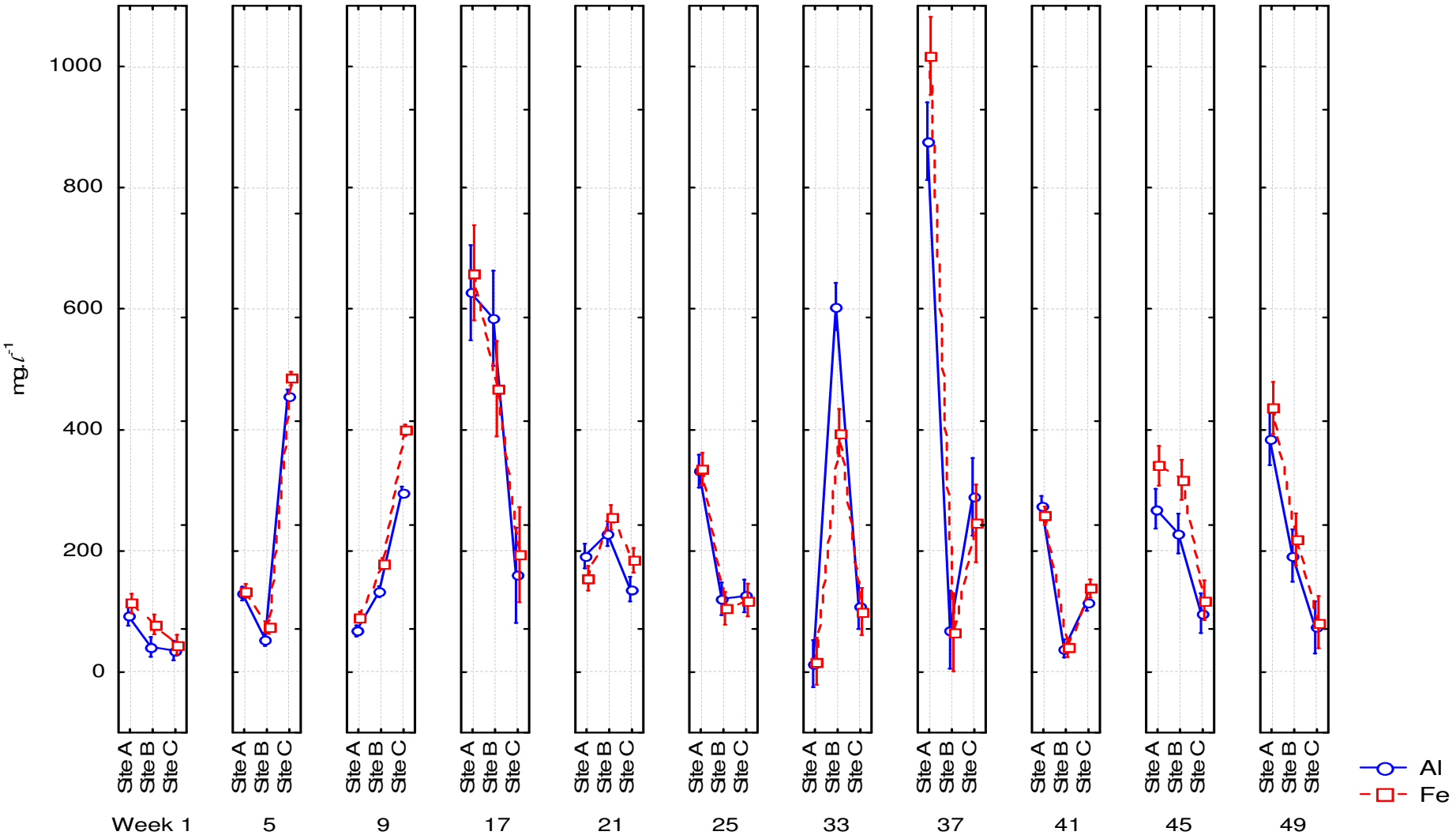

Figure 2.4

Mean Aluminium and Iron concentrations in biofilm samples from the Berg River

Table2.4. Mean metal concentrations of Cu, Mn and Ni recorded in biofilm samples for the different sampling weeks

Metals (**mg.**ℓ^{-1})									
Time (weeks)	Cu			Mn			Ni		
Sites	A	B	C	A	B	C	A	B	C
1	1.0±0.2	1.7±0.2	2.0±0.5	2.6±0.9	1.4±0.1	9.0±0.8	0.4±0.1	0.3±0.0	0.3±0.0
5	1.0±0.1	0.2±0.0	1.0±0.1	2.2±0.2	1.1±0.1	11.0±0.3	0.5±0.0	0.4±0.0	0.7±0.0
9	1.0±0.1	0.7±0.2	1.0±0.2	4.9±0.3	2.0±0.1	32.0±1.4	0.6±0.0	0.6±0.0	0.9±0.0
17	1.5±0.2	0.8±0.1	1.0±0.1	17.0±3.1	8.4±1.3	9.0±1.2	19.0±41.0	0.6±0.0	0.7±0.1
21	0.0±0.0	0.5±0.1	0.0±0.0	1.4±0.4	4.6±0.8	8.0±0.5	0.0±0.0	0.0±0.0	0.0±0.0
25	1.0±0.1	0.7±0.1	1.0±0.2	71.0±17.0	1.7±0.2	6.0±0.4	1.0±0.1	0.4±0.0	0.5±0.0
33	0.3±0.0	0.4±0.1	0.0±0.0	0.7±0.0	1.4±0.2	3.0±0.3	0.0±0.0	3.0±5.8	0.0±0.0
37	2.0±0.2	0.4±0.0	1.0±0.1	13.0±1.5	2.2±0.5	9.0±1.2	0.5±0.0	0.0±0.0	0.1±0.0
41	1.0±0.0	0.4±0.1	0.0±0.1	3.4±0.4	1.6±0.2	4.0±0.2	0.0±0.0	0.0±0.0	0.0±0.0
45	1.0±0.1	0.6±0.1	0.0±0.1	1.2±0.1	1.8±0.4	5.0±0.8	0.7±0.0	1.0±0.1	0.4±0.0
49	1.0±0.1	0.7±0.0	0.0±0.1	0.8±0.0	1.3±0.2	4.8±0.1	0.6±0.1	0.4±0.0	0.3±0.0

Table 2.5. Mean metal concentrations of Pb and Zn recorded in biofilm samples for the different sampling weeks

Metals (mg.ℓ^{-1})						
Time (weeks)	Pb			Zn		
Sites	A	B	C	A	B	C
1	0.4±0.1	0.0±0.0	0.2±0.0	2.0±0.5	2.0±0.2	1.0±0.1
5	0.4±0.0	0.2±0.0	1.61±0.1	2.0±0.2	2.0±0.1	5.0±0.2
9	1.3±0.2	0.6±0.0	1.2±0.1	2.0±0.1	3.0±0.2	6.0±0.3
17	0.3±0.0	0.9±0.1	0.5±0.1	5.6±1.0	3.0±0.5	2.0±0.3
21	1.3±0.2	0.7±0.1	0.4±0.1	0.0±0.0	2.0±0.4	1.0±0.2
25	0.0±0.0	0.2±0.0	0.3±0.1	8.4±1.0	1.0±0.1	2.0±0.2
33	0.2±0.1	0.3±0.0	0.2±0.0	0.5±0.0	1.0±0.2	1.0±0.1
37	0.7±0.1	0.2±0.0	0.7±0.1	8.0±0.1	1.0±0.1	2.0±0.3
41	0.7±0.1	0.0±0.0	0.2±0.0	3.0±0.7	1.0±0.2	1.0±0.3
45	0.9±0.1	0.6±0.1	0.1±0.1	5.0±0.6	3.0±0.5	1.0±0.1
49	1.4±0.4	0.5±0.0	0.0±0.0	6.0±1.4	3.0±0.2	1.0±0.1

Investigation into the metal contamination of the Plankenburg and Diep Rivers, Western Cape, South Africa

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Abstract

Metal contamination in the Plankenburg and Diep Rivers (Western Cape) was investigated over a 12 and 9 month period, respectively. Aluminium (Al), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), nickel (Ni) and zinc (Zn) concentrations were determined using the nitric acid digestion method and analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES). For both rivers the Al and Fe concentrations were higher than all the other metals analysed for in sediment and water samples. The highest concentrations recorded in the Plankenburg River was 13.6 $mg \cdot \ell^{-1}$ (water - Week 18, Site B) and 15 018 $mg \cdot kg^{-1}$ (sediment - Week 1, Site C) for Al and 48 $mg \cdot \ell^{-1}$ (water - Week 43, Site A) and 14 363.8 $mg \cdot kg^{-1}$ (sediment - Week 1, Site A) for Fe. The highest concentrations recorded in the Diep River was 4 $mg \cdot \ell^{-1}$ (water - Week 1, Site A) and 19 179 $mg \cdot kg^{-1}$ (sediment - Week 1, Site C) for Al and 513 $mg \cdot \ell^{-1}$ (water - Week 27, Site A) and 106 379.5 $mg \cdot kg^{-1}$ (sediment - Week 9, Site C) for Fe. For most of the metals analysed the concentrations were higher than the recommended water quality guidelines as stipulated by the Department of Water Affairs and Forestry (DWAF, 1996b), the Canadian Council for the Ministers of the Environment (CCME, 2001) and the 'World average' (Martin and Windom, 1991). Point sources of pollution could not conclusively be identified, but the industrial and residential areas could have influenced the increased concentrations. Metal concentrations should be routinely monitored and the guidelines should be updated and revised based on the current state of the rivers and pollution influences.

Keywords: ICP-AES, metal contamination, river water; sediment, water and sediment quality guidelines

Introduction

South Africa's major water sources are primarily used for agricultural activities (52%), industry, mining and power generation (12.5%) and domestic and municipal uses (12%), with a further 15% needed to maintain estuaries and rivers (Schutte and Pretorius, 1997; Holtzhausen, 2002). The quality of the water resources is, however, steadily declining due to an increase in urbanisation and industrialisation, with the major sources of pollution including industrial and agricultural effluents and domestic and commercial sewage [Department of Water Affairs and Forestry – chapter 2 (DWAF, 2004)]. In addition, population increases in South Africa are expected to lead to an increase in agricultural development, which will in turn lead to an increased demand for irrigation water (Natural Resources Management and Environment Department, 2007).

Point- and non-point source pollution contributes to a decline in the quality of the water when leaching occurs into the surrounding environment (Hills et al., 1998; Ho et al., 2003). Agricultural contamination was also shown by the Agency for Toxic Substances and Disease Registry (2000) to be due to the discharging of pesticides into rivers. In addition, pollutants such as micro-organisms, metals, oils and other toxic substances contribute to the decrease in water quality (Pegram et al., 1999).

Metals are present in the environment at trace amounts and certain metals, like iron (Fe), copper (Cu) and zinc (Zn) are essential for a variety of functions in organisms. It is important though, to ensure that these metals do not exceed normal concentrations, as they may have detrimental long-term effects on human health (Wright and Welbourne, 2002). Excessive consumption of cadmium (Cd) and lead (Pb) could result in neurological, bone and cardiovascular diseases, renal dysfunction, and various cancers, even at relatively low levels (Calderon, 2000; Jarup, 2002). The short-term

effects of high Cd concentrations also include diarrhoea, nausea, vomiting, renal failure, muscle cramps, salivation, sensory disturbances, convulsions, shock and liver injury (Hazards Centre and People's Science Institute, 2005). Short-term exposure to Cu fumes causes irritation of the eyes, nose and throat, and a flu-like illness called metal fume fever. Symptoms of metal fume fever include, fever, muscle aches, nausea, chills, dry throat and cough (US Department of Health and Human Services, 1978). Iron is an essential element, but ingestion of Fe concentrations above the permissible concentrations, may cause many gastrointestinal disturbances, including vomiting, diarrhoea and abdominal pain. The prolonged intake of high dose of Fe can result in liver damage and kidney failure (Hazards Centre and People's Science Institute, 2005).

High concentrations of metals usually deposit on and integrate in river sediment, which are organic or inorganic materials removed by erosion and transported by fluid flow to different locations (Prange and Dennison, 2000; Marchand et al., 2006). The highest metal content available for transport between sites is stored in the sediment-water interfaces (Maanan et al., 2004). Increased levels of heavy metals were reported in Mooi River sediment, South Africa (Wade et al., 2000). The release of mine water from a nearby goldmine into a tributary of the Mooi River presumably resulted in the increased levels of toxic metals in both the water and sediment. The results obtained did not change appreciably when compared to a previous study conducted by Witmann and Förstner (1977), where concentrations for water and sediment was recorded for Cu (5.4 $mg \cdot \ell^{-1}$ and 484 $mg \cdot kg^{-1}$) and Zn (26.0 $mg \cdot \ell^{-1}$ and 6440 $mg \cdot kg^{-1}$). The authors concluded that the high concentrations could be due to the fact that the slime dams receive discharges of high acidity from the mines.

A study investigating the aluminium (Al), Fe, Mn, Zn, chromium (Cr), Cu, Pb, Cd and mercury (Hg) bioaccumulation patterns in sediment, water and fish samples

collected on a quarterly basis from the Mhlathuze Estuary, South Africa, was conducted by Mzimela et al. (2003). The highest concentrations for Al (26 200 $mg \cdot \ell^{-1}$ and 13 928.6 $mg \cdot kg^{-1}$), Fe (23 500 $mg \cdot \ell^{-1}$ and 16 035.71 $mg \cdot kg^{-1}$) and Mn (266 $mg \cdot \ell^{-1}$ and 182.8 $mg \cdot kg^{-1}$) were recorded in water and sediment, respectively, in December, which coincided with an extremely high freshwater inflow from the Mhlathuze River. Metal concentrations were generally lower during April, which coincided with the reduced riverine runoff from the catchment of the estuary. In the fish tissue, Fe and Al were recorded in concentrations ranging from 450 and 3000 $\mu g \cdot g^{-1}$.

Jackson et al. (2007) investigated the degree of metal pollution (Al, Cu, Fe, Mn, Ni, Pb and Zn) at 4 different sampling points along the Berg River, as a recent decline in water quality has been reported. Inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis of water, sediment and biofilm samples revealed that the highest mean metal concentrations recorded were for Al (6 $mg \cdot \ell^{-1}$, 17 448.8 $mg \cdot kg^{-1}$ and 876.8 $mg \cdot \ell^{-1}$) and for Fe (14.6 $mg \cdot \ell^{-1}$, 26 473.3 $mg \cdot kg^{-1}$ and 1 017.5 $mg \cdot \ell^{-1}$). The increased concentrations of Al and Fe in comparison to the other metals analysed for, could be due to the leaching of metals into the river water from waste and household products associated with the informal settlement and the subsequent settling on sediment.

Cadmium, Hg and Zn concentrations in the Umtata, Buffalo, Keiskamma, and Tyume Rivers and in the Sandile and Umtata Dam were determined by Fatoki and Awofolu (2003). These catchments support rapidly growing populations and concerns arose regarding the quality of the surface waters. Cadmium levels in the Umtata River and the Umtata Dam were normal, but in the Keiskamma, Buffalo and Tyume rivers elevated Cd concentrations of 0.007 $mg \cdot \ell^{-1}$ to 0.009 $mg \cdot \ell^{-1}$, 0.008 $mg \cdot \ell^{-1}$ to 0.01 $mg \cdot \ell^{-1}$ and 0.008 $mg \cdot \ell^{-1}$ to 0.017 $mg \cdot \ell^{-1}$, were recorded in the respective rivers. These levels

exceeded the South African guideline of 0.005 $mg \cdot \ell^{-1}$ for Cd (DWAF, 1996a). According to the authors, use of river water with elevated Cd levels may have affected the health of the rural communities who use the river water prior to treatment, as Cd is extremely toxic and could cause adverse health effects (Friberg et al., 1986). Cadmium has been found to be toxic to fish and other aquatic organisms (Fianko et al., 2007). In humans, Cd can result in bone demineralisation, either through direct bone damage or indirectly as a result of renal dysfunction (Bernard, 2008). The recorded levels of Hg and Zn were normal in these rivers and did not exceed the recommended concentrations of metals in water as stipulated by the Department of Water Affairs and Forestry (DWAF, 1996b).

The Plankenburg River is 10 km long, and originates from a mountainous area in the Boland region, South Africa. It flows downstream through Stellenbosch and services various industrial and agricultural activities. The river also flows through the informal settlement of Kayamandi, where the population size in the settlement is roughly estimated to be about 22 000. Twenty percent of Kayamandi's inhabitants live in brick houses with in-house water connections and flush toilets. The remaining 80% of the inhabitants live in shacks that are densely populated and utilise portable toilets (DWAF, 2001). Service delivery to the settlement is inadequate and skips provided by the local municipality for refuse are not frequently emptied or removed. Farmers up- and downstream from this settlement utilise the river water for the irrigation of vineyards, as well as other crops. In addition, the river flows through Stellenbosch's industrial area, which includes amongst others a clothing factory, a well-known cheese factory, spray painting and mechanical workshops and yoghurt and dairy producing plants.

The Diep River is 65 km long, originates from the Riebeek-Kasteel Mountains, flows south-westerly, through Malmesbury and drains into Table Bay. Land in the upper catchment area is dominated by agricultural activities, while in the lower part of the

catchment land use is largely reserved for urban development, which includes formal and informal settlements, as well as industrial establishments, such as spray painting, chemical and clothing manufacturers, a wastewater treatment works and an oil refinery. The Diep River-Rietvlei system has silted up significantly over the past few years which has resulted from extensive erosion (Grindley and Dudley, 1988). It can therefore be regarded as a storage area for sediment-rich water during floods. The sedimentation rate is enhanced by vegetation in the vlei, especially where treated sewage water is being released.

The aim of this study was to investigate the spatial and temporal variation in the metal contamination in the Plankenburg and Diep Rivers in the Western Cape, South Africa. Metal concentrations in water and sediment samples were analysed using the nitric acid digestion method followed by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Saleh et al., 2000). The two rivers selected borders industrial areas, residential areas, agricultural areas and informal settlements. Increased metal pollutants also have a detrimental effect on human health (Wright & Welbourne, 2002), where exposure is mainly due to the ingestion of food and water contaminated with metals leaching into groundwater (Piver, 1992).

Materials and Methods

Sampling Sites

Four sampling sites were identified for the Plankenburg River (Stellenbosch) location: Site A (agricultural farming and residential areas); Site B (informal settlement of Kayamandi); Site C (substation in industrial area) and Site D (industrial area at Adam

Tas Bridge) (Fig. 3.1). Sampling of sites along the Plankenburg River started in May 2004 and continued for a period of one year until May 2005. Sites for the Diep River (Milnerton) location: Site A (Zoarvlei nature reserve - industrial as well as residential areas); Site B (Theo Marais sportsclub - industrial and residential area); Site C (Potsdam wastewater treatment works) and Site D (Rietvlei boating club and nature reserve) (Fig. 3.2). Sampling of these sites started in March 2005 and continued for a period of nine months until November 2005. Sampling at the Diep River initially started in May 2004, but due to adverse weather conditions, one of the original sampling points dried up and another site had to be selected to replace it. Site C was selected as effluent from a nearby oil refinery flows directly into a stormwater drain at the wastewater works. Results for the period before March 2005, was thus not reported on.

Sampling for metal concentration determination

Water samples were collected in sterile 250 mℓ narrow mouth square polypropylene bottles (Cole-Palmer Instrument Company). The sediment samples consisted of a combination of five different sub-samples collected from various points (±15 cm deep) in 250 mℓ plastic containers. The samples were stored at 4°C during transport. The temperature and pH was determined at each site using a YSI pH 100 portable pH millivolts and temperature instrument (YSI Environmental).

Metal concentration determination in water and sediment samples

The collected sediment samples were dried in an oven for three days and weighed using a fine analytical balance (RADWAG®). In order to determine the concentrations of Al, Zn, Cu, Fe, Pb, Ni and Mn (total metals), water (5 mℓ) and sediment samples (0.500 – 0.600 g; dry mass) were digested with 10 mℓ 55% Nitric acid at 40°C for 60 minutes and then at 120°C for 180 minutes, using a Grant dry-block heater. A blank (control) of 10 mℓ 55% nitric acid was analysed along with the collected samples to check for possible contamination. The samples were cooled to room temperature, filtered with Whatman No. 6 filter paper into 20 mℓ volumetric flasks, made up to a volume of 20 mℓ with distilled water and subsequently filtered for a second time using 0.45 μm cellulose nitrate ultrafiltration membrane filters (Whatman) (Odendaal and Reinecke, 1999). Metal concentrations were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis according to the procedure outlined in Saleh et al. (2000). Analytical efficiency was checked using standard reference material (100 g contaminated soil std). Metal concentrations are expressed in $mg \cdot \ell^{-1}$ (water) and $mg \cdot kg^{-1}$ (sediment). Recovery rates measured were between 91 and 102% for the metals analysed in accordance with confidence limits of published values for the reference materials.

Statistical analysis

Repeated measures ANOVA (RMA) was performed on all data obtained as outlined in Dunn and Clark (1987), using Statistica™. In each RMA, the residuals were analysed to determine if they were normally distributed. In all hypothesis tests, a significant level of

5% was used as standards. The results presented are the averages of five repeats for each particular sampling point at the different sampling sites for both water and sediment samples. For statistical analysis, the volumes (5 mℓ) and mass (0.500 – 0.600 g; dry mass) were taken into consideration for the calculation of the final metal concentrations in a given sample.

Results

Metal concentrations in water samples

Plankenburg River

Comparisons of the mean metal concentrations in water samples collected at Sites A, B, C and D along the Plankenburg River over time are presented in Tables 3.1, 3.2 and 3.5. The recorded Al and Fe concentrations were higher than all the other metals analysed for. The concentrations of Al ranged from 0.3 $mg \cdot \ell^{-1}$ (Week 1, Site A) to 13.6 $mg \cdot \ell^{-1}$ (Week 18, Site B), while for Fe the concentrations ranged from 0.3 $mg \cdot \ell^{-1}$ (Week 39, Site B) to 48 $mg \cdot \ell^{-1}$ (Week 43, Site A). Throughout the entire study period, the recorded concentrations for Al in water were higher than the recommended concentrations for Al of 0.1 $mg \cdot \ell^{-1}$ to 0.15 $mg \cdot \ell^{-1}$ (DWAF, 1996b) and 0.005 $mg \cdot \ell^{-1}$ to 0.1 $mg \cdot \ell^{-1}$ [Canadian Council of Ministers of the Environment (CCME, 2001)] (Table 3.5). The Fe concentrations also exceeded the guidelines of 0.3 $mg \cdot \ell^{-1}$ (CCME, 2001) and the 'World average' of 0.04 $mg \cdot \ell^{-1}$ (Martin and Windom, 1991). No Al guidelines were available for the 'World average' and no Al and Fe guidelines were available for the

Australian and New Zealand Environment and Conservation Council (ANZECC, 2000). No guidelines for Fe in river water samples were available from DWAF.

The mean metal concentrations recorded for Cu, Mn, Ni and Zn in the Plankenburg River are presented in Tables 3.1, 3.2 and 3.5. Lead was not detected in any of the analysed samples for the Plankenburg River. The concentrations of Cu, Ni and Zn in the Plankenburg River water samples varied throughout the study period. The Cu concentrations ranged from 0.3 mg·ℓ^{-1} (Weeks 27 and 52, Site D) to 2.2 mg·ℓ^{-1} (Week 1, Site A) (Tables 3.1, 3.2 and 3.5). The Cu concentrations were higher than the recommended concentrations of 0.002 – 0.012 mg·ℓ^{-1}, 0.002 – 0.004 mg·ℓ^{-1}, 0.0015 mg·ℓ^{-1} and 0.0001 – 0.00015 mg·ℓ^{-1} as stipulated by DWAF (1996b), the CCME (2001), Martin and Windom (1991) and ANZECC (2000), respectively (Table 3.5). Mean Mn concentrations ranged from values below the detection limit to 0.4 mg·ℓ^{-1} (Site C) (Tables 3.1 and 3.2) at different weeks during the sampling period and always fell within the recommended quality guideline of 1.3 mg·ℓ^{-1} as stipulated by DWAF (1996b) (Table 3.5). The recorded concentrations, were however higher than the 'World average' of 0.0015 mg·ℓ^{-1} (Martin and Windom, 1991) (Table 3.5). No environmental quality guideline for Mn was available from the CCME (2001) or ANZECC (2000). The mean metal concentrations for Ni recorded throughout the study period ranged from 0.1 mg·ℓ^{-1} (Sites B, C and D) to 0.5 mg·ℓ^{-1} (Site A) and were generally above the recommended concentrations of 0.025 – 0.15 mg·ℓ^{-1}, as stipulated by the CCME (2001), with the exception of weeks 27, 36, 43 and 52, where concentrations of 0.1 mg·ℓ^{-1} were recorded at sites B, C and D, respectively (Tables 3.1 and 3.2). The concentrations for Ni were significantly higher ($p < 0.05$) than the 'World average' guideline of 0.0005 mg·ℓ^{-1} (Martin and Windom, 1991) and the ANZECC (2000) guideline of 0.0001 – 0.0005 mg·ℓ^{-1} (Table 3.5). No environmental quality guideline for Ni was available from DWAF (1996b). The

mean metal concentrations recorded for Zn ranged from below the detection limit to 1.1 mg·ℓ$^{-1}$ (Tables 3.1, 3.2 and 3.5). Zinc concentrations fell within the recommended quality guideline of 0.03 mg·ℓ$^{-1}$, 0.036 mg·ℓ$^{-1}$, 0.0006 mg·ℓ$^{-1}$ and 0.0009 mg·ℓ$^{-1}$, as stipulated by the CCME (2001), DWAF (1996b), Martin and Windom (1991) and ANZECC (2000), respectively (Table 3.5), only once during the sampling period, when a concentration below the detection limit was recorded (Week 1, at Site B). During the subsequent sampling weeks, the recorded concentrations all exceeded the recommended range (Table 3.1 and 3.2).

Diep River

Comparisons of the mean metal concentrations in water samples collected at Sites A, B, C and D along the Diep River over time are presented in Tables 3.3, 3.4 and 3.5. At many sites the recorded Al, Zn and Fe concentrations were higher than all the other metals analysed for. The concentrations for Al ranged from below the detection limit during Week 9 at Site C to 4 mg·ℓ$^{-1}$ during Week 1 at Site A (Table 3.5). The concentrations of Zn ranged from 0.1 mg·ℓ$^{-1}$ to 4.4 mg·ℓ$^{-1}$ (Weeks 9 and 27, respectively, Site A) (Table 3.3 and 3.5). The concentrations for Fe ranged from 0.1 mg·ℓ$^{-1}$ to 513 mg·ℓ$^{-1}$ (Weeks 9 and 27, respectively, Site A) (Table 3.3 and 3.5). Throughout the entire study period, the overall concentrations for Al were mostly higher than the recommended concentrations of 0.1 mg·ℓ$^{-1}$ to 0.15 mg·ℓ$^{-1}$ (DWAF, 1996b) and 0.005 mg·ℓ$^{-1}$ to 0.1 mg·ℓ$^{-1}$ (CCME, 2001) (Table 3.5). The overall concentrations recorded throughout the study period for Zn were mostly higher than the recommended concentrations of 0.03 mg·ℓ$^{-1}$ (CCME, 2001), 0.036 mg·ℓ$^{-1}$ (DWAF, 1996b), 0.0006 mg·ℓ$^{-1}$ (Martin and Windom, 1991) and 0.0009 mg·ℓ$^{-1}$ (ANZECC, 2000) (Table 3.5). The

recommended concentrations of 0.3 mg·ℓ$^{-1}$ (CCME, 2001) and the 'World average' of 0.04 mg·ℓ$^{-1}$ (Martin and Windom, 1991) for Fe, were mostly higher than the overall concentrations recorded during the entire study period (Table 3.5). No Al guidelines were available for the 'World average' and no Al and Fe guidelines were available for the Australian and New Zealand Environment and Conservation Council (ANZECC, 2000). No guidelines for Fe in river water samples were available from DWAF.

The mean metal concentrations recorded for Cu, Mn and Ni in water samples in the Diep River are represented in Tables 3.3, 3.4 and 3.5. Lead was not detected in any of the samples analysed for in the Diep River. The recorded concentrations for Cu ranged from 0.1 mg·ℓ$^{-1}$ during Weeks 1 and 36, at Sites C and D, respectively, to 0.8 mg·ℓ$^{-1}$ during Week 32 at Site D (Table 3.4). These concentrations for Cu were higher than the recommended concentrations of 0.002 – 0.012 mg·ℓ$^{-1}$, 0.002 – 0.004 mg·ℓ$^{-1}$, 0.0015 mg·ℓ$^{-1}$ and 0.0001 – 0.00015 mg·ℓ$^{-1}$ as stipulated by DWAF (1996b), the CCME (2001), Martin and Windom (1991) and ANZECC (2000), respectively (Table 3.5). Similar to the result obtained for Mn in the Plankenburg River, Mn concentrations recorded in the Diep River, always fell within the recommended concentrations of 1.3 mg·ℓ$^{-1}$ (DWAF, 1996b) (Table 3.5), with the highest mean Mn concentration of 1.3 mg·ℓ$^{-1}$ recorded during Week 27 at Site A (Table 3.3). The Mn concentrations however mostly exceeded the 'World average' of 0.0015 mg·ℓ$^{-1}$ (Martin and Windom, 1991) (Table 3.5). The mean metal concentrations recorded for Ni fluctuated throughout the entire study period, ranging from values below the detection limit to 0.4 mg·ℓ$^{-1}$ during Weeks 23 and 32 at Sites D and C, respectively. The recorded concentrations exceeded the recommended concentration of 0.025 – 0.15 mg·ℓ$^{-1}$ (CCME, 2001), 0.0005 mg·ℓ$^{-1}$ (Martin and Windom, 1991) and the Australian and New Zealand

guidelines of 0.0001- 0.00015 mg·ℓ$^{-1}$ (ANZECC, 2000) during Weeks 5, 14, 18, 23, 27 and 32 at various sampling sites.

Metal concentrations in sediment samples

Plankenburg River

Comparisons of the mean metal concentrations for Al, Fe, Mn, Zn, Cu, Ni and Pb are presented in Figs. 3.3, 3.4 and 3.5, for sediment samples collected from various sites along the Plankenburg River. No recommended sediment quality guidelines for Al, Fe, Mn, Pb, Ni, Cu and Zn were available from DWAF (1996b) and the 'World average' (Martin and Windom, 1991), and only guidelines for Cu and Zn were available from the CCME (2001). Guidelines for Cu, Pb, Ni and Zn were available from ANZECC (2000). The highest Al concentration of 15 018 mg·kg^{-1} was recorded during Week 1 at Site C (Fig. 3.3). The lowest mean Al concentration of 1 609 mg·kg^{-1} was recorded during Week 52 at Site D (Fig. 3.3). The mean concentrations recorded for Fe during the entire study period ranged from 3 763 mg·kg^{-1} recorded at site D, during Week 52 to 19 179 mg·kg^{-1} recorded during Week 1 at Site C (Fig. 3.3).

The highest Mn concentration of 225 mg·kg^{-1} was recorded during Week 5 at Site A, while the lowest Mn concentration of 15.93 mg·kg^{-1} was recorded during Week 22 at Site B in the Plankenburg River (Fig. 3.4). The highest Zn concentration recorded in the Plankenburg River was 269.5 mg·kg^{-1} during Week 1 at Site B (Fig. 3.4). The highest recorded concentration was significantly higher ($p < 0.05$) than the recommended environmental quality guideline of 123 mg·kg^{-1} in freshwater sediment as stipulated by

the CCME (2001) and 200 mg·kg^{-1} as stipulated by ANZECC (2000). The mean metal concentrations recorded for Zn fluctuated throughout the study period, exceeding the recommended concentrations intermittently, with the exception of Site A, where all the concentrations fell below the recommended concentrations of the CCME (2001) and ANZECC (2000).

The highest Pb concentration in the Plankenburg River was recorded at 275 mg·kg^{-1} during Week 9 at Site C, with the lowest concentration of 7.38 mg·kg^{-1} recorded during Week 52 at Site C (Fig. 3.5). The highest Pb concentration exceeded the Australian and New Zealand quality guideline of 50 mg·kg^{-1} (ANZECC, 2000). The highest (11.7 mg·kg^{-1}) and lowest (0.62 mg·kg^{-1}) Ni concentrations were recorded during Weeks 1 and 52 at Sites C and D, respectively (Fig. 3.5). The highest Ni concentration was lower than the quality guideline as stipulated by ANZECC (2000).

The highest Cu concentration of 251.8 mg·kg^{-1} was recorded during Week 9 at Site C (Fig. 3.5). This concentration was significantly higher ($p < 0.05$) than the recommended environmental quality guideline of 35.7 mg·kg^{-1} in freshwater sediment as stipulated by the CCME (2001) and the Australian and New Zealand quality guideline of 65 mg·kg^{-1} (ANZECC, 2000). The Cu concentrations fluctuated throughout the entire study period. At Sites A, B and D of the Plankenburg River, concentrations were generally lower than the recommended guidelines, while Cu concentrations recorded at Site C were in most cases higher than the recommended guidelines (Fig. 3.5).

Diep River

Comparisons of the mean metal concentrations for Al and Fe, Mn and Zn and Cu, Ni and Pb are presented in Figs. 3.6, 3.7 and 3.8, respectively, for sediment samples

collected from various sites along the Diep River. No quality guidelines for Al, Cu, Fe, Mn, Ni, Pb and Zn were available from DWAF and the 'World average' (Martin and Windom, 1991) and guidelines for only Cu and Zn were available from the CCME (2001). Guidelines for Cu, Ni, Pb and Zn were available from ANZECC (2000). The highest mean Al concentration of 14 363.8 $mg \cdot kg^{-1}$ was recorded during Week 1 at Site A in the Diep River (Fig. 3.6). The lowest mean Al concentration was recorded at 175.5 $mg \cdot kg^{-1}$ during Week 1 at Site D (Fig. 3.6). The mean metal concentrations recorded for Fe ranged from 299.3 $mg \cdot kg^{-1}$ during Week 14 at Site B to 106 379.5 $mg \cdot kg^{-1}$ during Week 9 at Site C (Fig. 3.6).

The highest mean concentrations for Mn and Zn, were 1 353.5 $mg \cdot kg^{-1}$ and 1 081.2 $mg \cdot kg^{-1}$ during Week 1 at Sites C and A, respectively (Fig. 3.7). The highest mean Zn concentration of 1 081.2 $mg \cdot kg^{-1}$ was significantly ($p < 0.05$) higher than the recommended Canadian sediment quality guidelines of 123 $mg \cdot kg^{-1}$ (CCME, 2001) and the Australian and New Zealand guideline of 200 $mg \cdot kg^{-1}$ (ANZECC, 2000). The mean metal concentrations recorded for Zn fluctuated above and below the recommended concentration for most of the sampling sites, except for Site D at the Diep River, where all the concentrations fell below the recommended concentration.

The highest mean metal concentration of 643.06 $mg \cdot kg^{-1}$ recorded for Pb during Week 1 at Site A was higher than the recommended concentration of 50 $mg \cdot kg^{-1}$ as stipulated by ANZECC (2000). For Ni, the highest mean concentration of 15.81 $mg \cdot kg^{-1}$, recorded during Week 9, at Site C (Fig. 3.8), was lower than the ANZECC guideline of 21 $mg \cdot kg^{-1}$ (ANZECC, 2000). The highest Cu concentration recorded in the Diep River was 370.5 $mg \cdot kg^{-1}$ during Week 1 at Site B (Fig. 3.8). This concentration was significantly higher ($p < 0.05$) than the recommended Canadian environmental quality guideline of 35.7 $mg \cdot kg^{-1}$ in freshwater sediment as stipulated by the CCME (2001) and

65 mg·kg^{-1}, as stipulated by ANZECC (2000). The Cu concentrations also fluctuated above and below the recommended guideline at Sites A, B and C. Copper concentrations did not exceed the recommended guideline at Site D (Fig. 3.8).

Discussion

Metal concentrations in water samples

For all the sites investigated along the Plankenburg River, the results for metal concentrations fluctuated throughout the study period and no specific point sources of pollution could be identified. The different sites were situated close to agricultural areas and residential areas (Site A), the informal settlement of Kayamandi (Site B), the substation in the industrial area of Stellenbosch (Site C) and the industrial area at the Adam Tas Bridge (Site D). In a previous investigation into the metal contamination of the Berg River, Western Cape, Jackson et al. (2007) showed that Mn, Al and Fe were recorded at higher concentrations at an agricultural area situated along this river system. Correspondingly, the agricultural area (Site A) situated along the Plankenburg River was found to be one of the possible sources of Cu, Zn, Ni and Fe pollution, as elevated concentrations of these metals were recorded at this particular site. Similar to farmers in the Paarl/Wellington area for the Berg River, the farmers in Stellenbosch use the same pesticides, such as Mancozeb^{TM} and Copperflo, among others, to treat their crops. Copper is a major component of Copperflo, while Mancozeb^{TM} is composed of 60% Mn and Zn (Acrobat$^{®}$MZ, 2005). Vermeulen et al. (2001) stated that 1343 x 10^3 kg Mancozeb^{TM} per annum is utilised in agricultural areas in the Western Cape. The Fisheries and Aquaculture Department (Botswana) compiled a review in 2007, on heavy

metals in aquatic systems, where they identified the major sources of pollution and most commonly documented pollutants in aquatic systems. They found that fertilisers and biocides, which include pesticides and herbicides, are a major source of Cd, Hg, Pb, Al, arsenic (As), chromium (Cr), Cu, Mn, Ni, Zn and tin (Sn) pollution.

A stormwater drain flowing directly from the informal settlement of Kayamandi enters the river at Site B. Effluent from household products and waste could leach into the river via this stormwater drain and influence the metal concentrations at this site. Site C is situated close to the industrial area which includes amongst others spraypainters, panel-beaters and yoghurt manufacturers. All the metals analysed were found at elevated concentrations at Site C and effluent or waste from the surrounding areas may enter the river due to the leaching of waste from the surrounding factories into the river system. The increased and continued pollution of the Plankenburg River can adversely affect the primary uses of the river water, such as supplying water for irrigation. The concentrations recorded during the study generally fell within the guidelines for irrigation water for DWAF (1996c) and ANZECC (2000) for all the metals analysed, except for Fe, where the highest concentrations of 48 $mg \cdot \ell^{-1}$ exceeded the recommended guidelines of 20 $mg \cdot \ell^{-1}$ (DWAF, 1996c) and 10 $mg \cdot \ell^{-1}$ (ANZECC, 2000). The increased Fe concentration could result in a deposition of an Fe coating on plants, which can interfere with photosynthesis, leading to plant damage and death. This can in turn negatively impact the export of fruits and wine due to the production of below par products, which could influence South Africa's economy (DWAF, 2001).

As with the results for the Plankenburg River, the point sources of pollution at the Diep River could not be conclusively identified. The different sites were situated close to the Zoarvlei Nature Reserve - industrial and residential areas (Site A), the Theo Marais Sportsclub – industrial and residential areas (Site B), the Potsdam Wastewater treatment

works (Site C) and the Rietvlei Boating club and Nature Reserve (Site D). No direct correlation between rainfall, pH or temperature could be drawn at both rivers, as the concentrations recorded in the water samples fluctuated throughout the study period. From the results it was concluded that Sites A and B contributed to the Al, Fe and Zn pollution in the Diep River, especially during Weeks 1 and 27. According to the Fisheries and Aquaculture Department (2007) of Botswana, industrial waste, such as pigments, paints, alloys, solders and batteries, are the primary sources of Pb, Zn, Mn, Al, Cu and Fe in the environment. In the Diep River, the pollution incidences at the various sites could have been influenced by the leaching of industrial effluent from the surrounding industries into the river. These industries include numerous panel-beaters, Al works, chemical manufacturers (cleaning materials), petrol stations and cold storage facilities. As with the Plankenburg River, pollution could possibly be attributed to improper waste discharge and the leaching of effluent and waste products into the river.

Metal concentrations in sediment samples

Comparison of the overall results obtained at the various sites, showed that the point sources of pollution at the Plankenburg River could not conclusively be identified as concentrations at the various sites fluctuated throughout the entire study period. The increased concentrations for Al and Fe at the different sites, relative to the other metals analysed for, could be ascribed to elevated Al and Fe pollution at the different sampling times. For both Al and Fe, the highest mean metal concentrations were recorded at Site C of the Plankenburg River, which is situated close to the industrial area in Stellenbosch. At Site A (located in the agricultural area), the sources of Al, Mn and Fe contamination of the Plankenburg River, could be due to the leaching of pesticides, fertilisers and

algicides, utilised in the surrounding farming areas, into the river (Jackson et al., 2007). The concentrations recorded for Cu and Zn at the agricultural area fell within the recommended guidelines of the CCME (2001) and ANZECC (2000). The concentrations of Fe and Al were however, recorded in significantly high ($p < 0.05$) concentrations. Iron and Al are also two of the most abundant naturally occurring elements in the environment [Agency for Toxic Substances and Disease Registry (ATSDR), 1995]. Iron is the most abundant element in the Earth's crust (35%) with Al as the third most abundant element, constituting 7.3%. At Site B (the informal settlement), Fe, Zn and Pb could have leached into the river via a nearby stormwater drain, through which waste from the settlement is discarded. The galvanised sheeting used in housing materials in the informal settlement is composed of Fe coated with Zn, to provide resistance to abrasion (Surfacequery, 2007). These results were similar to that obtained from a report compiled by the Nairobi River Basin Programme Phase II Pollution Monitoring Stakeholders in 2003. The water and sediment quality of the Moitoine- and Ngong Rivers was monitored and results showed that Fe and Zn concentrations were high at the site situated in the Kibera informal settlement. The authors attributed this to the use of Fe sheets galvanised with Zn as roofing and building materials in the settlement. The industrial area situated along the banks of the Plankenburg River, houses different industries, which include amongst others spray painters, a cheese factory and panel-beaters, from which effluent could be discharged into the river, accounting for the increased concentrations of metals.

A comparison of the overall results obtained at the various sites along the Diep River could not conclusively identify any definite point source of metal contamination. As with results obtained at the Plankenburg River the elevated metal concentrations at specific sites during the sampling period could be attributed to pollution incidences. No

direct correlation between rainfall, pH or temperature could be drawn at both rivers, as concentrations recorded in the sediment samples fluctuated throughout the study period. Increased concentrations could be due to the leaching of industrial waste into the river at Sites A and B, which are situated close to the industrial area. These industries include amongst others spray painting, paint manufacturers, a pharmaceutical company, chemical manufacturing companies and concrete manufacturers. The highest mean concentrations recorded for Al along the Diep River, were at Sites A and C, which is situated close to the industrial area and the oil refinery, respectively. The highest mean concentrations for Fe recorded along the Diep River were at Site C (Potsdam Wastewater Treatment Works), which is situated close to an oil refinery.

Aluminium products are also used in production equipment and as containers for chemicals and food beverage products in the area. Corrosion of Al is a result of the combination of sulphur dioxide, chlorides, phosphates, nitrates and other industrial emissions with precipitation or dew, resulting in increased Al concentrations in the sediment samples as Al could leach into the surrounding environment. The surrounding industrial activities could all have a significant impact on the water source and the surrounding environment. Waste from a nearby oil refinery could also enter the river via stormwater drains at Site C, the Potsdam Wastewater Treatment Works.

According to the Fisheries and Aquaculture Department (2007) the metals associated with oil refinery discharge are Fe, Ni, Pb, Mn and Zn. Mwamburi (2003) found increased concentrations of Fe, Mn, Zn, Cr and Al in sediment samples of the Kasat River, Kenya. The increases in metal concentrations in comparison with unpolluted sites could be correlated to the direct waste input into the Kasat River from municipal and industrial sources. Singh et al. (2005) studied the concentrations of Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn in water and bed sediments of the Gomti River (India).

Water and sediment samples were collected from 10 different locations and analysed using ICP-AES. The heavy metal concentrations found in the river water were 0.0001 to 0.0005 $mg \cdot \ell^{-1}$ (Cd), 0.0015 to 0.0688 $mg \cdot \ell^{-1}$ (Cr), 0.0013 to 0.0.0043 $mg \cdot \ell^{-1}$ (Cu), 0.0791 to 0.3190 $mg \cdot \ell^{-1}$ (Fe), 0.0038 to 0.0.0973 $mg \cdot \ell^{-1}$ (Mn), 0.0066 to 0.011 $mg \cdot \ell^{-1}$ (Ni), 0.0158 to 0.0276 $mg \cdot \ell^{-1}$ (Pb) and 0.0144 to 0.0298 $mg \cdot \ell^{-1}$ (Zn). In the sediment samples, the concentrations were 0.7 to 7.9 $\mu g \cdot g^{-1}$ (Cd), 6.1 to 0.60 $\mu g \cdot g^{-1}$ (Cr), 3.7 to 35.7 $\mu g \cdot g^{-1}$ (Cu), 5051.5 to 8291.5 $\mu g \cdot g^{-1}$ (Fe), 134.9 to 320.5 $\mu g \cdot g^{-1}$ (Mn), 13.9 to 37.4 $\mu g \cdot g^{-1}$ (Ni), 21.3 to 92.2 $\mu g \cdot g^{-1}$ (Pb) and 15.7 to 99.4 $\mu g \cdot g^{-1}$ (Zn). The authors concluded that the high concentrations were due to a discharge of industrial effluent from various sources, including municipal waste, untreated sewage and agrochemical runoff from nearby cities and villages into the river water. Based on the geoaccumulation indices, the Gomti River sediments from Neemsar to Jaunpur was polluted with Pb (moderately), Cd (moderately polluted to highly polluted) and Ni (sediment was highly polluted).

Davies et al. (2006) evaluated the accumulation of Cr, Cd and Pb in water, sediment and periwinkle (*Tympanotonus fuscatus var radula*; shell and soft tissues) from 4 stations along Elechi Creek (Nigeria), which receive effluent from heavily industrialised and highly populated settlements. Chromium, Cd and Pb concentrations in sediment, water and periwinkles was determined using Buck Scientific Atomic Absorption/ Emission Spectrophotometry. The results showed that the concentrations of Cr were highest in both the sediment and water samples at all the sampling sites, where concentrations of 0.01 $mg \cdot kg^{-1}$ were recorded. The concentrations of these metals were higher in the periwinkles, which is consumed by the surrounding human population. The authors concluded that for future use, the metal concentrations in sediment must be monitored on a regular basis. As with the results recorded in the present study, the

concentrations recorded in the sediment samples exceeded the concentrations recorded in the water samples.

The effect of anthropogenic inputs on the accumulation of metals in sediment at the Hugli River (India), were studied by Sarkar et al. (2004). Of the 8 stations studied, all the elements (Al, Fe, Mn, Zn, Cr, Pb, Ni, Sn, gallium (Ga), vanadium (V), bismuth (Bi), cerium (Ce) and As) analysed for, displayed elevated concentrations at the Gangasagar site (mouth of the river). This was presumably due to the metal-containing effluent or discharge from upstream located oil refineries, fertilisers, pesticides, a sulphuric acid plant, a battery manufacturing plant, tanneries and thermal power plants. In the present study, the exact point sources of pollution could also not conclusively be identified. The industrial and residential areas, waterworks and oil refinery could all have contributed to the metal contamination of the river. The elevated levels of metals could therefore be attributed to anthropogenic sources. In the present study, the recorded concentrations for metals in sediment samples for Cu and Zn at both rivers exceeded the recommended concentrations of the CCME (2001), ANZECC (2000) and Micó et al. (2007), while the Pb concentration exceeded the recommended guidelines of ANZECC (2000) and the baseline values determined by Micó et al. (2007).

Baseline values for heavy metals were proposed by Micó et al. (2007) to identify soil contamination in Alicante, Spain. Cadmium, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn concentrations were determined using atomic absorption spectrometry. The baseline values identified were 0.7 $mg \cdot kg^{-1}$, 11 $mg \cdot kg^{-1}$, 36 $mg \cdot kg^{-1}$, 28 $mg \cdot kg^{-1}$, 19,822 $mg \cdot kg^{-1}$, 402 $mg \cdot kg^{-1}$, 31 $mg \cdot kg^{-1}$, 28 $mg \cdot kg^{-1}$ and 83 $mg \cdot kg^{-1}$ for Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn, respectively. The authors concluded that the baseline concentrations would be beneficial to compare the concentrations of metals for which there are no recommended quality guidelines, such as Al, Fe and Mn. The values could also provide a basis to

identify contaminated sites. The concentration of Fe in the soil recorded by Micó et al. (2007) was comparable to the concentrations of Fe recorded in the Plankenburg River, but lower than the concentration for Fe recorded in the Diep River. The Mn concentrations recorded at the Plankenburg River was lower than the Micó et al. (2007) results, but the Diep River result exceeded to baseline concentration recorded by Micó et al. (2007). The highest Cu, Pb and Zn concentrations recorded at both rivers were significantly higher ($p < 0.05$) than the baseline concentration stipulated by Micó et al. (2007), while for Ni, the recorded concentrations at both rivers were below the baseline concentrations of Micó et al. (2007). Results from the present study show that the metal concentrations in the river systems should routinely be analysed. The national guidelines should be updated or revised to accurately reflect the current state of the rivers and pollution influences.

Conclusions

The major conclusions of the study include the following:

- Aluminium and Fe concentrations were higher than all the other metals analysed for in the water samples collected from the Plankenburg River, which exceeded the guidelines stipulated by DWAF and the CCME (Al and Fe) and the 'World average' (Fe).
- Concentrations of Cu and Zn (with the exception of Week 1, Site B) in the Plankenburg River water samples exceeded the guidelines stipulated by the CCME, DWAF, ANZECC and the 'World average'.
- Concentrations of Mn fell within the DWAF guidelines, as well as the 'World average'. No guidelines for Mn were available from the CCME.

- No Pb could be detected in any of the Plankenburg and Diep River water samples.
- The highest mean metal concentrations in sediment samples were recorded for Al and Fe at Site C (substation in the industrial area) in the Plankenburg River.
- The highest mean metal concentrations in water samples were recorded for Al, Fe and Zn at Site A (industrial area) in the Diep River, which exceeded the guidelines stipulated by DWAF, the CCME, ANZECC and the 'World average', and for Fe and Zn, the baseline values of Micó et al. (2007).
- Concentrations for Cu in water samples from the Diep River exceeded the recommended concentrations for ANZECC, DWAF, the 'World average' and the CCME, while Ni concentrations fluctuated above and below the recommended guidelines at Sites A, B, C and D. Manganese concentrations fell below the recommended guideline during the sampling period, with the exception of Week 27, where the Mn concentration was 1.3 $mg \cdot \ell^{-1}$, at Site A.
- The highest mean Al concentration in sediment samples from the Diep River was recorded at Site A (industrial area) and the highest mean Fe concentration was recorded at Site C (wastewater treatment works). The highest Fe concentration was significantly higher ($p < 0.05$) than the baseline value obtained by Mico et al. (2007).
- Possible sources of contamination of the Plankenburg River could be ascribed to the leaching of household waste into the river water from the informal- and formal residential settlements, as well as the leaching of industrial effluent from the industries situated close to the river.

- In addition, contamination of the Plankenburg River could also have been due to the excessive use of pesticides and insecticides on farms bordering the river system and the discarding of these pesticides into the rivers.
- Possible sources of contamination of the Diep River could have been the leaching of industrial waste from various industries into the sampled sites along the banks of the river, as well as waste from the nearby oil refinery.
- Metal concentration analysis should be routinely performed to ensure an accurate assessment of the current state of the rivers, and based on these results quality guidelines should be adapted accordingly.

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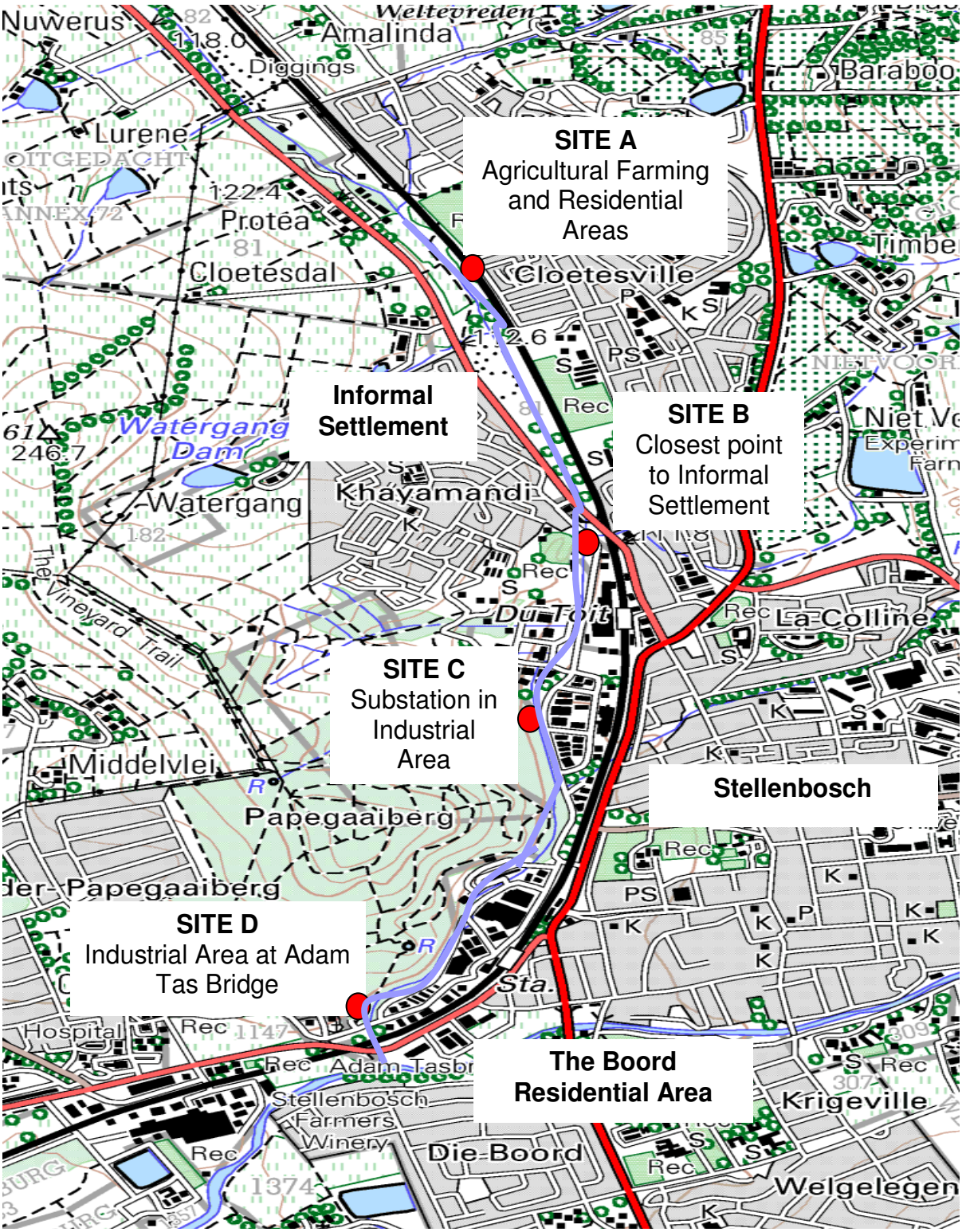

Figure 3.1

Map of the Plankenburg River indicating the different sampling points: Site A - agricultural farming and residential areas; Site B - close to the informal settlement of Kayamandi; Site C – Substation in the industrial area and Site D - industrial area at Adam Tas Bridge.

Figure 3.2

Map of the Diep River indicating the different sampling points: Site A - Zoarvlei Nature Reserve (industrial as well as residential areas); Site B - Theo Marais Sportsclub (Industrial and residential area); Site C – Potsdam Wastewater Treatment Works (close to an Oil Refinery and residential areas of Milnerton) and Site D - the Rietvlei Boating Club and Nature Reserve.

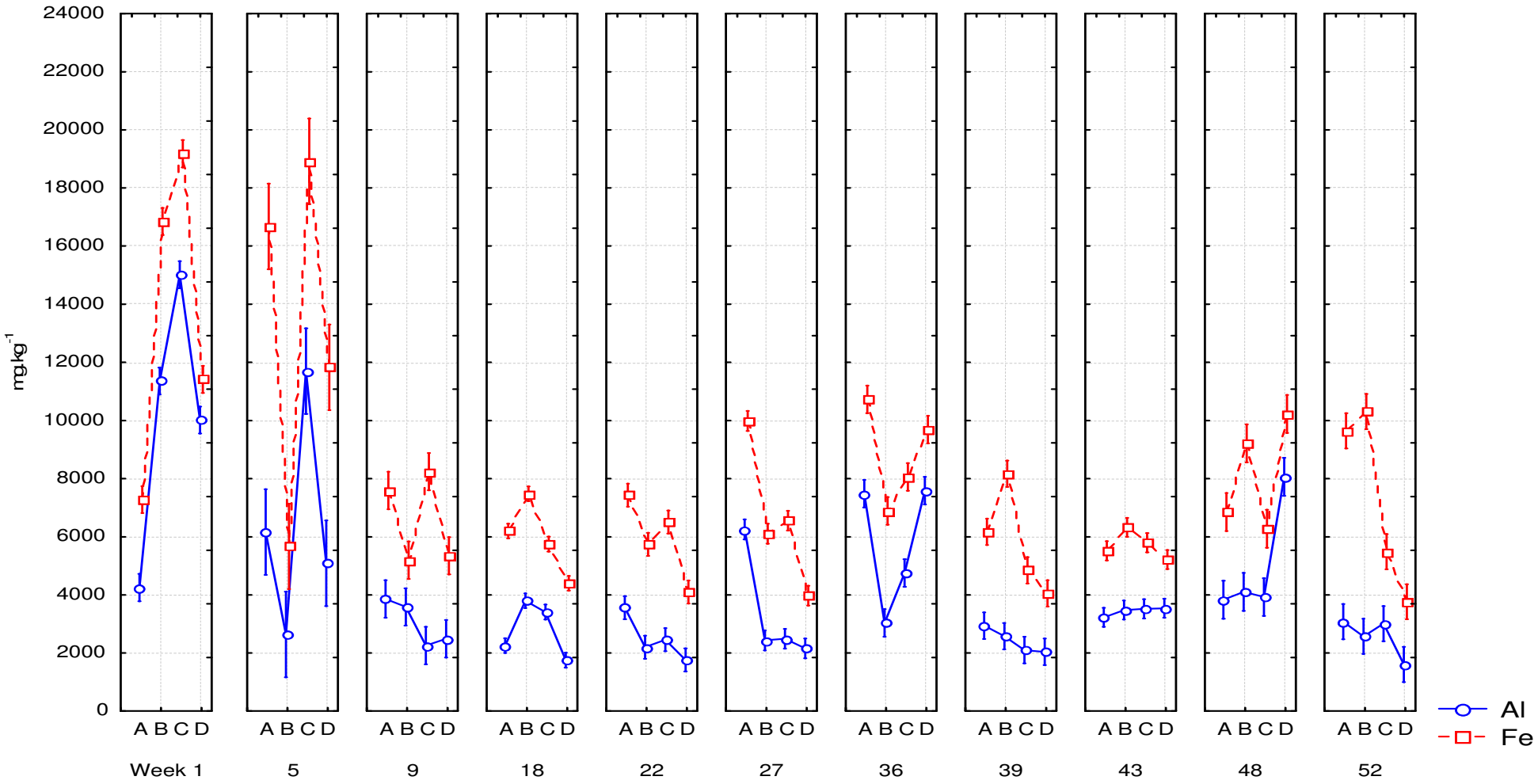

Figure 3.3

Metal concentrations (mg·kg^{-1}) (Al and Fe) in sediment samples obtained from 4 sites (A to D) in the Plankenburg River

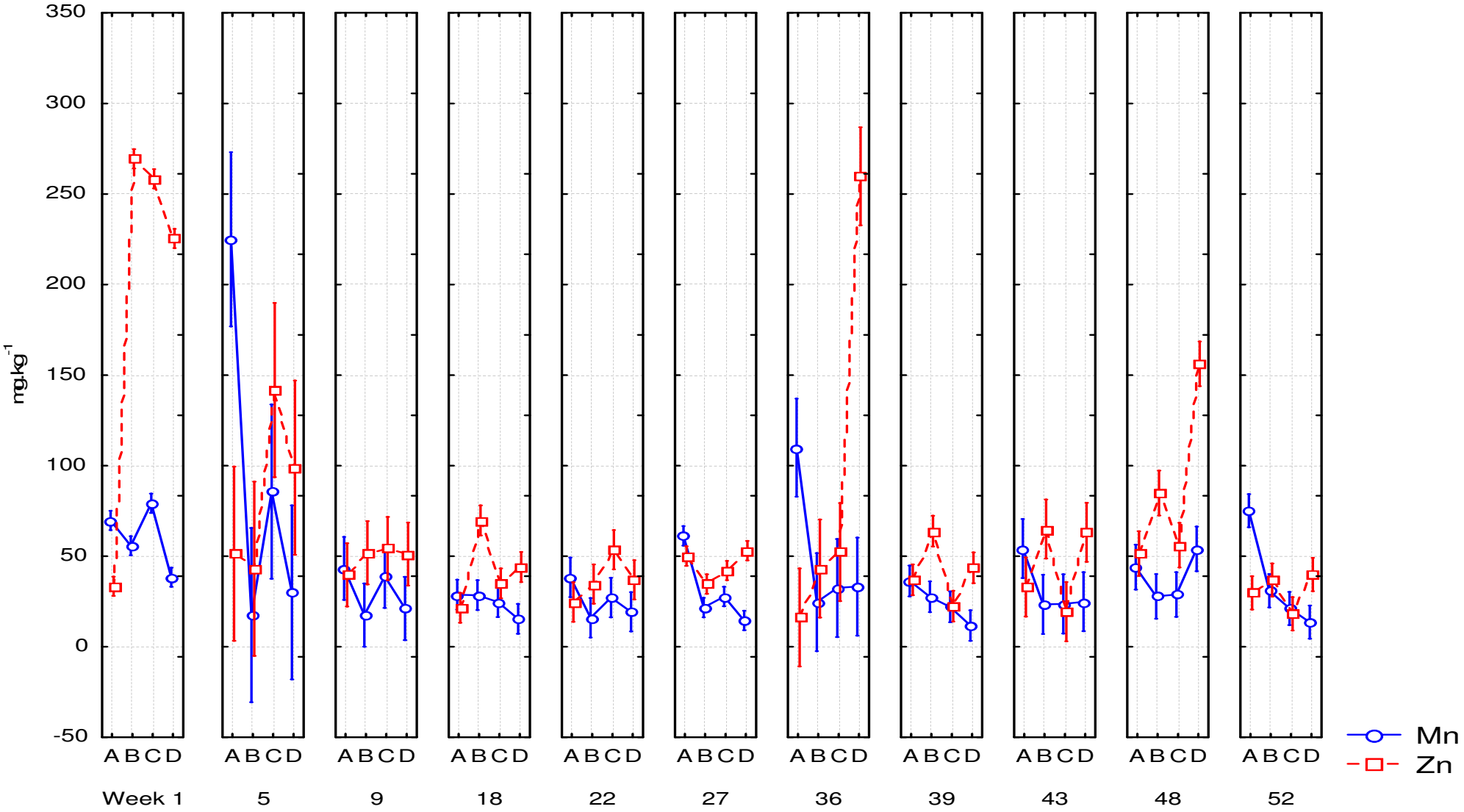

Figure 3.4

Metal concentrations (mg·kg^{-1}) (Mn and Zn) in sediment samples obtained from 4 sites (A to D) in the Plankenburg River

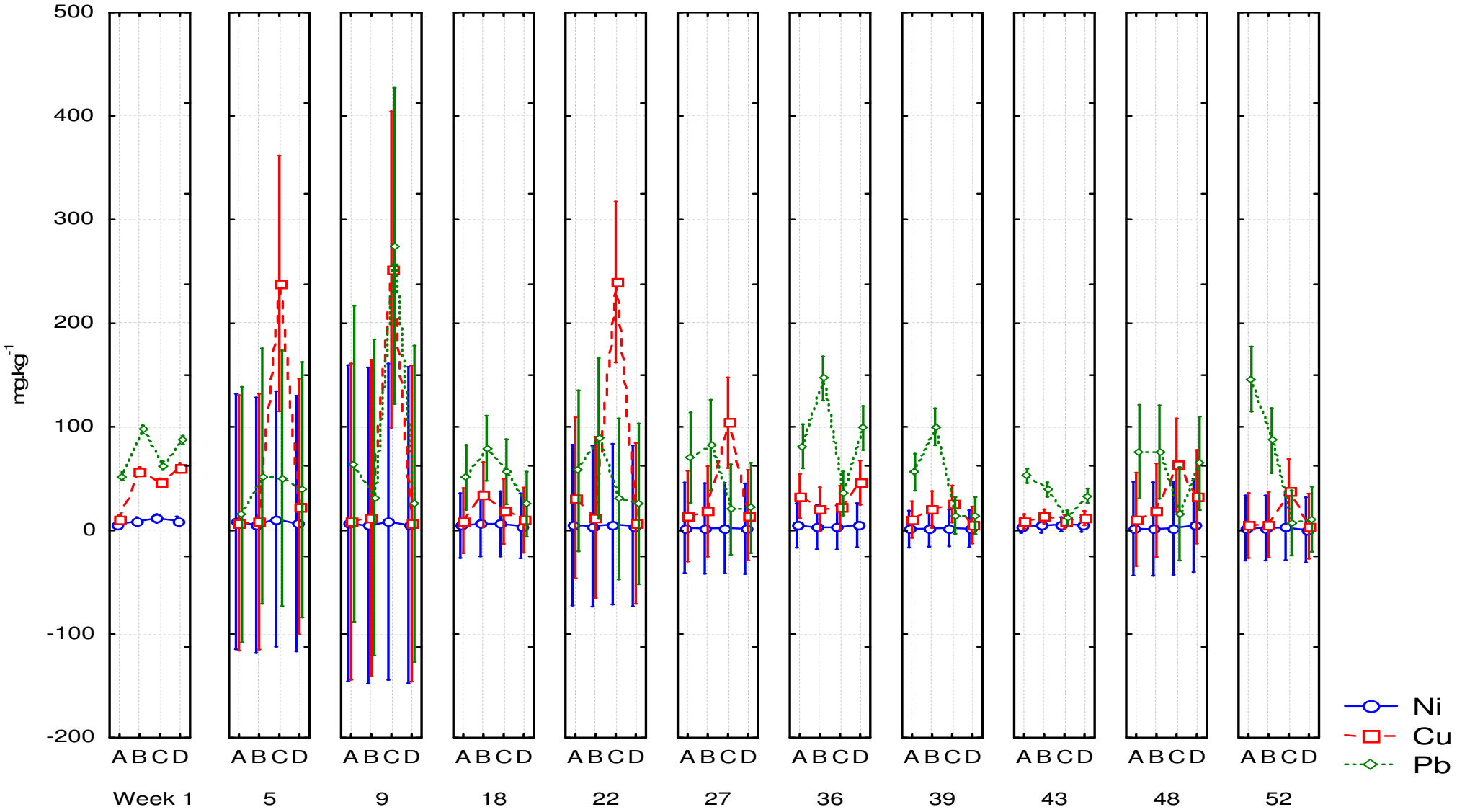

Figure 3.5

Metal concentrations (mg·kg^{-1}) (Cu, Ni and Pb) in sediment samples obtained from 4 sites (A to D) in the Plankenburg River

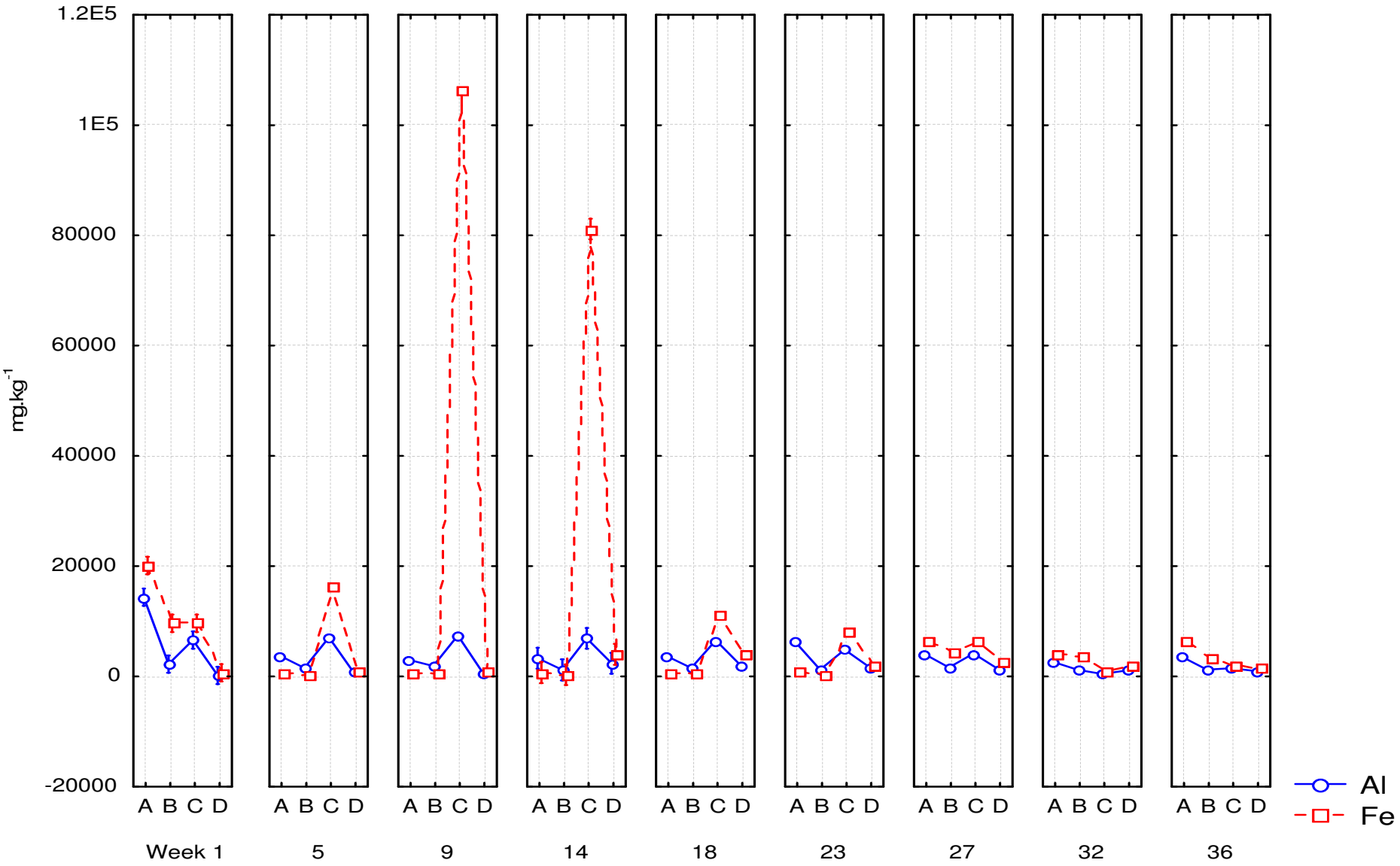

Figure 3.6

Metal concentrations (mg·kg^{-1}) (Al and Fe) in sediment samples obtained from 4 sites (A to D) in the Diep River

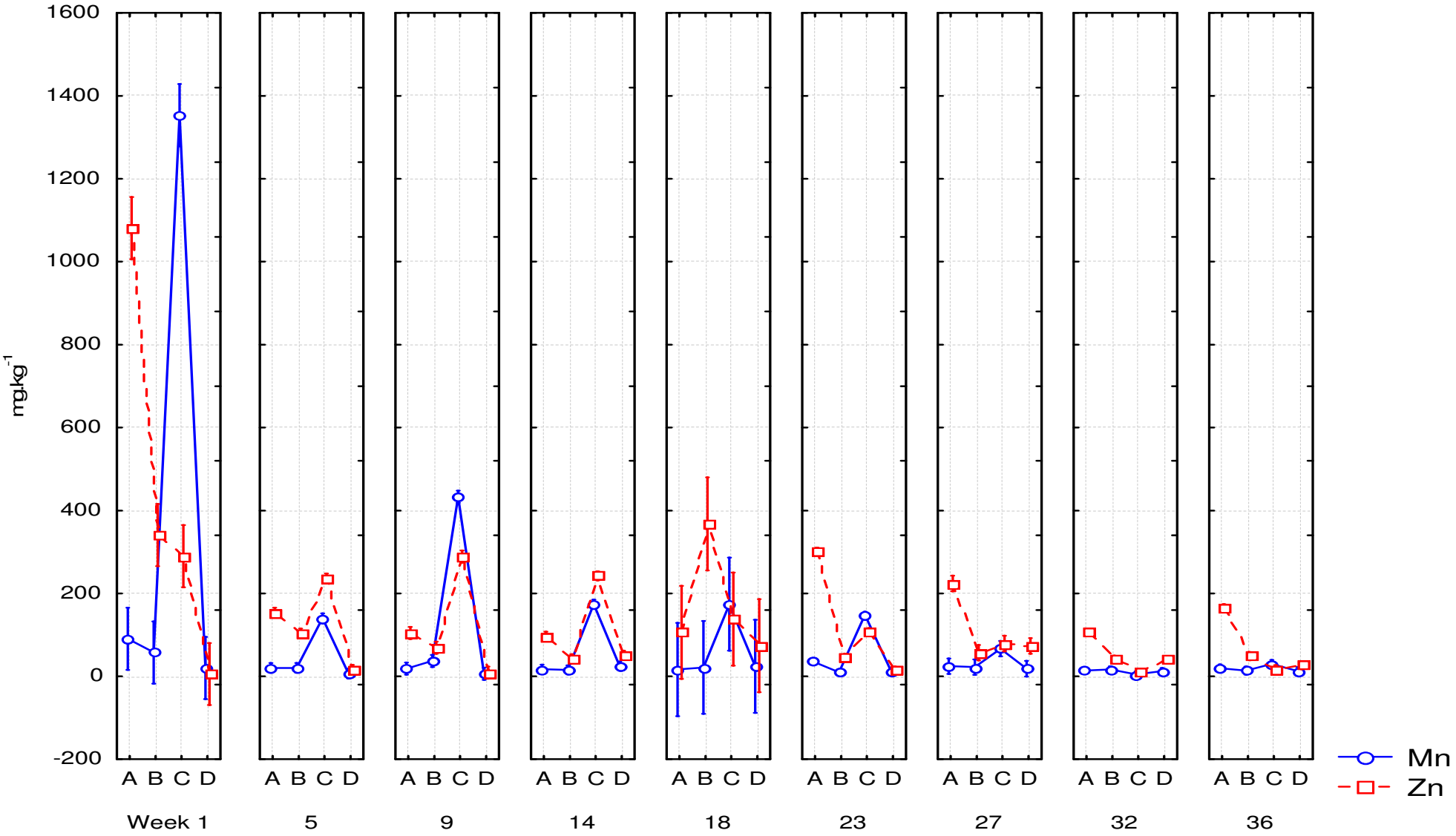

Figure 3.7

Metal concentrations (mg·kg^{-1}) (Mn and Zn) in sediment samples obtained from 4 sites (A to D) in the Diep River

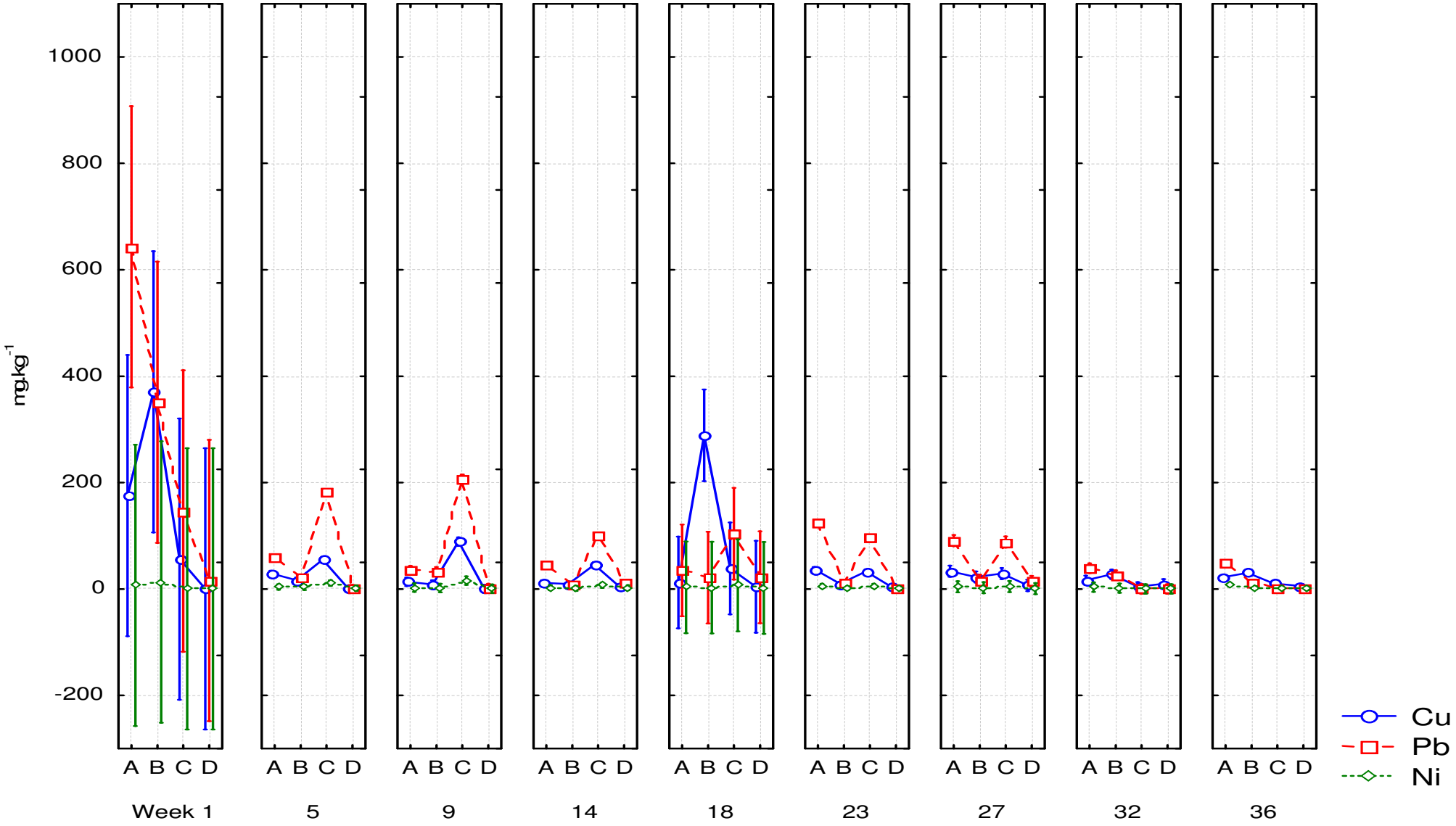

Figure 3.8

Metal concentrations (mg·kg^{-1}) (Cu, Pb and Ni) in sediment samples obtained from 4 sites (A to D) in the Diep River

Table 3.1 Metal concentrations (mg·ℓ$^{-1}$) (±SD*) in water samples obtained from the Plankenburg River (Sites A and B)

	Site A						Site B					
Weeks	**[Cu]**	**[Mn]**	**[Ni]**	**[Zn]**	**[Al]**	**[Fe]**	**[Cu]**	**[Mn]**	**[Ni]**	**[Zn]**	**[Al]**	**[Fe]**
1	1.1±0	0.2±0	0.5±0	0.3±0	0.3±0.1	5.1±0.2	1.7±1	[a]0±0	0.2±0	[a]0±0	0.5±0.3	4.4±0.7
5	0.8±0	0.2±0	0.2±0	0.3±0	0.5±0.1	15.3±6	0.6±0	0.1±0	0.2±0	0.1±0	0.6±0.1	9.7±1.9
9	0.8±0	0.1±0	0.2±0	0.4±0	0.8±0.1	26.5±5.4	0.6±0	0.1±0	0.2±0	0.4±0	1±0.1	25.8±6.8
18	0.4±0	0.1±0	0.3±0	0.2±0	0.8±0.1	10.9±3.3	0.5±0	0.2±0	0.2±0	0.4±0	13.6±4.2	0.5±0
22	0.5±0	0.02±0	0.2±0	0.4±0	0.9±0	12.13.6	0.5±0	0.04±0	0.3±0	0.1±0	1.4±0.2	5.1±0.6
27	0.4±0	0.1±0	0.2±0	0.2±0	0.6±0.1	5.8±1.7	0.4±0	0.1±0	0.1±0	0.2±0	0.3±0.1	4.9±1.2
36	0.5±0	0.1±0	0.3±0	0.4±0	0.7±0.1	29.8±14.9	0.5±0	[a]0±0	0.2±0	0.2±0	0.7±0.2	0.5±0.2
39	0.4±0	0.3±0	0.3±0	0.1±0	0.7±0.1	5±1.1	0.3±0	0.2±0	0.3±0	0.1±0	0.6±0.2	0.3±0.1
43	0.4±0	0.3±0	0.2±0	0.6±0	1.6±0.3	48±8.8	0.8±0	0.2±0	0.2±0	1±0	1.1±0.3	0.8±0.3
48	0.6±0	[a]0±0	0.3±0	0.1±0	0.4±0	10.44.8	0.4±0	[a]0±0	0.3±0	0.1±0	0.7±0.2	8.5±4.7
52	0.4±0	0.3±0	0.2±0	0.3±0	0.6±0.1	18.8±5.5	0.4±0	0.2±0	0.2±0	0.2±0	0.8±0.2	19.3±5.4

* standard deviation (SD)
[a] = values below the detection limit

Table 3.2 Metal concentrations (mg·ℓ$^{-1}$) (±SD*) in water samples obtained from the Plankenburg River (Sites C and D)

	Site C						Site D					
Weeks	[Cu]	[Mn]	[Ni]	[Zn]	[Al]	[Fe]	[Cu]	[Mn]	[Ni]	[Zn]	[Al]	[Fe]
1	2.2±1	0.2±0	0.5±0	0.3±0	0.6±0.3	5.1±0.3	1±0	0.1±0	0.4±0	0.3±0	0.5±0.1	5±0.2
5	0.5±0	0.1±0	0.2±0	0.3±0	0.7±0.2	19.2±2	0.6±0	0.1±0	0.3±0	0.3±0	0.6±0.4	15.9±1.4
9	0.6±0	0.2±0	0.2±0	0.4±0	1±0.1	20.6±3.6	0.6±0	0.1±0	0.2±0	0.4±0	0.8±0.1	17.1±3.8
18	0.4±0	0.1±0	0.3±0	0.2±0	0.8±0.1	10.2±2	0.4±0	[a]0±0	0.2±0	0.1±0	1±0.1	0.4±0.1
22	0.6±0	0.1±0	0.2±0	0.2±0	1.1±0.1	5.2±0.5	0.5±0	[a]0±0	0.2±0	0.1±0	0.9±0	4.4±0.8
27	1.1±2	0.4±1	0.1±2	1±1	1.6±2.5	25.7±12.6	0.3±0	0.1±0	0.1±0	0.3±0	0.4±0.1	10.1±7.5
36	0.5±0	[a]0±0	0.1±0	0.2±0	1±0.1	0.5±0.1	0.4±0	[a]0±0	0.2±0	0.2±0	6.8±0.7	0.4±0
39	0.6±0	0.4±0	0.3±0	1.1±0	0.9±0.1	0.6±0.2	0.7±0	0.1±0	0.2±0	0.5±0	2.1±0.2	31.3±13.7
43	0.5±0	0.3±0	0.2±0	0.5±0	0.8±0.1	0.5±0.1	0.6±0	[a]0±0	0.1±0	0.3±0	1.2±0.1	25.1±4.7
48	0.5±0	[a]0±0	0.2±0	0.1±0	0.7±0.1	8.1±2	0.6±0	[a]0±0	0.2±0	0.2±0	0.6±0	6.6±1.2
52	0.4±0	0.1±0	0.2±0	0.2±0	0.4±0.2	12.6±6.2	0.3±0	0.1±0	0.1±0	0.2±0	0.4±0	4.6±0.5

* standard deviation (SD)
[a] = values below the detection limit

Table 3.3 Metal concentrations (mg·ℓ^{-1}) (±SD*) in water samples obtained from the Diep River (Sites A and B)

	Site A						Site B					
Weeks	**[Cu]**	**[Mn]**	**[Ni]**	**[Zn]**	**[Al]**	**[Fe]**	**[Cu]**	**[Mn]**	**[Ni]**	**[Zn]**	**[Al]**	**[Fe]**
1	0.5±0.1	[a]0±0	0.1±0.1	1.8±0.1	4±0.9	72±12.7	0.2±0	[a]0±0	[a]0±0	0.7±0.3	0.8±0.3	13.2±4.4
5	0.5±0.1	0.2±0	0.3±0.1	0.5±0	3.5±0.5	19.9±3	0.4±0.2	[a]0±0	0.2±0.1	0.2±0	0.7±0.4	11.1±4.9
9	0.4±0.1	[a]0±0	[a]0±0	0.1±0.1	0.5±0	0.1±0.2	0.3±0	0.1±0	[a]0±0	0.5±0.5	0.3±0.4	7.3±2.9
14	0.5±0	0.3±0	0.1±0.1	1±0.2	1.1±0.6	99.9±16	0.6±0.1	0.3±0.1	[a]0±0	1.1±0.2	1.8±0.3	105.9±23.4
18	0.4±0.1	0.1±0.1	0.2±0.1	0.6±0.2	0.7±0.2	69±32.8	0.6±0	[a]0±0	[a]0±0	0.3±0.1	1.3±0.2	5.4±2.6
23	0.5±0.1	[a]0±0	0.2±0.1	0.3±0.1	1.1±0.2	4.7±0.8	0.4±0	0.3±0.2	[a]0±0	1.1±0.5	1.7±0.2	113.6±57.1
27	0.3±0	1.3±0.5	0.2±0.2	4.4±1.6	1±0.3	513±204.1	0.3±0.1	0.8±0.4	0.2±0.1	2.6±0.9	0.9±0.1	317.1±148
32	0.4±0.2	[a]0±0	0.1±0.1	0.4±0.1	0.5±0.3	12.8±8.4	0.4±0.1	[a]0±0	[a]0±0	0.5±0.1	0.3±0.1	11.1±2.3
36	0.5±0.1	[a]0±0	[a]0±0	0.4±0.1	0.7±0.2	0.7±0.4	0.1±0.1	0.1±0	[a]0±0	0.3±0.1	0.5±0.1	12±2

* standard deviation (SD)
[a] = values below the detection limit

Table 3.4 Metal concentrations (mg·ℓ^{-1}) (±SD*) in water samples obtained from the Diep River (Sites C and D)

	Site C						Site D					
Weeks	**[Cu]**	**[Mn]**	**[Ni]**	**[Zn]**	**[Al]**	**[Fe]**	**[Cu]**	**[Mn]**	**[Ni]**	**[Zn]**	**[Al]**	**[Fe]**
1	0.1±0	0±0.1	0.1±0.2	0.6±0.4	0.6±0.1	15.4±16.9	0.1±0	[a]0±0	[a]0±0	0.4±0.2	1±0.1	9.4±4.1
5	0.5±0.3	[a]0±0	0.2±0.1	0.2±0	0.6±0.4	11±9.4	0.6±0.1	[a]0±0	0.1±0.1	0.2±0	0.9±0.2	3.9±0.7
9	0.3±0.1	0.1±0	[a]0±0	0.2±0	[a]0±0	3.9±1.2	0.7±0	[a]0±0	[a]0±0	0.4±0.1	0.6±0.1	2.6±2.6
14	0.5±0.1	0.2±0.1	0.2±0.2	1±0.4	1.1±0.2	89.9±31.9	0.4±0	0.2±0.1	[a]0±0	0.9±0.1	1.2±0.2	98.9±13.1
18	0.6±0.1	[a]0±0	0.3±0.3	0.5±0.1	1.1±0.2	4.4±1.2	0.5±0.1	[a]0±0	[a]0±0	0.4±0.1	1.2±0.1	4.2±2.1
23	0.4±0	0.2±0.1	0.2±0.2	0.9±0.4	1.3±0.2	82.6±46.5	0.2±0	[a]0±0	0.4±0.1	0.5±0.1	1.3±0.2	10.5±5.3
27	0.6±0.3	a0±0	0.1±0.1	2.5±4.4	0.3±0.2	11±2.3	0.4±0.2	[a]0±0	0.1±0.1	0.5±0	0.4±0.2	16.2±3.4
32	0.3±0.1	[a]0±0	0.4±0.2	0.6±0.2	0.3±0.1	12.1±8	0.8±0.2	[a]0±0	0.2±0.2	0.6±0.1	0.5±0.1	6.4±1.6
36	0.1±0	0.1±0	[a]0±0	0.4±0.1	0.4±0	0.2±0.5	0.1±0.1	0.1±0	[a]0±0	0.8±0.2	1.1±1.1	23.9±10.9

* standard deviation (SD)
[a] = values below the detection limit

Table 3.5 Concentrations obtained in water of the Plankenburg and Diep Rivers compared to recommended safe concentrations as stipulated by the Department of Water Affairs and Forestry (1996b), the Canadian Council of Ministers of the Environment Quality Guidelines (2001), the 'World average' (Martin and Windom, 1991) and the Australian and New Zealand Environment and Conservation Council (ANZECC, 2000)

Metal	Recommended safe concentrations as stipulated by DWAF (1996b) ($mg \cdot \ell^{-1}$)	Environmental quality guidelines as stipulated by CCME (2001) ($mg \cdot \ell^{-1}$)	'World average' for metal concentrations in freshwater by Martin and Windom (1991) ($mg \cdot \ell^{-1}$)	Water quality guidelines as stipulated ANZECC (2000) ($mg \cdot \ell^{-1}$)	Mean metal concentrations obtained in water ($mg \cdot \ell^{-1}$) (Plankenburg River)	Mean metal concentrations obtained in water ($mg \cdot \ell^{-1}$) (Diep River)
Al	0.1 – 0.15	0.005 – 0.1	N/A	N/A	0.3 – 13.6	[a]0 – 4
Cu	0.002 – 0.012	0.002 – 0.004	0.0015	0.0001 – 0.00015	0.3 – 2.2	0.1 – 0.8
Fe	N/A	0.3	0.04	N/A	0.3 – 48	0.1 – 513
Mn	1.3	N/A	0.0015	N/A	[a]0 – 0.4	[a]0 – 1.3
Ni	N/A	0.025 – 0.15	0.0005	0.0001 – 0.00015	0.1 – 0.5	[a]0 – 0.4
Pb	N/A	0.001 – 0.007	N/A	N/A	[a]0 – [a]0	[a]0 – 0
Zn	0.036	0.03	0.0006	0.0009	[a]0 – 1.1	0.1 – 4.4

N/A = Data not available
[a] = values below the detection limit

Identification of Metal-tolerant Organisms Isolated from the Plankenburg River, Western Cape, South Africa.

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Abstract

The ability of biofilms to resist pollutants make them advantageous for use in bioremediation. The objective of this investigation was to isolate metal-tolerant micro-organisms from a site along the Plankenburg River. Microbial biofilms cultivated in multi-channelled flow cells were exposed to varying concentrations of aluminium (Al), iron (Fe), copper (Cu), manganese (Mn), nickel (Ni) and zinc (Zn), stained with the BacLight™ viability probe, visualised using Epifluorescence Microscopy and analysed using ScionImage. Exposure to the highest Al, Fe, Cu and Mn concentrations increased the percentages of dead cells. A difference in live and dead cells exposed to the varying Zn and Ni concentrations was not evident. When exposed to the lowest concentrations, no notable could be detected in comparison with the untreated control. Possible metal-tolerant micro-organisms were identified from the exposed flow cells using Polymerase Chain Reaction (PCR) and Deoxyribonucleic Acid (DNA) sequencing, followed by Clustal X alignment and phylogenetic analysis. Phylogenetic analysis identified a variety of organisms, including *Bacillus* sp., *Pseudomonas* sp., *Delftia tsuruhatensis* strain A90, *Kocuria kristinae* strain 6J-5b, *Comamonas testosteroni* WDL7, *Stenotrophomonas maltophilia* strain 776, *Staphylococcus* sp. MOLA:313, *Micrococcus* sp. TPR14, *Sphingomonas* sp. 8b-1 and *Microbacterium* sp. PAO-12. Two major clusters could be distinguished based on their Gram-reactions.

Keywords: biofilms, Deoxyribonucleic Acid (DNA) sequencing, Epifluorescent Microscopy, flow cells, Polymerase Chain Reaction (PCR)

Introduction

Population growth and urbanisation, results in increased water resource utilisation. Continued deposition of point- and non-point source pollutants, including industrial effluents, agricultural runoff, leaking sewers, on-site sanitation at informal housing and waste irrigation (Department of Environmental Affairs and Tourism 1996), amongst others, adversely affects the surrounding environment.

Metal contamination in the environment can also be attributed to the natural occurrence of metals in soil, atmospheric deposits (Radenac et al. 2001) and the corrosion of building materials (Maanan et al. 2004). The metals most commonly associated with most river water systems are lead (Pb), copper (Cu), iron (Fe), cadmium (Cd), aluminium (Al), mercury (Hg), arsenic (As) and manganese (Mn) (Wright and Welbourne, 2002, Jackson et al. 2007).

Zinc (Zn), nickel (Ni), As, Hg, cobalt (Co) and Mn concentrations were studied in water and fish samples isolated from the Aba River, Nigeria, into which waste from various industries are discharged. Atomic Absorption Spectrometry (AAS) analysis, revealed elevated concentrations of Zn, Mn and As in fresh fish and elevated Ni and Hg concentrations in frozen fish found in a nearby market (Allinnor 2005).

In water and the environment, micro-organisms exist mostly as biofilm communities attached to surfaces (Teitzel and Parsek, 2003). Microbial biofilms exhibit high affinities for contaminants due to the ability of the exopolymers to bind and sequester antimicrobial agents from the surrounding environment (Hunt 1986). Biofilms have been shown by Roane and Pepper (2000) to be one of the most effective treatments for the removal of metals from metal-contaminated water

Flow cell systems have been used to cultivate microbial biofilms *in vivo* (Caldwell et al. 2002). They are multi-channelled to allow for experimental replication and simplified handling. Teitzel and Parsek (2003) used a flow cell system to visualise the behaviour of biofilm-bound micro-organisms in response to Cu and Zn. The CLSM analysis revealed that the majority of cells in the outer layers were dead, in comparison to the untreated control, where the majority of cells were alive.

Microbial composition can be determined genetically (Christensen et al. 1998), through the amplification of the 16S or 23S rRNA region of the genomic DNA, using specific primers (Amann et al. 1995). The diversity of tolerant micro-organisms depends on nucleotide sequences variations (Martin 2002), ranging from 20% to 80% G+C (Ochman et al. 2005) among individual species sharing common ancestry. This genetic variation can then be visualised with phylogenetic trees (Martin 2002).

Chien et al. (2007) studied the microbial diversity in soil contaminated with effluent from a chemical industrial factory, using 16S rDNA. The organisms isolated were *Polyangium* spp., *Sphingomonas* spp., *Variovorax* spp., *Hafina* spp., *Clostridia*, *Acidobacteria*, the enterics and some uncultured strains. *Acinetobacter, Enterobacter and Stenotrophomonas* spp. also exhibited the ability to tolerate high concentrations of Cd.

The objective of this investigation was to isolate metal-tolerant micro-organisms from a metal contaminated site along the Plankenburg River. The micro-organisms were cultured and isolated in flow cell systems after exposure to varying metal concentrations and identified using the Polymerase Chain Reaction (PCR) technique and analysed phylogenetically.

Materials and Methods

Site description

A previous study identified four sampling sites along the Plankenburg River (Fig. 4.1) (Jackson et al. 2008). These sites included Site A (Agricultural Farming and Residential Areas); Site B (Closest point to Informal Settlement); Site C (Substation in Industrial Area) and Site D (Industrial Area at Adam Tas Bridge). Results from this study showed that the highest concentrations of metals were recorded at Site C (Substation in the industrial area), which explains why the particular site was selected to investigate the efficiency of the bioreactor systems to reduce metal concentrations in the river water. Ten litres of river water was then collected from Site C (Fig. 4.1) in one ten litre plastic container and transported at 4°C.

Metal concentrations in river water

To determine the concentrations of Al, Cu, Fe, Mn, Ni and Zn in water (5 ml), samples were digested with 10 ml 55% nitric acid at 40°C for 60 minutes and then at 120°C for 180 minutes, using a Grant dry-block heater. A blank (control) of 10 ml 55% nitric acid was analysed along with the collected samples to check for possible contamination. The samples were cooled to room temperature, filtered with Whatman No. 6 filter paper into 20 ml volumetric flasks, made up to a volume of 20 ml with distilled water and subsequently filtered for a second time using 0.45 µm cellulose nitrate ultrafiltration membrane filters (Whatman) (Odendaal and Reinecke, 1999). Metal concentrations were determined using Inductively Coupled Plasma Atomic Emission

Spectrometry (ICP-AES) analysis according to the procedure outlined in Saleh et al. (2000).

Flow Cell Technique

Six multi-channelled (eight channels) flow cells were constructed from Perspex, a glass coverslip and silicone tubing. The flow channels were 5 mm wide, 30 mm long, 3 mm deep and were 4 mm in distance from the next channel. The glass coverslip (50 mm x 75 mm) was kept in place on top of the flow cell with marine silicone glue and provided an attachment surface for the microbial growth. Silicone tubing (1.6 mm) was used for the flow through the respective channels from the reservoirs (influent - collected river water) to the outlet (effluent). After construction, the flow cells were sterilised with a solution of sodium hypochlorite and flushed with distilled water (Fig. 4.2). The collected river water was pumped through the flow cell systems, using a Watson Marlow peristaltic pump (205S) (Watson Marlow Limited, Cornwall, England), to ensure a constant flow rate at 2 rpms (revolutions per minute). After a three-week period, which allowed for maximum biofilm growth, the channels were exposed to varying metal concentrations. Each flow cell was exposed to different concentrations of Al, Fe, Cu, Mn, Ni and Zn (Table 4.1), based on concentrations recorded during a previous study (Jackson et al. 2008). The first two channels of each flow cell were not exposed to any metals and served as the controls. Each flow cell channel was exposed to the respective metal concentrations for six hours, while the peristaltic pump was switched off and the tubing was clamped.

150 **Exposure to the Baclight™ viability probe**

151 After the six hour metal exposure period, one of each channel spiked with the
152 respective metal concentrations, as well as one of the unexposed control channels
153 were stained with the multifluor LIVE/DEAD Baclight™ fluorescent viability probe
154 (invitrogen – Molecular Probes™, Oregan, USA). The stain was prepared by mixing
155 4 µl of the green fluorescing probe (SYTO 9) with 4 µl of the red fluorescing probe
156 (propidium iodide) and 1 ml of distilled water. The probe was allowed to attach to the
157 cell surfaces of the biofilm organisms for a period of 20 minutes. After the attachment
158 period, the pump was switched on to remove any excess dye. The channels not
159 exposed to the probe, were flushed by turning the pump up to its maximum flow rate
160 of 90 rpms in order to dislodge the attached biofilm growth. This microbial
161 suspension was collected into individual 50 ml Schott bottles.

162

163 **Microscopy and Image Analysis**

164 Epifluorescence microscopy was used in conjunction with the LIVE/DEAD BacLight™
165 probe (invitrogen – Molecular Probes™, Oregan, USA) in order to provide total cell
166 counts, using images based on the relative abundance of micro-organisms
167 (Boulos et al. 1999). The images were visualised and captured using The Zeiss
168 Axiovert 200 motorised fluorescence microscope, which contains the Dapi, Alexa,
169 Cy3, GFP and Cy5 laser lines. It is also equipped with a monochrome Zeiss Axiocam
170 HR digital camera. The 100X, 1.4 NA oil immersion objective was used to visualise
171 the biofilm, along with an argon laser exciting the fluorophores. Ten randomly
172 captured images along the surfaces of the attached coverslip were obtained from
173 each of the exposed channels using the Axiovision 4.6 Software Programme (Zeiss

imaging systems), which were further used for viewing and simple image processing. Ten images were randomly captured along the surfaces of the coverslips and the percentage area covered by living and non-living biomass was determined using ScionImage Analysis (Scioncorp.com). These techniques were performed in duplicate and the results obtained were the averages of the replicates.

DNA Extraction and Agarose Gel Electrophoresis

The flushed material obtained from the unstained flow cell channels for each respective metal, was spread plated onto nutrient agar plates and incubated at 37ºC for two days. Single colonies were then selected in order to isolate pure cultures and DNA was extracted using the High pure PCR template preparation kit, as per manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Ten microlitres of the extracted DNA samples were electrophoretically analysed on a 0.8% molecular grade agarose gel containing 12 µl of 0.5 µg/ml ethidiumbromide (EtBr), using 1 x Tris-acetate- ethylenediamine tetraacetic acid (TAE) electrophoresis buffer at 90 volts for one hour.

Polymerase Chain Reaction (PCR)

Amplification of target DNA by PCR was performed in a total reaction volume of 50 µl containing 10 mM dNTP Mix (1 µl), 25 mM $MgCl_2$ (3 µl), 10X Taq Buffer with $(NH_4)_2SO_4$ (5 µl), 10 µM forward (fDD2 - CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG) (5 µl), 10 µM reverse (rPP2 - CCAAGCTTCTAGACGGITACCTTGTTACGACTT) (5 µl) (Rawlings 1995), Taq DNA polymerase (1 µl) (5u/5 µl) (Fermentas Life Sciences, EU), 1 µl of a concentrated

DNA sample and 29 µl sterile distilled. The amplification process included an initial denaturation step of 94°C for 2 minutes, followed by 30 cycles of amplification (1 minute at 94°C, one minute at 57°C and two minutes at 72°C). This was followed by a final extension step of 72°C for 10 minutes. Ten microlitres of the subsequent PCR amplicons were then electrophoretically analysed on a 0.8% molecular grade agarose gel containing 12 µl of 0.5 µg/ml EtBr, using 1 x TAE electrophoresis buffer at 100 volts for one hour, to determine whether amplification was successful.

Sequencing of 16S rRNA

The amplified PCR products (1200 bp) were purified using a High Pure PCR product purification kit, as per manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The concentrations of the DNA samples were determined using spectrophotometry and 15 µl of concentrated DNA (50 to 100 ng/µl depending on the length) samples were loaded onto 96-well microtitre plates, dried in a speed vac, with medium heat for 30 to 60 minutes (depending on the volumes), and sent for sequencing. The sequencing lab used the Applied Biosystems Big Dye Terminator v3.1.

Phylogenetic Analysis

The resultant sequences were identified with a similarity search using Blastn from the National Centre for Biotechnology Information (NCBI) (Altschul et al. 1997). Contiguous sequences were formed for the forward and reverse sequences of a particular organism, using the CAP3 Sequence Assembly Programme (Huang and Madan 1999). The contiguous sequences were aligned with Clustal X (1.81) (Higgins

and Sharpe 1988) using default parameters and the BLOSUM matrix, which corrects for multiple base changes. There were 37 organisms isolated overall, but many of the isolates were identical, therefore the 13 representative organisms on the tree were used for phylogenetic analyses. An unrooted tree was constructed using the neighbour-joining program of Saitou and Nei (1987). Phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis, Version 3.1 (MEGA version 3.1) (Kumar et al. 2004). To estimate the node reliability, bootstrap values were obtained from 1000 randomly generated trees. Trees were visualised using MEGA version 3.1 (Felsenstein 1985, Efron et al. 1996, Kumar et al. 2004).

Results and Discussion

Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) was used to determine the initial concentrations of metals in the collected river water prior to the flow cell set-up. These concentrations were compared to the Department of Water Affairs and Forestry (DWAF 1996) and the Canadian Council of the Ministers of the Environment (CCME 2001) recommended guidelines (Table 4.2). The Al, Cu, Fe, Ni and Zn concentrations were 11.56 $mg.l^{-1}$, 0.06 $mg.l^{-1}$, 12.2 $mg.l^{-1}$, 0.17 $mg.l^{-1}$ and 0.4 $mg.l^{-1}$, respectively (Table 4.2). These concentrations were above the recommended concentrations as stipulated by DWAF (1996) and the CCME (2001). The Mn concentration of 0.29 $mg.l^{-1}$, was below the recommended concentration of 1.3 $mg.l^{-1}$ (DWAF 1996).

Figures 4.3A, 4.4A, 4.5A, 4.6A, 4.7A and 4.8A represent the percentages of live and dead organisms (epifluorescence microscopy in conjunction with the ScionImage

statistical programme) within the biofilm cultivated in the flow cells. In the graphs, the letter G (green) indicates the live cells and the letter R (red), the dead cells. Figures 4.3B, 4.4B, 4.5B, 4.6B, 4.7B and 4.8B are images captured (untreated control and images captured after exposure to the respective metal concentrations) with epifluorescence microscopy. The results recorded in the control channels (which were left unexposed), showed that the initial percentages of live cells, for all the metals analysed, were higher than that of the dead cells.

The following percentages were recorded for the channels exposed to Al concentrations; the control channels [48.12% alive (G), 43.21% dead (R)], the highest concentration of 900 $mg.l^{-1}$ [40.63% (G) and 47.68% (R)], for 500 $mg.l^{-1}$ [42.31% (G) and 44.52% (R)] and when exposed to 10 $mg.l^{-1}$ [43.38% (G) and 42.59% (R)] (Figs. 4.3A and 4.3B).

The percentages recorded for live and dead cells exposed to varying concentrations of Cu were; 44.02% (G) and 40.96% (R) in the control channel, 41.85% (G) and 44.67% (R) for the highest concentration of 10 $mg.l^{-1}$, 40.86% (G) and 41.14% (R) for 1 $mg.l^{-1}$ and when exposed to the lowest concentration of 0.5 $mg.l^{-1}$, the percentages were 41.66% (G) and 41.04% (R) (Figs. 4.4A and 4B).

For Fe, the ratio of live and dead cells in the untreated control was 46% live (G) and 40.88% dead (R), respectively. The percentages obtained when the channels were exposed to the highest Fe concentration of 1000 $mg.l^{-1}$ was 41.66% (G) and 44.47% (R), when exposed to 500 $mg.l^{-1}$, the percentages were 42.96% (G) and 44.25% (R), and when exposed to the lowest concentration of 10 $mg.l^{-1}$, the percentages recorded were 43.32% (G) and 42.40% (R) (Figs. 4.5A and 4.5B).

The percentages of live and dead cells recorded after exposure to Mn in the untreated control were 43% (G) and 41.07% (R), respectively. Upon exposure to the highest concentration (80 $mg.l^{-1}$), the number of live cells were 44.04% (G) and the number of dead cells were 50.23% (R). When exposed to the two lower concentrations of 15 $mg.l^{-1}$ and 1.5 $mg.l^{-1}$, the percentages of live and dead cells were 42.94% (G) and 43.39% (R) and 41.99% (G) and 41.18% (R), respectively (Figs. 4.6A and 4.6B).

The percentage of cells in the untreated flow cell control channels used to evaluate the various Ni concentrations, were 47.34% (G) and 44.27% (R), ratio of live and dead cells, respectively. The percentages recorded in the channels exposed to the highest Ni concentration of 20 $mg.l^{-1}$, was 43.68% (G) and 43.75% (R), while the percentages recorded when exposed to 1 $mg.l^{-1}$, were 44.91% (G) and 42.89% (R) and when exposed to 0.5 $mg.l^{-1}$, 41.49% of the cells were alive (G) and 40.95% of the cells were dead (R) (Figs. 4.7A and 4.7B).

The channels exposed to the varying Zn concentrations yielded the following results in the untreated control channel; 45.06% live cells (G) and 41.32% dead cells (R). The percentages recorded in the flow cell channels exposed to the highest Zn concentrations (40 $mg.l^{-1}$) was 41.27% (G) and 41.82% (R), while the percentages recorded when exposed to 1 $mg.l^{-1}$, was 41.37% (G) and 41.44% (R). The percentages recorded when exposed to 0.5 $mg.l^{-1}$, was 42.58% (G) and 41.15% (R), respectively (Figs. 4.8A and 4.8B).

When compared to the untreated controls, the percentages obtained in the channels of the flow cells exposed to the highest concentrations of Al (900 $mg.l^{-1}$), Cu (10 $mg.l^{-1}$), Fe (1000 $mg.l^{-1}$) and Mn (80 $mg.l^{-1}$) (Figs. 4.3A, 4.4A, 4.5A, 4.6A), showed

293 an increase in the number of dead cells of 4.47%, 3.71%, 3.59% and 9.16%,
294 respectively. When the channels were exposed to the lowest concentrations of
295 10 mg.l^{-1} (Al), 0.5 mg.l^{-1} (Cu), 1.5 mg.l^{-1} (Mn) and 0.5 mg.l^{-1} (Zn), the ratio of live to
296 dead cells was similar to that of the untreated control. When exposed to the highest
297 concentrations of Zn (40 mg.l^{-1}) and Ni (20 mg.l^{-1}), no significant differences between
298 the live and dead cell percentages, were observed as the percentages of dead cells
299 only decreased by 0.52% and 0.5%, respectively (Figs. 4.7A and 4.8A). In a previous
300 study, conducted by Bhadra et al. (2007), the effect of high concentrations of metals
301 (Zn and Ni) in the river water affected the numbers of Zn and Ni resistant bacteria,
302 therefore, the higher metal concentrations resulted in an increase in resistance
303 mechanisms of the isolates. Table 4.2 represents the metal concentrations recorded
304 in the water samples, and showed that the initial Zn concentration in the river water
305 prior to treatment was 0.4 mg.l^{-1}, which was significantly higher ($p < 0.05$) than the
306 recommended guideline of 0.036 mg.l^{-1} (DWAF 1996) and 0.03 mg.l^{-1} (CCME 2001).
307 The initial Ni concentration in the river water prior to treatment was 0.17 mg.l^{-1}, which
308 was significantly higher ($p < 0.05$) than the recommended guideline of 0.025 mg.l^{-1} to
309 0.15 mg.l^{-1} (CCME 2001).

310 Results show that flow cells are ideal to provide results instantaneously while
311 providing multiple replications and also allow for the comparison of untreated controls
312 and treated channels (Nancharaiah et al. 2005). In the case of exposure of the
313 respective flow cell channels to the highest Al and Fe concentrations, Figs. 4.3B and
314 4.5B showed that the organisms tended to clump together in response to the metal
315 exposure. The ability of extracellular polymeric substances to bind metals and
316 pollutants also contribute to the clumping of cells (McLean et al. 1990). Metals can

alter the number, biochemical activity, diversity and community structure of micro-organisms in many different ways (Ellis et al. 2003). The resistance of micro-organisms to the metals could be attributed to many factors, including, lateral gene transfer (Sobecky et al. 1998), co-contamination with organic material (Toes et al. 2008) and trace elements in cells, inhibiting normal physiological functions (Hultberg et al. 1997). Metals, such as Mn, Fe, Cu, Ni, Zn and cobalt (Co) can cause direct and indirect oxidative stress, which result in the accumulation of reactive oxygen species (Salzano et al. 2007). Previous research performed by Teitzel and Parsek (2003) showed that cells at the biofilm-bulk liquid interface were exposed to the high concentrations of various metals. The two fluorophores used (SYTO 9 and Propidium Iodide) stains the living cells green and the dead cells red, respectively. When the cells fluoresce yellow, it means that the two images are superimposed and it is impossible to distinguish live cells from dead cells (Figs. 4.3B to 4.8B). Teitzel and Parsek (2003) also reported that in minimal media with short exposure times, biofilms have a demonstrable resistance to the heavy metals Cu^{2+}, Zn^{2+} and Pb^{2+}.

Table 4.3 represents the names of organisms isolated from the flow cells after exposure to varying metal concentrations. An agarose gel electrophoresis photograph showing selective results of the PCR fragments (1200 bp) is presented in Fig. 4.9. Figure 4.9 clearly shows that the 1200 bp PCR amplicon was routinely amplified. Overall 37 organisms were isolated, but many of the isolates were identical, therefore only 13 organisms were used in the construction of the phylogenetic tree. The phylogeny of the 13 representative organisms in GenBank: www.ncbi.nlm.nih/gov/Genban/submit.html, were analysed using the Neighbour-

joining algorithm in Clustal X (Fig. 4.10). The tree was aligned to determine evolutionary relatedness between the various isolates.

Most of the isolates from all the channels were identified as *Staphylococcus* sp. MOLA:313, *Delftia tsuruhatensis* strain A90, *Pseudomonas fluorescens* isolate TC222, *Pseudomonas beteli* strain RRLJ SMAR, *Bacillus* sp. ZH6 and *Stenotrophomonas maltophilia* strain 776. Two major clusters could be distinguished from the tree (Fig. 4.10) based on their Gram-reaction. From the phylogenetic tree it could be seen that the Gram-negative organisms, *Proteobacteria* (*Pseudomonas* sp., and *Stenotrophomonas* sp.) clustered together, whereas the Gram-positive organisms, low and high G + C Gram-positives (*Bacillus* sp., *Micrococcus* sp. and *Microbacterium* sp.) clustered together. In the first cluster, two clades could clearly be distinguished; the first clade consisted of *Pseudomonas* sp. and the second clade, of a *Pseudomonas* sp. and a *Stenotrophomonas* sp. The isolates, *Delftia tsuruhatensis* st. A90 and *Sphingomonas* sp. 8b-1 were rooted to clades one and two, but judging by the length of the nodes, did not seem to belong to any of the groups. In the second cluster, two distinct clades could be distinguished. The first clade consisted of WDL7 *Comamonas testosterone*, ZH6 *Bacillus* sp. and MOLA 313 *Staphylococcus* sp. The second clade consisted of TPR1 *Micrococcus* sp., 6J-5b *Kocuria kristinae* st and PAO-12 *Microbacterium* sp.

In previous research it was shown that several Gram-positive (*Arthrobacter* sp. and *Corynebacterium* sp.) and Gram-negative (*Alcaligenes* sp.) organisms were shown to be resistant to Pb, Hg, Cd, Cu, Co and Zn (Trajanovska et al. 1997), which could be correlated to the results obtained in the present study. The major difference between the two clusters, was that the Gram-positive organisms, *Staphylococcus*

MOLA:313, *Micrococcus* sp. TPR1 and *Kocuria kristinae* st. 6J-5b were resistant to Mn (Fig. 4.10 and Table 4.4), and that the clade consisting of the *Pseudomonas* sp. all exhibited tolerance to Ni exposure. Table 4.4 represents the organisms isolated from the different flow cells after exposure to the varying metal concentrations. The organisms in the table below were present after exposure to Al, Cu, Fe, Mn, Ni and Zn concentrations and were presumed to be metal-tolerant organisms. Organisms such as *Comamonas testosteroni* WDL7, *Microbacterium* sp. PAO-12, *Sphingomonas* sp. 8b-1, *Kocuria kristinae* strain 6J-5b and *Micrococcus* sp. TPR14 exhibited tolerance to specific metals, which included Cu, Ni, Zn and Mn, respectively (Table 4.4). The other isolates showed resistance to a range of different metals.

In a previous study by Bhadra et al. (2007), *Pseudomonas, Bacillus, Moraxella, Enterobacter, Serratia, Morganella* and *Acinetobacter* species were used to study Ni and Zn resistance, as these organisms possess inducible Ni or Zn resistance genetic systems. Their results indicated that the Ni and Zn resistance was induced at concentrations as low as 5 µm Zn^{2+} (325 ppb) for Ni resistance in *Pseudomonas* sp. and Zn resistance were inducible by a concentration as low as 4 µm Zn^{2+}. Hussein et al. (2004) showed that Cd and Ni could be bounded to their *Pseudomonas* species isolated from wastewater by as much as 0.5 and 0.56 $mg.kg^{-1}$ biomass, respectively, while Cu and Cr uptake values ranged between 0.01 to 0.24 $mg.kg^{-1}$ biomass. In the present study, it was shown that *Pseudomomas* sp. was isolated from the flow cell channels exposed to Ni and Zn (Table 4.4).

The clusters also contained more Gram-negative than Gram-positive organisms (Fig. 4.10). Duxbury and Bicknell (1983) and Drancourt et al. (2000), amongst others, suggested that Gram-negative organisms predominated in metal-polluted

388 environments, and also showed that it was reasonable to expect a certain degree of
389 overlap between Gram-positive and Gram-negative bacteria, as some of these
390 species are resistant to the same metals. Possible metal-tolerance mechanisms of
391 organisms could be that Gram-positive bacteria possess a high metal absorption
392 capacity Yilmaz (2003).

393 After the metals bind to the organism, it must either cross the cell wall of the
394 Gram-positive organism or the outer membrane of the Gram-negative organism. The
395 Gram-positive bacteria have no receptor molecules to block or hinder the transport of
396 pollutants across the membrane and are therefore less resistant to attack (Cloete
397 2003). The Gram-negative organisms have developed a means of resistance to more
398 specific pollutants because of the narrow porin channels, which slow down the
399 penetration of substances, contributing to the resistance of Gram-negative organisms
400 to pollutants (Nikaido 1996). The efficiency of metal ions depend on their ability to
401 bind to proteins and prevent replication of the bacterial cells (Kar et al. 1992). Studies
402 on Cu, Cd, Zn, Cr, Ni and As resistant bacteria have identified metal resistance genes
403 to be located on the plasmid (Trajanovska et al. 1997) and can be inducible in the
404 presence of the particular metal (Cloete 2003). The *Staphylococcus* sp. were shown
405 to carry genes for Hg, Cd, As, Pb and Zn resistance (Nakahara et al. 1977). In their
406 studies, they also found that most of the metal-resistant isolates were multiply metal
407 resistant and also multiply drug resistant (Nakahara et al. 1977), as micro-organisms
408 usually contain a cluster of genes involved in both metal and antibiotic resistance
409 (Alonso et al. 2000). The high degree of antibiotic resistance may also be associated
410 with higher levels of tolerance to various heavy metals (Hassen et al. 1998).

411 Cloete (2003) showed that the presence of some metals might induce resistance to a
412 broader spectrum of metals.

413 *Bacillus*, *Pseudomonas*, *Xanthomonas*, *Stenotrophomonas*, *Sphingomonas* sp.
414 and *Janthinobacterium lividum*, were shown to display resistance to Zn, *Bacillus* sp.
415 was shown to be resistant to Cu, while *Variovorax* sp. were shown to be resistant to
416 silver (Ag), Zn and Cu (Piotrowska-Seget et al. 2005, Kuffner et al. 2008). In the
417 present study, isolates, such as *Comamonas testosteroni* WDL7 showed resistance to
418 Cu, *Kocuria kristinae* strain 6J-5b showed resistance to Mn, and Bacillus sp. ZH6,
419 *Stenotrophomonas maltophilia* strain 776 and *Staphylococcus* sp. MOLA:313 all
420 showed resistance to Al, Fe and Zn (Table 4.4).

421 Piotrowska-Seget et al. (2005) studied metal-tolerant bacteria occurring in heavily
422 polluted soil and mine spoil in Katowice, Poland. The authors performed minimum
423 inhibitory concentration studies to determine the resistance mechanisms of the
424 different bacterial isolates. They found that *Pseudomonas gladioli*, was resistant to Zn
425 and Cu concentrations of 10 mM and 5 mM, respectively. In the present study, the
426 isolate, *Pseudomonas beteli* str. RRLJSMAR, showed resistance to Cu concentrations.
427 Another study conducted by Chien et al. (2007) evaluated the bacterial diversity in soil
428 in order to determine bacterial response to media amended with Cd, Cr, Ni, Zn, Pb,
429 and Cu. *Stenotrophomonas* sp., isolated from a site contaminated with high
430 concentrations of Cd (3 $mg.kg^{-1}$) and Cr (115 $mg.kg^{-1}$), was able to grow on media
431 containing Cd concentrations of up to 4 mM, and was also able to remove up to 80%
432 dissolved ions upon completion of the stationary growth phase. In addition it was also
433 able to resist other metals, such as, Cu, Cr, Ni, Pb and Zn at levels of more than 2 mM
434 (Chien et al. 2007).

Three *Bacillus* isolates (*B. cereus, B. megaterium and B. sphaericus*), recovered from a uranium waste pile in Germany, were evaluated for their ability to accumulate metals, including Al, Cd, Cu, Fe, Mn, Ni, Pb, and Zn, amongst others. Results revealed that the *Bacillus* sp. were able to accumulate large amounts of Pb, Cd, Cu, Al, Mn, Ni and Zn (Selenska-Pobell et al. 2006).

Yilmaz (2003) showed that *Bacillus circulans* could tolerate high concentrations of Cu, Mn, Ni, Zn, Co and Cd, and that the increased metal resistance resulted in a decrease in bacterial growth. *Pseudomonas* sp. was shown to be resistant to Cu, Cd, Cr, Pb, Ni and vanadium (V) (Muraleedharan et al. 1991, Raja et al. 2006, Shirdam 2006). The present study showed that different micro-organisms with the ability to resist varying concentrations of metals were isolated from the treated flow cells following metal exposure. Isolates, such as *Pseudomonas* sp., *Bacillus* sp. and *Staphylococcus* sp., were shown to be tolerant to a wider range of metals (Cu, Mn, Ni, Zn, Al and Fe), while others, such as *Comamonas testosteroni* WDL7 and *Kocuria kristinae* strain 6J-5b showed resistance to only Cu and Mn, respectively. These organisms can thus potentially be employed for future use in remediation processes.

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Figure 4.9. Agarose gel electrophoresis photograph of the PCR products obtained with 16SrRNA universal forward and reverse primers (fDD2 and rPP2) of organisms isolated from flow cells after exposure to varying metal concentrations. Lane one represents the marker (lambda DNA/HindIII), lane two, the negative control and lanes three to 20, represent the selected isolates showing the 1200 bp amplicons.

Figure 4.10. An unrooted phylogenetic tree of organisms isolated from flow cells after exposure to varying metal concentrations. The tree of 13 isolates was constructed using the Neighbour-joining algorithm of Clustal X. Bootstrap values are constructed using the shown at the nodes.

Table 4.1 Representation of the different concentrations of metals (Al, Cu, Fe, Mn, Ni and Zn) to which the six respective flow cells channels were exposed.

Metals	Flow cell channels one and two	Flow cell channels three and four	Flow cell channels five and six	Flow cell channels seven and eight
Al	Untreated control	900 $mg.l^{-1}$	500 $mg.l^{-1}$	10 $mg.l^{-1}$
Cu	Untreated control	10 $mg.l^{-1}$	2.5 $mg.l^{-1}$	0.5 $mg.l^{-1}$
Fe	Untreated control	1000 $mg.l^{-1}$	500 $mg.l^{-1}$	10 $mg.l^{-1}$
Mn	Untreated control	80 $mg.l^{-1}$	15 $mg.l^{-1}$	1.5 $mg.l^{-1}$
Ni	Untreated control	20 $mg.l^{-1}$	1 $mg.l^{-1}$	0.5 $mg.l^{-1}$
Zn	Untreated control	40 $mg.l^{-1}$	1 $mg.l^{-1}$	0.5 $mg.l^{-1}$

Table 4.2 Metal concentrations obtained in water of the Plankenburg River compared to recommended safe concentrations as stipulated by DWAF (1996) and the CCME (2001).

Metal	Recommended safe concentrations as stipulated by DWAF (1996) ($mg.l^{-1}$)	Environmental quality guidelines as stipulated by CCME (2001) ($mg.l^{-1}$)	Mean meal concentrations obtained in water ($mg.l^{-1}$) (Plankenburg River)
Al	0.1 – 0.15	0.005 – 0.1	11.56
Cu	0.002 – 0.012	0.002 – 0.004	0.06
Fe	N/A	0.3	12.2
Mn	1.3	N/A	0.29
Ni	N/A	0.025 – 0.15	0.17
Pb	N/A	0.001 – 0.007	0
Zn	0.036	0.03	0.4

744 **Table 4.3** Table of 13 isolates, their names presented on the phylogenetic tree and
745 accession numbers.
746

Name presented on tree	Organism	Accession #
12A 10 *Pseudomonas* sp.	*Pseudomonas* sp. 12A_10	gb|AY689075.1|
TC222 *P. fluorescens* isolate	*Pseudomonas fluorescens* isolate TC222	dbj|AB238774.1|
12A *Pseudomonas* sp.	*Pseudomonas* sp. 12A	gb|AY689075.1|
A90 *D. tsuruhatensis* st	*Delftia tsuruhatensis* strain A90	gb|EF421404.1|
RRLJSMAR *P. beteli* st	*Pseudomonas beteli* strain RRLJ SMAR	gb|DQ299947.1|
776 *S. maltophilia* st	*Stenotrophomonas maltophilia* strain 776	gb|EU430096.1|
8b-1 *Sphingomonas* sp.	*Sphingomonas* sp. 8b-1	gb|DQ378211.1|
WDL7 *C. testosteroni*	*Comamonas testosteroni* WDL7	AF538929
ZH6 *Bacillus* sp.	*Bacillus* sp. ZH6	gb|EU236752.1|
MOLA 313 *Staphylococcus* sp.	*Staphylococcus* sp. MOLA:313	emb|AM945546.1|
PAO-12 *Microbacterium* sp.	*Microbacterium* sp. PAO-12	gb|EF514877.1|
TPR1 *Micrococcus* sp.	*Micrococcus* sp. TPR14	gb|EU373424.1|
6J-5b *K. kristinae* st	*Kocuria kristinae* strain 6J-5b	gb|EU379300.1|

747
748 **Table 4.4** Isolated organisms and the metals to which they were exposed.
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Microorganisms	Metals
Comamonas testosteroni WDL7	Cu
Microbacterium sp. PAO-12	Ni
Sphingomonas sp. 8b-1	Zn
Kocuria kristinae strain 6J-5b	Mn
Micrococcus sp. TPR14	Mn
Pseudomonas beteli strain RRLJ SMAR, *Pseudomonas* sp. 12A, *Pseudomonas fluorescens* isolate TC222, *Pseudomonas* sp. 12A_10	Cu, Ni
Delftia tsuruhatensis strain A90	Ni, Cu
Bacillus sp. ZH6	Zn, Ni, Al, Fe
Staphylococcus sp. MOLA:313	Zn, Mn, Al, Fe
Stenotrophomonas maltophilia strain 776	Zn, Ni, Cu, Al, Fe

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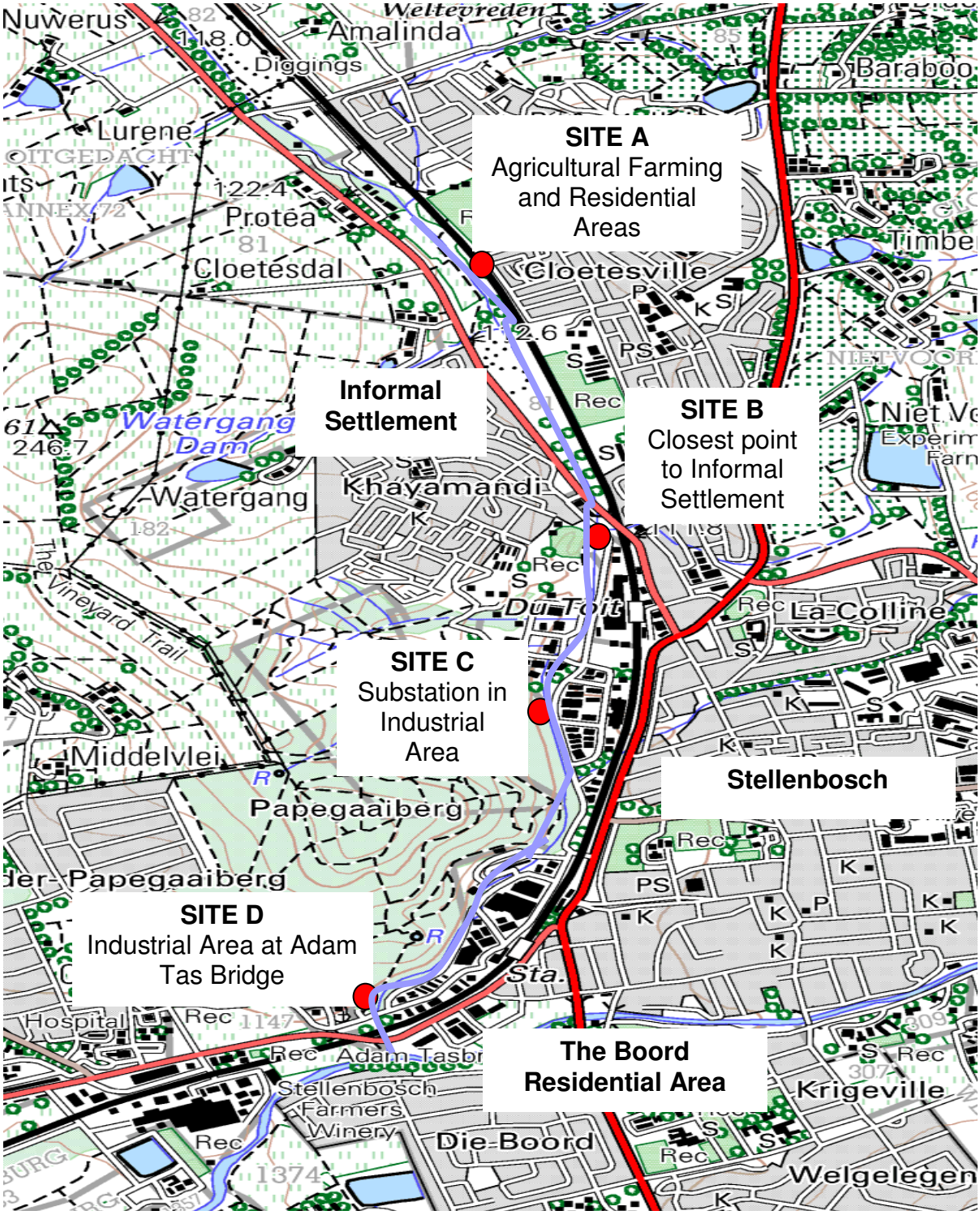


Figure 4.1. Map of the Plankenburg River indicating the different sampling points: Site A – Agricultural Farming and Residential Areas; Site B – Closest point to Informal Settlement; Site C – Substation in Industrial Area and Site D – Industrial Area at Adam Tas Bridge.

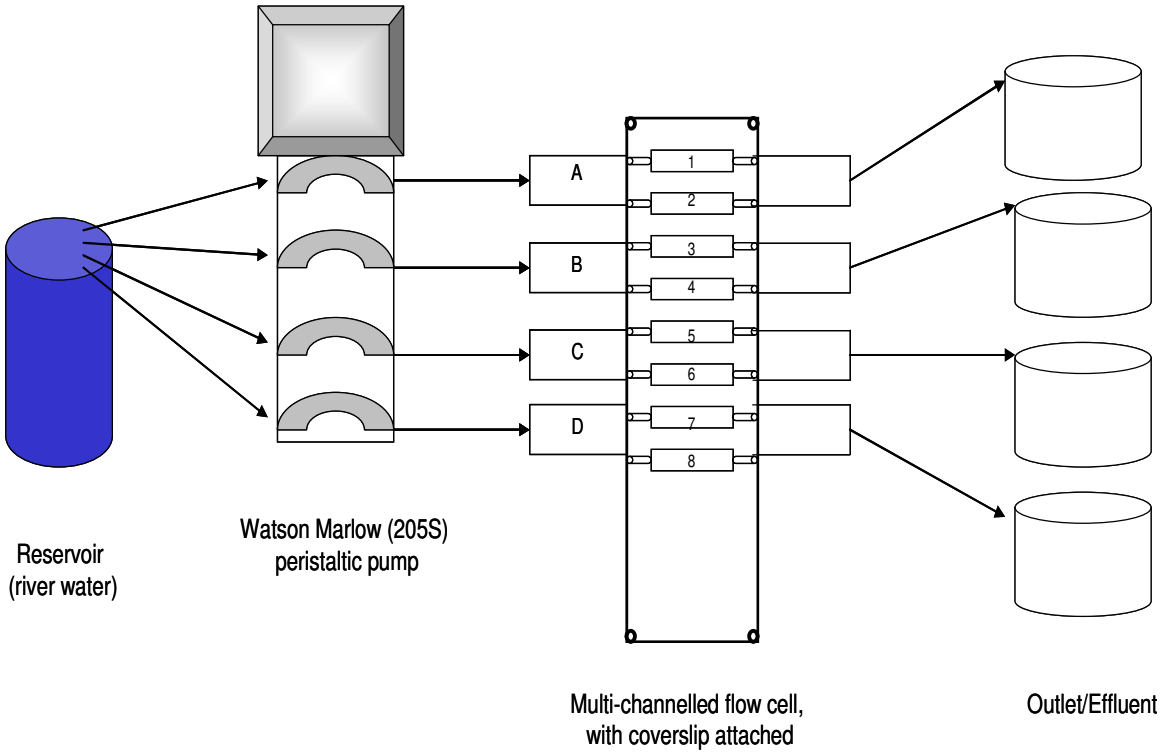


Figure 4.2. Multi-channel flow cell system to isolate metal-tolerant organisms. A – untreated control (channels 1 and 2), B – highest concentration (channels 3 and 4), C – second lowest concentration (channels 5 and 6) and D – lowest concentration (channels 7 and 8).

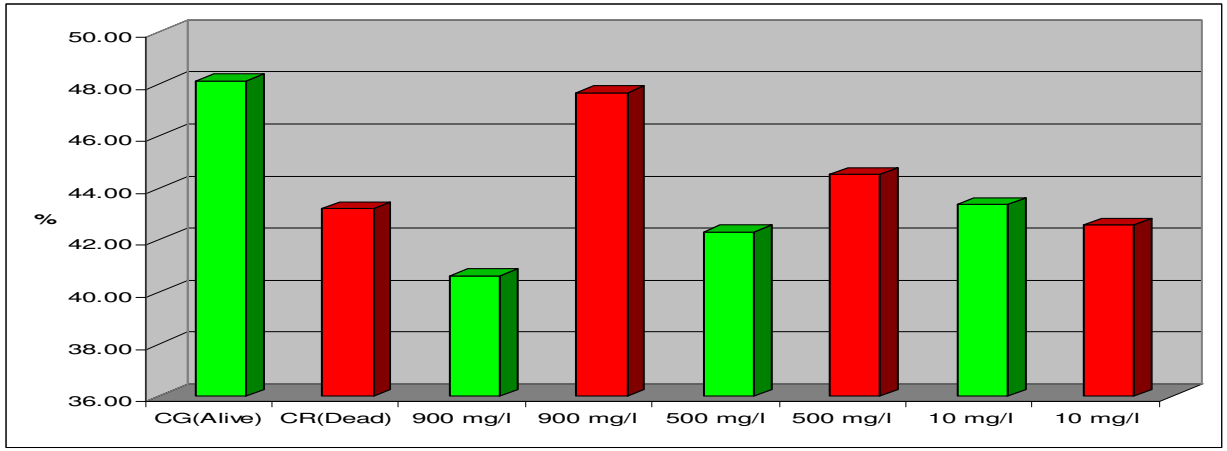


Figure 4.3A. Represents the percentages of living and dead organisms in response to exposure to various Al concentrations.

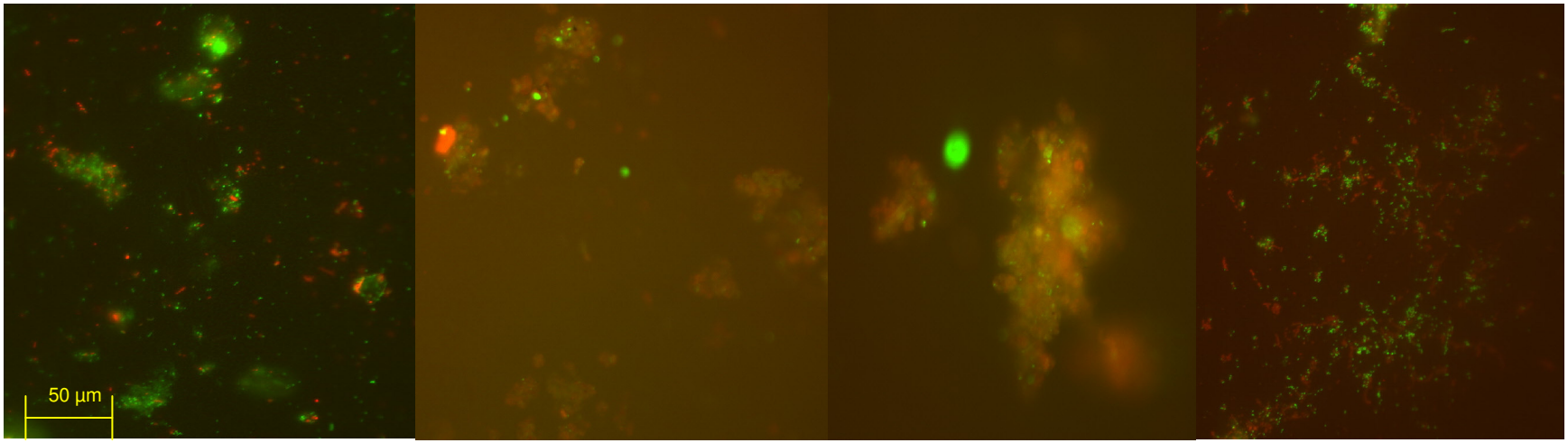


Figure 4.3B. Epifluorescent images of biofilm exposed to (i) Control, (ii) 900 mg.l^{-1}Al, (iii) 500 mg.l^{-1} Al and (iv) 10 mg.l^{-1} Al.

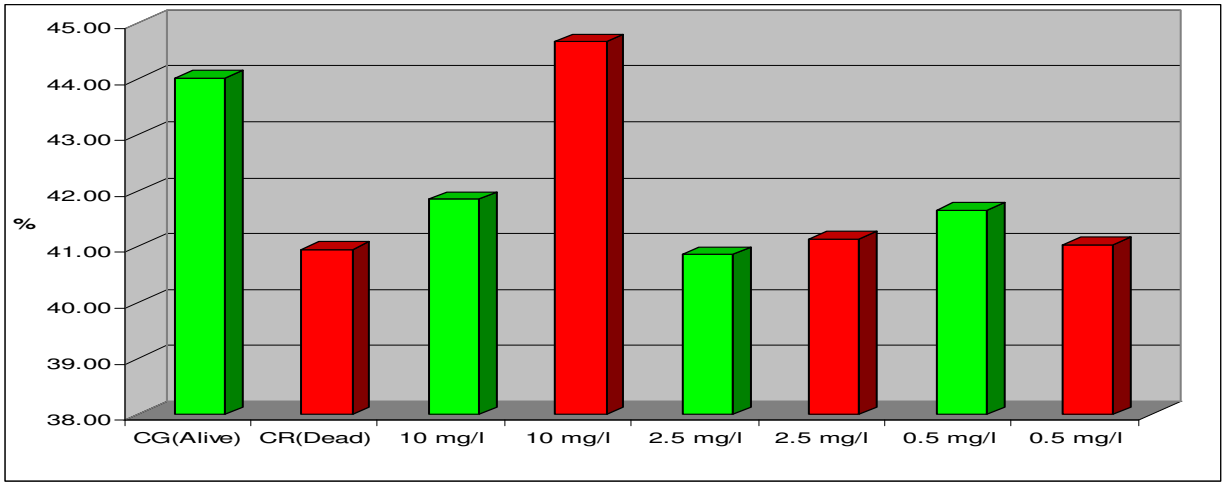
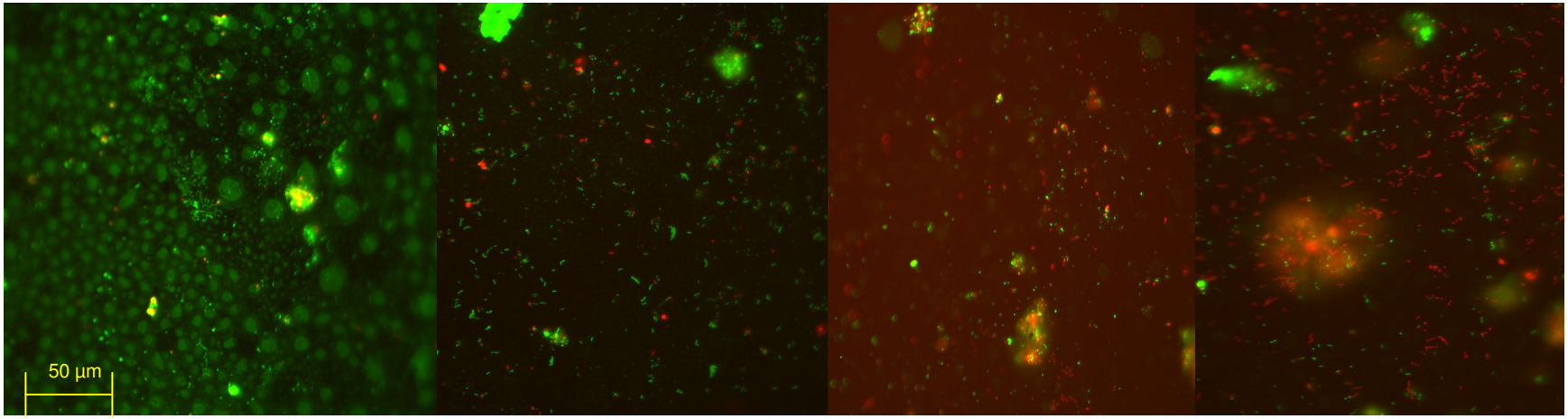


Figure 4.4A. Represents the percentages of living and dead organisms in response to exposure to various Cu concentrations.



(i) (ii) (iii) (iv)

Figure 4.4B. Epifluorescent images of biofilm exposed to (i) Control, (ii) 10 mg.l^{-1} Cu, (iii) 2.5 mg.l^{-1} Cu and (iv) 0.5 mg.l^{-1} Cu.

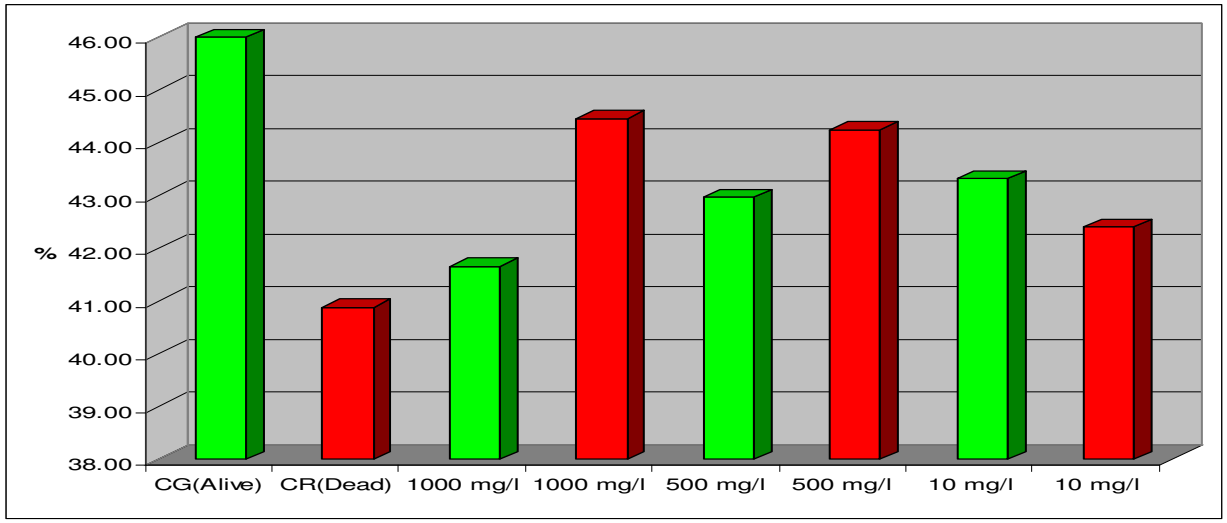
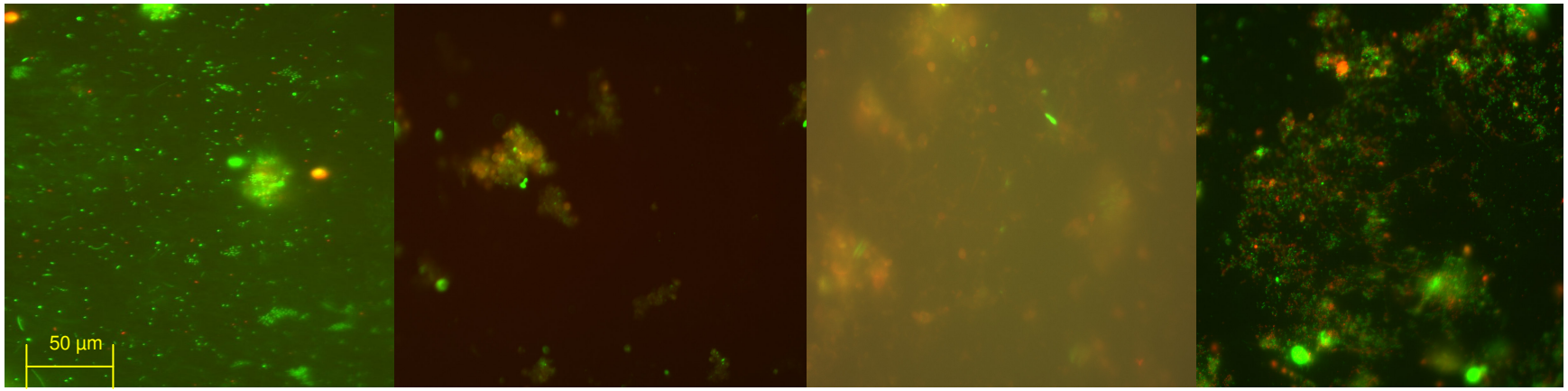


Figure 4.5A. Represents the percentages of living and dead organisms in response to exposure to various Fe concentrations.



(i) (ii) (iii) (iv)

Figure 4.5B. Epifluorescent images of biofilm exposed to (i) Control, (ii) 1000 mg.l^{-1} Fe, (iii) 500 mg.l^{-1} Fe and (iv) 10 mg.l^{-1} Fe.

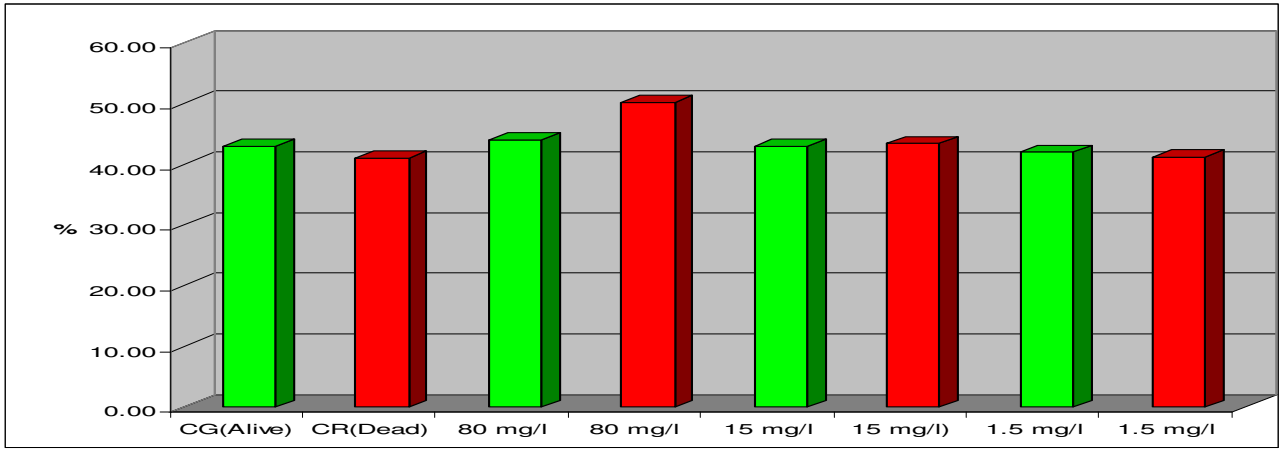
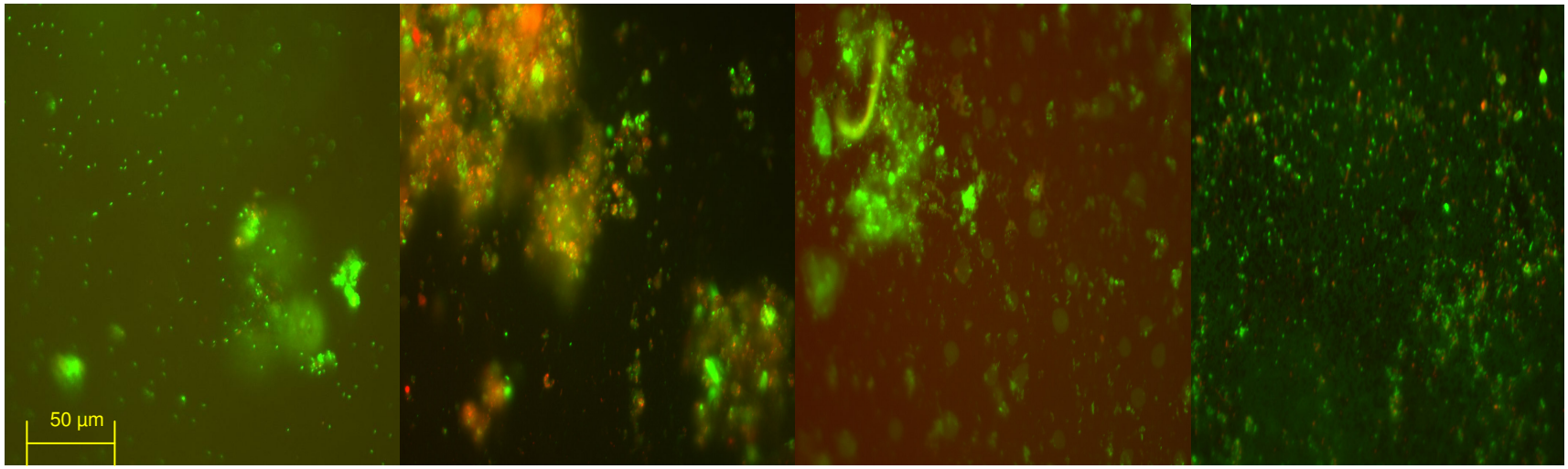


Figure 4.6A. Represents the percentages of living and dead organisms in response to exposure to various Mn concentrations.



(i) (ii) (iii) (iv)

Figure 4.6B. Epifluorescent images of biofilm exposed to (i) Control, (ii) 80 mg.l^{-1} Mn, (iii) 15 mg.l^{-1} Mn and (iv) 1.5 mg.l^{-1} Mn.

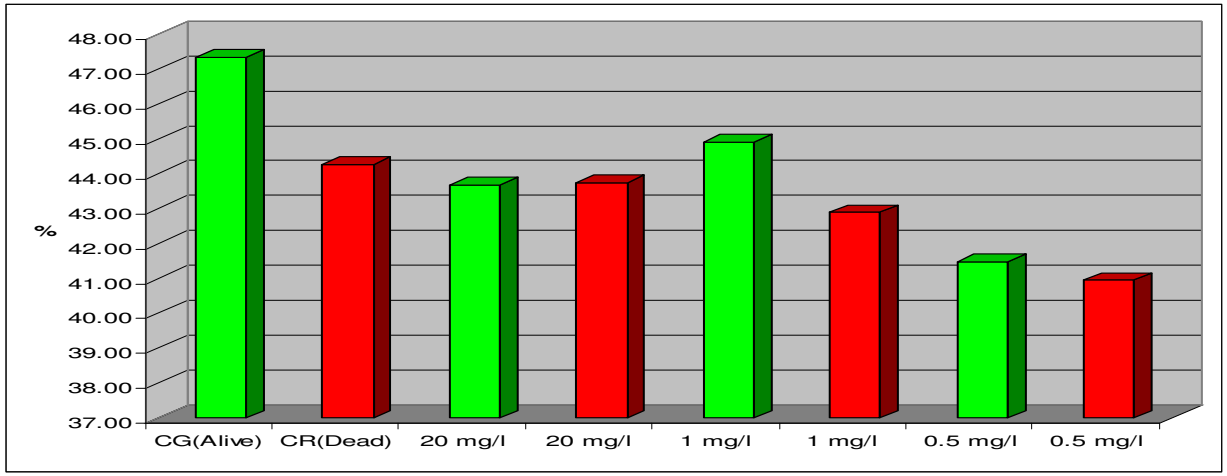
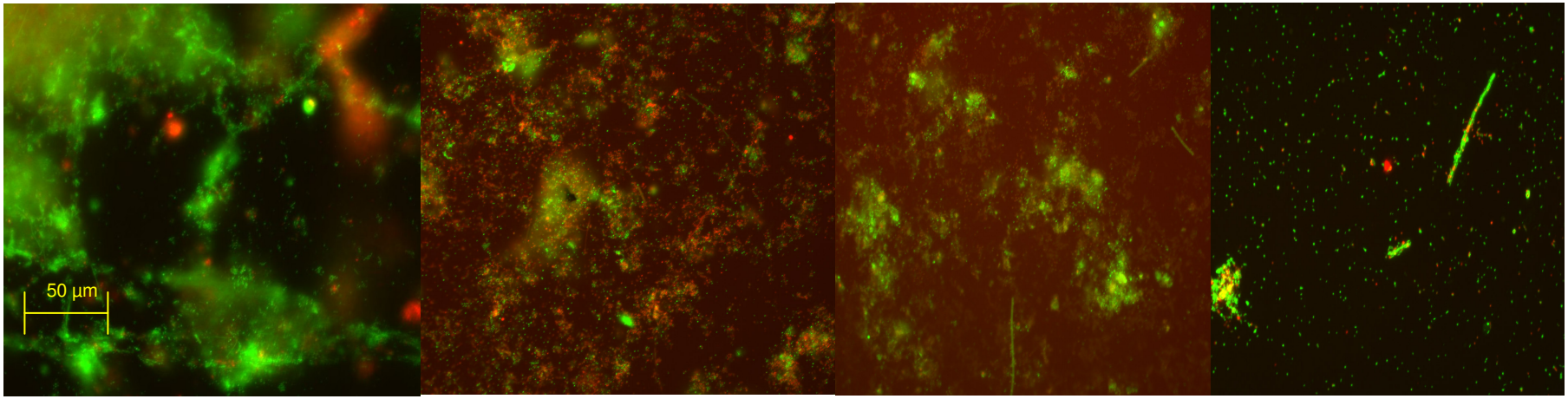


Figure 4.7A. Represents the percentages of living and dead organisms in response to exposure to various Ni concentrations.



(i) (ii) (iii) (iv)

Figure 4.7B. Epifluorescent images of biofilm exposed to (i) Control, (ii) 20 mg.l^{-1} Ni, (iii) 1 mg.l^{-1} Ni and (iv) 0.5 mg.l^{-1} Ni.

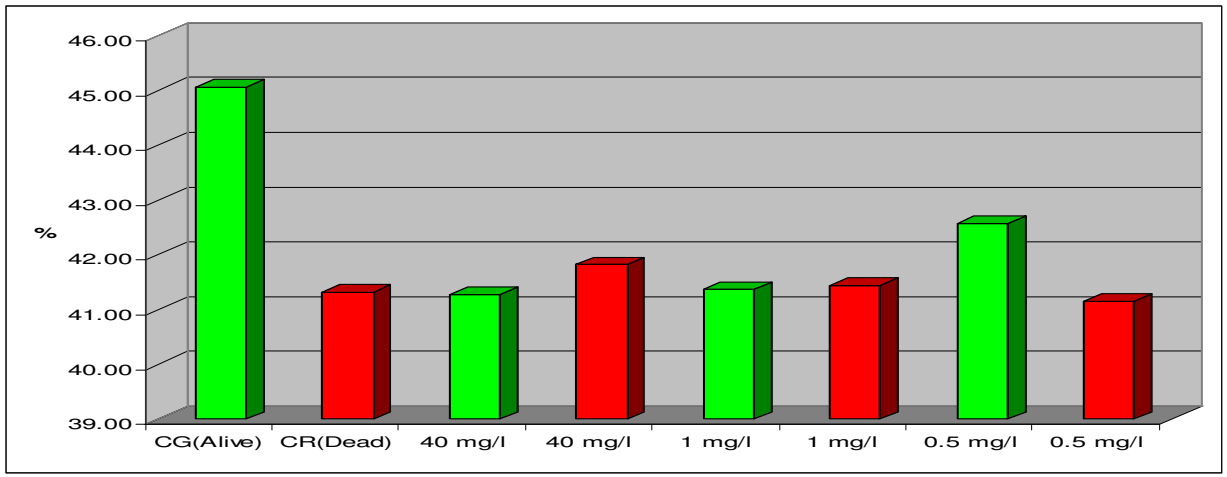
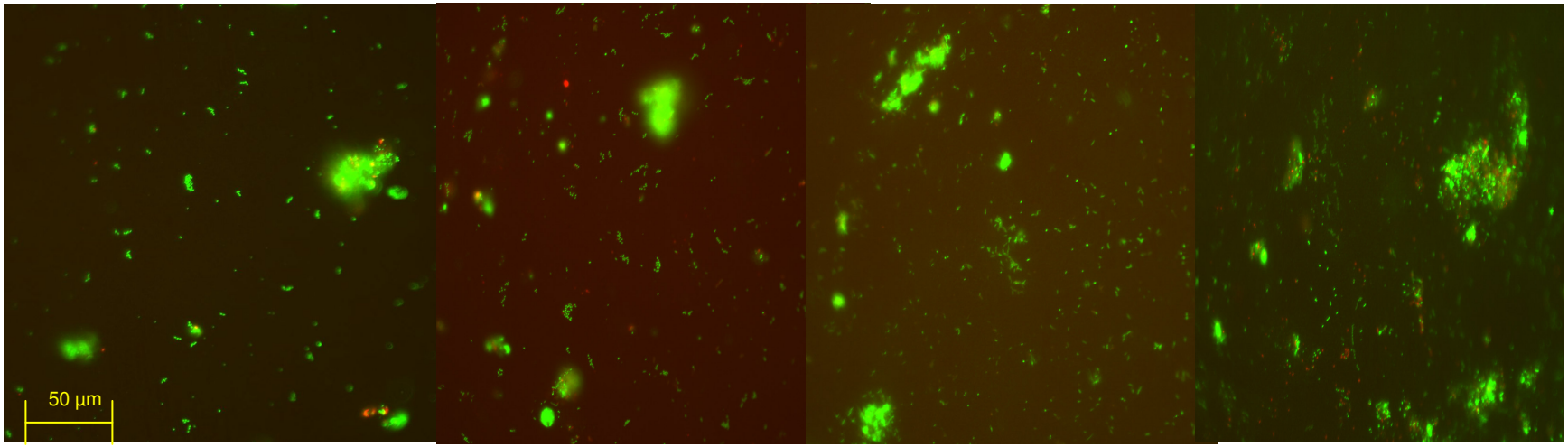


Figure 4.8A. Represents the percentages of living and dead organisms in response to exposure to various Zn concentrations.



(i) (ii) (iii) (iv)

Figure 4.8B. Epifluorescent images of biofilm exposed to (i) Control, (ii) 40 mg.l^{-1} Zn, (iii) 1 mg.l^{-1} Zn and (iv) .05 mg.l^{-1} Zn.

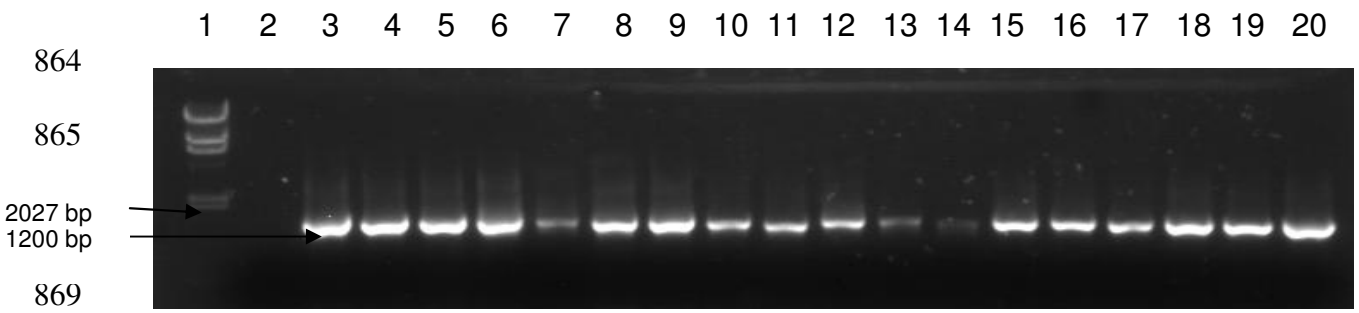


Figure 4.9. Agarose gel electrophoresis photograph of the PCR products obtained with 16SrRNA universal forward and reverse primers (fDD2 and rPP2) of organisms isolated from flow cells after exposure to varying metal concentrations. Lane one represents the marker (lambda DNA/HindIII), lane two, the negative control and lanes three to 20, represent the selected isolates showing the 1200 bp amplicons.

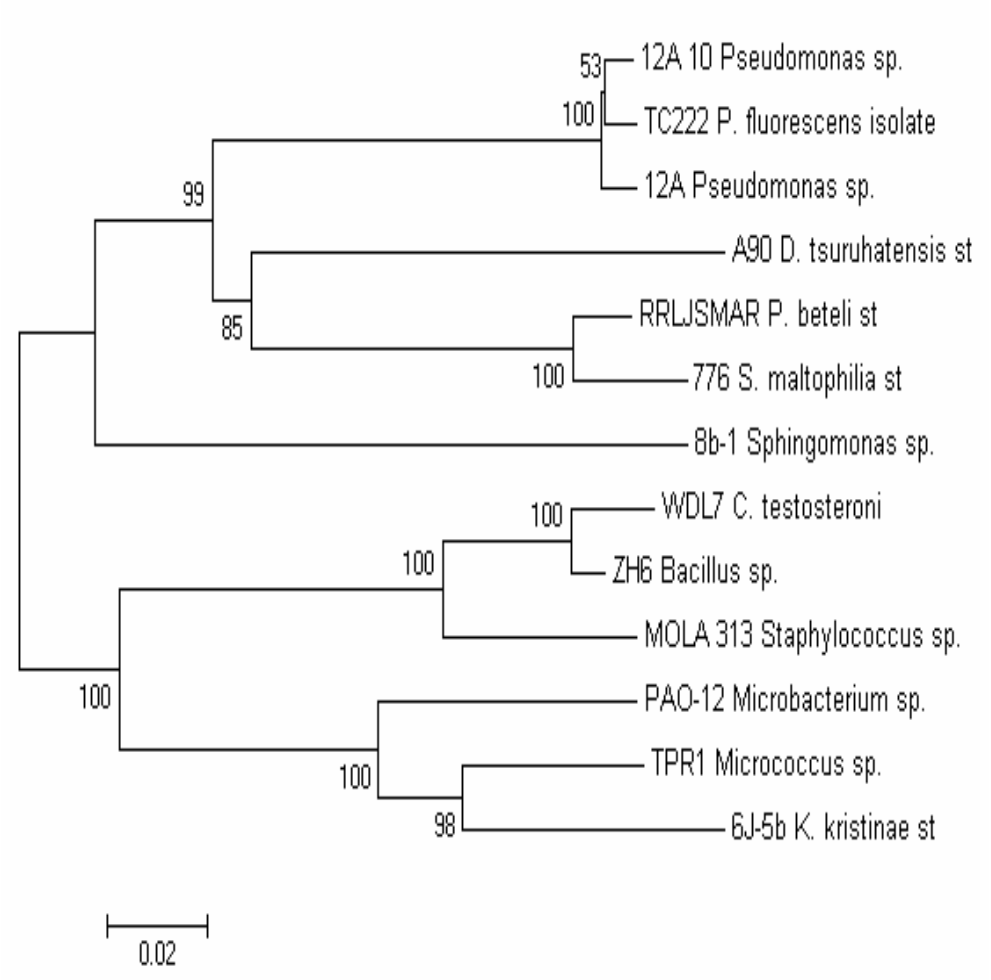


Figure 4.10. An unrooted phylogenetic tree of organisms isolated from flow cells after exposure to varying metal concentrations. The tree of 13 isolates was Neighbour-joining algorithm of Clustal X. Bootstrap values are constructed using the shown at the nodes.

Bioremediation of Metal Contamination in the Plankenburg River, Western Cape, South Africa

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As most of South Africa's water resources are stored in dams, rivers and abstraction schemes, the bioremediation of river water could serve as a possible alternative to the growing dilemma of water shortages. Two laboratory-scale and one on-site bioreactor system was developed to decrease the metal concentrations in the river water. The final concentrations for Al, Ni and Zn (bioreactor one) and Mn (bioreactor two), decreased to below their recommended concentrations in water samples, as stipulated by the Department of Water Affairs and Forestry (DWAF) and the Canadian Council for the Ministers of the Environment (CCME). The metal concentrations recorded in biofilm suspensions removed from bioreactor two and the on-site bioreactor, revealed concentrations higher than those recorded in the corresponding water samples, except for Fe. The metal-tolerant organisms isolated from the bioballs collected from laboratory-scale bioreactor two (*Bacillus*, *Pseudomonas*, *Micrococcus* and *Stenotrophomonas*, amongst others), could possibly be utilised for bioremediation purposes. The bioreactor system will however be optimised to improve its efficiency.

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Abstract

Three bioreactors (two laboratory-scale and one on-site) were evaluated for their efficiency to reduce metal concentrations in water collected from the Plankenburg River, South Africa. Water (bioreactors one, two and on-site) and bioballs (bioreactors two and on-site) collected throughout the study periods, were digested and analysed using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Aluminium (Al), nickel (Ni), and zinc (Zn) concentrations decreased from 0.41 $mg.l^{-1}$ to 0.06 $mg.l^{-1}$ (85%), 0.2 $mg.l^{-1}$ to 0.07 $mg.l^{-1}$ (65%) and 75 $mg.l^{-1}$ to 0.02 $mg.l^{-1}$ (97%), respectively (bioreactor one). Aluminium [(1.55 $mg.l^{-1}$ to 0.38 $mg.l^{-1}$ (75%)], copper (Cu) [57% (from 0.33 $mg.l^{-1}$ to 0.14 $mg.l^{-1}$)], iron (Fe) [71.99 $mg.l^{-1}$ to 40.4 $mg.l^{-1}$ (44%)] and manganese (Mn) [57% (0.07 $mg.l^{-1}$ to 0.03 $mg.l^{-1}$)] concentrations also decreased in the water samples from bioreactor two. In the on-site, six-tank bioreactor system, concentrations for Fe, Cu, Mn and Ni decreased, while Zn and Al concentrations increased. The concentrations recorded in biofilm samples were higher than the corresponding water samples. The bioballs employed in the bioreactor were thus shown to be efficient attachment surfaces for biofilm development and subsequent metal accumulation. Potentially metal-tolerant organisms (*Pseudomonas* sp., *Sphingomonas* sp., and *Bacillus* sp.) were also identified using phylogeny.

Keywords: bioreactor, Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), metals, phylogeny, river water

1. Introduction

The quality and quantity of the essential element water, is important for the continued sustenance of not only the world's human population, but also for its application in industrial and agricultural sectors, amongst others. In South Africa, water resources are generally collected in dams or water abstraction schemes. These water sources are then primarily used for agricultural activities, industry, mining and power generation, domestic and municipal uses, with 15% of the available water resources required to maintain estuaries and rivers (Langwaldt and Puhakka, 2000).

Pollution, by metals and microbes (Pegram et al., 1999), amongst others, greatly influences the quality of the water sources, and leads to the continued search for new and improved methods to not only clean up contaminated systems, but also to achieve this aim in an environmentally friendly and cost-effective manner.

The ubiquitous nature of biofilms allows these viable and metabolically active microorganisms (Ehrlich, 1998) to survive and proliferate in a variety of different environments, due to its protective polysaccharide coating. Biofilms have a high metal-binding capacity as toxicants are absorbed by cell surface polymers, or extracellular polymeric substances (EPS), which have been shown to be responsible for the interaction of toxicants with the biofilm community (Henriques and Love, 2007). Biofilms are thus applied in the effective remediation or removal of pollutants such as metals, from contaminated areas (Roane and Pepper, 2000).

Bioremediation is a process by which microbial degradation processes are used in technical and controlled treatment systems (Langwaldt and Puhakka, 2000). Bioremediation can also be applied as green technologies, due to its negligible effects on the environment, and its proven cost-efficiency (Adriaens et al., 2006). Bioreactors, which can be applied in bioremediation strategies, are basically tanks in which living organisms carry out biological reactions. Their efficiency is based on the ability of bacteria to attach to inert packing, such as granular activated carbon, at interfaces to generate high biomass (Bouwer and McCarty, 1982; Teitzel and Parsek, 2003). The reactor should also be easy to maintain and operate (Evangelho et al., 2001; Teitzel and Parsek, 2003), and should be able to function under aerobic and anaerobic conditions (Langwaldt and Puhakka, 2000).

Bioreactors have been utilised in many studies to remove or reduce metal concentrations in wastewater and various types of effluent. A mixture of sewage and synthetic gold milling effluent was treated using a trickling filter bioreactor (Evangelho et al., 2001). More than 90% of the free cyanide, thiocyanate, copper (Cu) and zinc (Zn) was removed post-treatment, after analysis with nitric acid digestion and Atomic Absorption Spectrometry (AAS). A rotating-disk biofilm reactor was also used to determine the heavy metal toxicities for biofilms and planktonic cells after exposure to metal concentrations of 0.015 mM to 225 mM Cu, lead (Pb) and Zn, respectively (Teitzel and Parsek, 2003). When comparing the results obtained for resistance of planktonic to biofilm cells, the authors found that the biofilm cells exhibited a two-fold and 600-fold increase in resistance to Pb and Cu, respectively.

The structure and distribution of microorganisms in the contaminated area, as well as possible tolerance, is dependant on the types of pollutants in the specific areas (Marín-Guirao et al., 2005). Organisms that have been isolated from

contaminated sites can also be identified and used in bioreactor systems to improve the removal efficiency of the contaminants.

Amann et al. (1995) showed that specific microorganisms can be identified genetically through the amplification of the 16S or 23S rRNA region of the genomic DNA, using primers to identify the organisms. Toes et al. (2008) investigated the effects of heavy metal pollution (Cd and Cu) on the microbial diversity in muddy- and sandy sediments, using Denaturing Gradient Gel Electrophoresis (DGGE) profiles of bacterial 16S rRNA genes and phylogenetic analyses. Phylogenetic trees showed an abundance of members of the *Flavobacteriaceae* and the a- and c-*Proteobacteria* in the sediments.

The objective of this investigation was to assess the efficiency of a bioreactor system to remove, or decrease the concentrations of metal contaminants at a site along the Plankenburg River, Western Cape, South Africa. Potential metal-tolerant microorganisms were also isolated from the attachment material.

2. Materials and methods

2.1. Site description

A previous study identified four sampling sites along the Plankenburg River, Stellenbosch, South Africa (Fig. 5.1) (Jackson et al., 2008a). These sites included Site A (Agricultural Farming and Residential Areas); Site B (Closest point to Informal Settlement); Site C (Substation in Industrial Area) and Site D (Industrial Area at Adam Tas Bridge). Results from this study showed that the highest concentrations of metals were recorded at Site C (Substation in Industrial Area), which explains why the particular site was selected to investigate the efficiency of the bioreactor systems to reduce metal concentrations in the river water.

2.2. Laboratory-scale bioreactor set up

River water (200 L) was collected from Site C at the Plankenburg River (preliminary results identified this site as a source of metal pollution). Laboratory-scale bioremediation systems were then evaluated to reduce the concentrations of metals in water collected from this site (Jackson et al., 2008a). One bioreactor system was established in the laboratory (two-week period) to minimise the influence of environmental factors. The running time for a second bioreactor was extended to three weeks and it was moved outside to determine the effect of environmental factors on the bioremediation system. The river water was fed through the horizontal bioreactors (35 cm x 30 cm x 100 cm) (Fig. 5.2A) at a flow rate of 1000 L/h, using an Ecopool 6 pump, at a retention time of three minutes. Each of the three compartments of the bioreactor was filled with Bioballs™ (Fig. 5.2B), which is composed of acrylonitrile, butadiene and styrene (ABS). These Bioballs™ serve as attachment material for microbial organisms to adhere to. Each Bioball™ has a surface area of 20 cm^3 and were compactly packed in the different sections of the reactor.

2.3. Sampling of laboratory-scale bioreactors

Sixty millilitres of water was collected in two 50 ml Greiner bio-one tubes (Cellstar®) on a daily basis from the effluent line (Fig. 5.2). Three Bioballs™, were also collected, from each of the three different compartments from reactor two, and stored in sterile whirl-pack bags. The collected samples were stored at 4°C until further use.

2.4. On-site bioreactor

A large-scale on-site reactor was then established at Site C along the Plankenburg River (Fig. 5.3). The system consisted of six 500 L tanks stacked next to each other, with river water flowing from one tank to another along a gradient. The river water was pumped at a flow rate of 1000 L/h and a retention time of 120 minutes. Each of the six tanks were compactly packed with Bioballs™ to maximise the biofilm attachment area (Fig. 5.2B). The bioreactor is still in operation, however results for the first two months only, are presented.

2.5. Sampling of on-site bioreactor

Sixty millilitres of water was collected in two 50 ml Greiner bio-one tubes (Cellstar®), from the effluent line twice a week. Six Bioballs™ from each of the six tanks were also collected and stored in sterile whirl-pack bags. The collected samples were stored at 4°C during transport and until further use.

2.6. Sonication of collected biofilm samples

Bacterial growth was removed from the representative bioballs collected from each container, by sonication. Collected material samples (~100 g) were sonicated for 10 min in 30 ml sterile water in a Branson 5510 sonication bath (Bransonic® Ultrasonic Cleaner). The procedure was repeated at least twice, with fresh sterile d.H_2O added after each sonication step. The sonicated samples were combined resulting in a total of 60 ml bacterial suspension. The biofilm suspension obtained was used for further analysis.

2.7. Metal concentrations in water and biofilm samples

To determine the concentrations of Al, Zn, Cu, Fe, Pb, Ni and Mn in water (5 ml) and biofilm (5 ml) samples were digested with 10 ml 55% nitric acid at 40°C for 60 minutes and then at 120°C for 180 minutes, using a Grant dry-block heater. A blank (control) of 10 ml 55% nitric acid was analysed along with the collected samples to check for possible contamination. The samples were cooled to room temperature, filtered with Whatman No. 6 filter paper into 20 ml volumetric flasks, made up to a volume of 20 ml with distilled water and subsequently filtered for a second time using 0.45 µm cellulose nitrate ultrafiltration membrane filters (Whatman). Metal concentrations were determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) analysis according to the procedure outlined in Saleh et al. (2000).

2.8. Statistical analysis

Repeated measures ANOVA (RMA) was performed on all data obtained as outlined in (Dunn and Clark, 1987), using Statistica™. In each RMA, the residuals were analysed to determine if they were normally distributed. In all hypothesis tests, a significant level of 5% was used as standards. The results presented are the averages of five repeats for each particular sampling point at the different sampling sites. For statistical analysis, the volumes (5 ml) were taken into consideration for the calculation of the final metal concentrations in a given sample.

2.9. DNA extraction and Agarose Gel Electrophoresis

One hundred microlitres of the sonicated biofilm samples were spread plated onto nutrient agar plates and incubated at 37°C for two to three days, to isolate pure bacterial cultures. This procedure was performed in duplicate. Single colonies were then selected on the basis of their morphological differences and DNA was extracted using the High pure PCR template preparation kit as per manufacturer's instructions (Roche Diagnostics). Ten microlitres of the extracted DNA samples were electrophoretically analysed on a 0.8% molecular grade agarose gel containing 12 µl of 0.5 µg/ml ethidiumbromide (EtBr), using 1 x Tris-acetate-ethylenediamine tetraacetic acid (TAE) electrophoresis buffer at 90 volts for one hour.

2.10. Polymerase Chain Reaction (PCR)

Amplification of target DNA by PCR was performed in a total reaction volume of 50 µl containing 10 mM dNTP Mix (1 µl), 25 mM $MgCl_2$ (3 µl), 10X Taq Buffer with $(NH_4)_2SO_4$ (5 µl), 10 µM forward (fDD2 - CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG) (5 µl), 10 µM reverse (rPP2 - CCAAGCTTCTAGACGGITACCTTGTTACGACTT) (5 µl) (Rawlings, 1995), Taq DNA polymerase (1 µl) (5u/5 µl) (Fermentas Life Sciences, EU), 1 µl of a concentrated DNA sample and 29 µl sterile distilled. The amplification process included an initial denaturation step of 94°C for 2 minutes, followed by 30 cycles of amplification (1 minute at 94°C, one minute at 57°C and two minutes at 72°C). This was followed by a final extension step of 72°C for 10 minutes. Ten microlitres of the subsequent PCR amplicons were then electrophoretically analysed on a 0.8% molecular grade agarose gel containing 12 µl of 0.5 µg/ml EtBr, using 1 x TAE electrophoresis buffer at 100 volts for one hour, to determine whether amplification was successful.

2.11. Sequencing of 16S rRNA

The amplified PCR products (1200 bp) were purified using a High Pure PCR product purification kit, as per manufacturer's instructions (Roche Diagnostics). The concentrations of the DNA samples were determined using spectrophotometry and 15 µl of concentrated DNA (50 to 100 ng/µl) samples were loaded onto 96-well microtitre plates, dried in a speedy vac, with medium heat for 30 to 60 minutes (depending on the volumes), and sent for sequencing. The sequencing lab used the Applied Biosystems Big Dye Terminator v3.1.

2.12.Phylogenetic analysis

The resultant sequences were identified with a similarity search using Blastn from the National Centre for Biotechnology Information (NCBI) (Altschul et al., 1997). Contiguous sequences were formed for the forward and reverse sequences of a particular organism, using the CAP3 Sequence Assembly Programme (Huang and Madan, 1999). The contiguous sequences were aligned with Clustal X (1.81) (Higgins and Sharpe, 1988) using default parameters and the BLOSUM matrix, which corrects for multiple base changes. The 20 isolates (Fig. 5.8) and 45 isolates (Fig. 5.9) were representative of the organisms isolated overall. An unrooted tree was constructed using the neighbour-joining program of Saitou and Nei (1987). Phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis, Version 3.1 (MEGA version 3.1) (Kumar et al., 2004). To estimate the node reliability, bootstrap values were obtained from 1000 randomly generated trees. Trees were visualised using MEGA version 3.1 (Kumar et al., 2004).

3. Results and Discussion

3.1. Laboratory-scale Bioreactors

Figures 5.4 (bioreactor one), 5.5 (bioreactor two) and 5.6 (on-site bioreactor) represent the mean metal concentrations for Al, Cu, Fe, Mn, Ni and Zn recorded over the respective study periods.

3.2. Laboratory-scale bioreactor one

In laboratory-scale bioreactor one, the final concentrations ($mg.l^{-1}$) on day 15 for Al, Ni and Zn were lower ($p < 0.05$) than the initial concentrations recorded (Fig. 5.4). Concentrations for Al decreased, from 0.41 $mg.l^{-1}$ to 0.06 $mg.l^{-1}$ (85%). The post-treatment Al concentration of 0.06 $mg.l^{-1}$ was lower than the recommended concentrations of 0.1 $mg.l^{-1}$ to 0.15 $mg.l^{-1}$ (DWAF, 1996) and 0.005 $mg.l^{-1}$ to 0.1 $mg.l^{-1}$ (CCME, 2001). The recorded concentrations for Ni, decreased from 0.2 $mg.l^{-1}$ to 0.07 $mg.l^{-1}$ (65%), and also fell within the recommended concentrations of 0.025 $mg.l^{-1}$ to 0.15 $mg.l^{-1}$ (CCME, 2001). Zinc concentrations decreased from 0.75 $mg.l^{-1}$ to 0.02 $mg.l^{-1}$ (97%), which was lower than the recommended concentrations of 0.036 $mg.l^{-1}$ (DWAF, 1996) and 0.03 $mg.l^{-1}$ (CCME, 2001).

The final concentrations for Cu (Fig. 5.4) and Fe (results not presented) on day 15 increased in comparison to the concentrations recorded in the initial sample. The metal concentrations for Cu increased from 0.15 $mg.l^{-1}$ to 0.21 $mg.l^{-1}$, and was significantly higher ($p < 0.05$) than the recommended concentration of 0.002 $mg.l^{-1}$ to 0.012 $mg.l^{-1}$ (DWAF, 1996) and 0.002 $mg.l^{-1}$ to 0.004 $mg.l^{-1}$ (CCME, 2001). The concentration for Fe increased from 4.98 $mg.l^{-1}$ to 7.06 $mg.l^{-1}$, and was also significantly higher ($p < 0.05$) than the recommended concentration of 0.3 $mg.l^{-1}$ (CCME, 2001). No Mn concentrations were recorded in any of the bioreactor one samples throughout the study period. Comparison of the initial and final water sample concentrations indicated that there were reductions in the concentrations of most of the metals analysed for. The final concentrations for Al, Ni and Zn (65% to 97% reduction) decreased to below their recommended concentrations, whereas the

concentrations recorded for Cu and Fe increased and were significantly higher ($p < 0.05$) than the recommended guidelines (CCME, 2001; DWAF, 1996). In a previous study by Jackson et al. (2007b), the authors evaluated the efficiency of a laboratory-scale bioreactor, with particular emphasis on Chemical Oxygen Demand (COD) reduction in winery effluent as well as metal reduction in the Plankenburg River. The authors reported a reduction in the concentrations of Al (0.75 $mg.l^{-1}$ to 0.18 $mg.l^{-1}$) and Ni (0.19 $mg.l^{-1}$ to 0.06 $mg.l^{-1}$), while the result for Fe was similar to the result obtained in the present study. The final Al concentrations were, however still higher than the recommended concentrations.

3.3. Laboratory-scale bioreactor two

The recorded concentrations for Cu, Mn (Fig. 5.5), Fe and Al (results not shown), decreased ($p < 0.05$) after the three-week experimental procedure. Concentrations for Al decreased from 1.55 $mg.l^{-1}$ to 0.38 $mg.l^{-1}$ (75%), which was higher than the recommended concentrations for Al (CCME, 2001; DWAF, 1996). For Cu, the concentrations decreased by 58% (from 0.33 $mg.l^{-1}$ to 0.14 $mg.l^{-1}$), which also exceeded the stipulated guidelines for Cu (CCME, 2001; DWAF, 1996). The metal concentrations for Fe decreased from 71.99 $mg.l^{-1}$ to 40.4 $mg.l^{-1}$ (44%) and was significantly higher ($p < 0.05$) than the recommended concentration of 0.3 $mg.l^{-1}$ (CCME, 2001). The initial concentrations for Al, Cu and Fe in bioreactor two were however significantly higher ($p < 0.05$) than the initial concentrations recorded in bioreactor one. Manganese concentrations decreased by 57% (0.07 $mg.l^{-1}$ to 0.03 $mg.l^{-1}$). The Mn concentrations recorded before and after treatment, were however, lower than the recommended guideline, which is 1.3 $mg.l^{-1}$ (DWAF, 1996).

Negligible increases in Ni and Zn concentrations were observed. Nickel concentrations increased from below the detection limit to 0.03 $mg.l^{-1}$, and was lower than the recommended concentrations of 0.025 $mg.l^{-1}$ to 0.15 $mg.l^{-1}$ (CCME, 2001), while recorded Zn concentrations increased from 0.62 $mg.l^{-1}$ to 0.64 $mg.l^{-1}$, and was significantly higher ($p < 0.05$) than the recommended concentrations of 0.036 $mg.l^{-1}$ (DWAF, 1996) and 0.03 $mg.l^{-1}$ (CCME, 2001).

The results recorded in the biofilm samples (results not shown) revealed a negligible increase in the mean metal concentrations for Al, Cu, Fe and Mn from compartments (i) to (iii) (Fig. 5.2). The Al, Cu, Fe and Mn concentrations in the biofilm increased from 2.2 $mg.l^{-1}$ to 2.9 $mg.l^{-1}$, 0.23 $mg.l^{-1}$ to 0.36 $mg.l^{-1}$, 5.43 $mg.l^{-1}$ to 6.59 $mg.l^{-1}$ and 0.08 $mg.l^{-1}$ to 0.10 $mg.l^{-1}$, respectively. Nickel and Zn concentrations from compartments (i) to (iii) decreased from 0.21 $mg.l^{-1}$ to 0.17 $mg.l^{-1}$ and 1.01 $mg.l^{-1}$ to 0.78 $mg.l^{-1}$, respectively (results not shown). Although the concentration of Fe in the bioballs was lower than that of the water samples, the concentration recorded in the biofilm samples increased during the course of the study period.

Shirdam et al. (2006) showed that metal accumulation was two to three times higher in immobilised cells than in free-floating cells. Jackson et al. (2007a) studied metal accumulation in water, biofilm and sediment samples collected from the Berg River. On average metal concentrations of 6 $mg.l^{-1}$ (Al) and 14.6 $mg.l^{-1}$ (Fe) were recorded in water samples, compared to 876.8 $mg.l^{-1}$ for Al and 1017.5 $mg.l^{-1}$ for Fe in biofilm samples. Research has shown that the extracellular polymeric substances (EPS) exhibits a high metal absorption capacity (Suh et al., 1999).

3.4. On-site Bioreactor

The recorded concentrations for Al, Cu, Mn and Ni in river water are presented in Fig. 5.6. The concentrations for Cu ranged from 0.16 $mg.l^{-1}$ (initial) to 0.01 $mg.l^{-1}$ (final) (94%), with the final concentrations falling within the recommended concentrations according to DWAF (1996). The initial Mn concentration of 0.12 $mg.l^{-1}$ decreased to 0.01 $mg.l^{-1}$ (92%). Both the initial (day one) and the final (day 67) concentrations fell within the recommended concentration of 1.3 $mg.l^{-1}$ (DWAF, 1996). Nickel concentrations decreased from 0.1 $mg.l^{-1}$ to 0.01 $mg.l^{-1}$ (90%), and fell within the recommended concentrations (CCME, 2001; DWAF, 1996). Iron concentrations decreased from 4.2 $mg.l^{-1}$ to 0.5 $mg.l^{-1}$ (88%) (result not shown), which was still higher ($p < 0.05$) than the recommended concentration (DWAF, 1996). In contrast the concentrations for Al increased from 0.42 $mg.l^{-1}$ to 0.66 $mg.l^{-1}$. Both the initial and final concentrations exceeded the recommended concentrations as stipulated by DWAF (1996) and the CCME (2001). The only other metal, besides Al, that showed an increase in the concentration post-treatment, was Zn, which increased from 0.66 $mg.l^{-1}$ to 0.8 $mg.l^{-1}$ (result not shown). Both the initial and final Zn concentrations exceeded the stipulated guidelines (CCME, 2001; DWAF, 1996).

The mean metal concentrations recorded for Al, Cu, Fe and Zn in the biofilm suspension removed from the bioballs for the on-site bioreactor (Fig. 5.3) were higher ($p < 0.05$) in tank A in comparison to the mean metal concentrations recorded in tank F over the entire study period. The concentrations for Al, Cu, Fe and Zn, recorded in tanks A and F, were; 47 $mg.l^{-1}$ to 9 $mg.l^{-1}$, 0.8 $mg.l^{-1}$ to 0.09 $mg.l^{-1}$, 83 $mg.l^{-1}$ to 52 $mg.l^{-1}$ and 3 $mg.l^{-1}$ to 2 $mg.l^{-1}$, respectively (results not shown). The concentrations of Mn in tank A and F, increased from 0.5 $mg.l^{-1}$ to 0.7 $mg.l^{-1}$ over the study period, while Ni concentrations remained the same at 0.1 $mg.l^{-1}$ (results not shown). The increased metal concentration in tank A and in the biofilm samples could indicate the efficiency of the bioballs to remove metals from the river water, which could also explain the decreased metal concentrations in bioballs collected from tank F. The concentrations recorded in the biofilm suspension removed from the bioballs collected from the compartments in bioreactor two, as well as the biofilm suspension collected from the six-tank, on-site bioreactor, revealed concentrations higher than those recorded in the corresponding water samples (Al, Cu, Mn, Ni and Zn), except for Fe in bioreactor two, where the concentration of Fe in the water was higher. Cylindrical bioreactors were used to determine the efficiency of a yeast biomass (*Saccharomyces cervisiae*) to remove, or reduce the concentrations of Ni, Zn, Cu, Cd and chromium (Cr) from electroplating effluent (Stoll and Duncan, 1997). The results showed that for all the metals analysed, the two-tank system efficiently reduced the metal concentrations between 17% and 18%. Costley and Wallis (2001) investigated the efficiency of a rotating biological contactor bioreactor to reduce Cu, Cd and Zn from heavy metal contaminated industrial wastewater, using three-week old biofilm material in a batch reactor. The system was able to reduce Cd, Cu and Zn concentrations by 30.4%, 81.1% and 49.6%, respectively. In the present study, an on-site six-tank bioreactor system was evaluated for its ability to remove metals from river water. The on-site bioreactor system was able to reduce Cu, Fe, Mn and Ni concentrations in the water samples by 88% to 94%. Overall, the removal efficiency of metals from the river water in the on-site bioreactor proved to be high.

3.5. Identification of organisms isolated from bioballs in Bioreactor two

Figures 5.7a and 5.7b are representatives of the agarose gel electrophoresis photos captured after PCR amplification. The size of the PCR product (1200 bp) in comparison with the Lambda DNA/Hind III marker can clearly be seen.

The phylogeny of the representative organisms in GenBank, were analysed using the Neighbour-joining algorithm in Clustal X (Figs. 5.8 and 5.9). The organisms used to construct the phylogenetic trees were representative of the isolated organisms of which many were identical. Tables 5.1 and 5.2 represent organism names and accession numbers for Figs. 5.8 and 5.9. These figures also represent the micro-organisms isolated from the biofilm suspension removed from the bioballs collected from the bioreactor three days (to allow for biofilm attachment) after it was started (Fig. 5.8) and the organisms isolated from the bioballs collected during the bioreactor run (Fig. 5.9), up until the final day of sample collection.

When comparing the isolates in Fig. 5.9 to the isolates originally present in Fig. 5.8, organisms such as *Aeromonas* sp., *Acinetobacter* sp., *Janthinobacter* sp., *Burkholderiaceae* sp., *Leptothrix* sp., *Luteococcus* sp., *Brevibacillus* sp., *Sphingomonas* sp., *Microbacterium* sp., *Delftia* sp., *Brachybacterium* sp., *Kocuria* sp., amongst others, were present in Fig. 5.9 (after 15 days), but absent in Fig. 5.8 (after 3 days). Species, such as *Hydrogenophaga* sp., *Chelatobacter* sp., *Corynebacterium* sp., *Ochrobactrum anthropi* strain W-7 and *Crocebacterium* sp. were present in the sample collected after 3 days, but were absent at the end of the bioreactor run. Bacterium PTO3, *Pseudomonas* sp., *Variovorax* sp., *Bacillus* sp., *Sphingomonas* sp. and *Brevundimonas* sp. were present throughout the bioreactor run (Figs. 5.8 and 5.9).

In Figs.5.8 and 5.9, the Gram-negative (such as *Pseudomonas*, *Variovorax*, *Acinetobacter,* and *Burkholderia*, amongst others) organisms exceeded the Gram-positive (such as *Micrococcus*, *Bacillus* and *Staphylococcus*, amongst others) organisms and tended to cluster together in both Figs. 5.8 and 5.9. This result was similar to the study by Duxbury and Bicknell (1983), who showed that Gram-negative organisms predominated in metal-polluted areas, such as the river water used in the current study, where high concentrations of metals were recorded. Eusébio et al. (2007) also showed a predominance of Gram-negative bacteria to Gram-positive bacteria, recorded at 87% and 13%, respectively. Out of a total of 331 aerobic heterotrophic bacterial strains, only 20 were Gram-positive, while the rest of the samples were dominated by Gram-negative bacteria, such as *Burkholderia* sp., *Pedobacter* sp., *Janthinobacter* sp., *Duganella* sp., and *Sphingomonas* sp. Männistö et al. (2001) also showed that the presence of many *Pseudomonas* isolates exhibited strong inhibition against certain Gram-positive species.

Hydrogenophaga sp. along with *Acidovorax* sp. belong to the “Knallgas” goup of hydrogen-oxidising organisms (Aragno and Schlegel, 1992). The *Hydrogenophaga* sp. have been shown to be quite common in activated sludge, preceding wastewater treatment and they, along with *Comamonas* isolates, may dominate the biofilm in the early stages of development and during periods of nutrient limitation, such as methanol, but tend not to be present at later stages in the reactor runs (Lemmer et al., 1997). In a study by Xia et al. (2008), *Ochrobactrum* sp. were involved in the fouling of membranes and also played a major role in the development of the mature biofilm. A lack of nitrogen or ammonia as a nitrogen source, necessary for the growth of nitrate-reducing organisms could have contributed to the loss of the above-

mentioned bacteria in the later stages of the bioreactor run. Similarly, in the present study, species such as *Hydrogenophaga* sp. and *Ochrobactrum anthropi* strain W-7 were present in the sample collected after 3 days, but were not present in the samples collected at the end of the bioreactor run. The surviving populations present both in the beginning and at the end of the bioreactor run belonged predominantly to the genera, *Pseudomonas* and *Bacillus*. In previous studies, the organisms predominantly isolated were *Pseudomonas* and *Bacillus* spp., presumably because of their ability to survive under both aerobic and anaerobic conditions and utilise nitrogen as an alternative electron acceptor if necessary (Eusébio et al., 2007).

Stenotrophomonas sp. was shown by Chien et al. (2007) to be resistant to high concentrations of Cd, and to some extent to Cr, Cu, Pb, Ni and Zn. Kim et al. (2007) showed that *Bacillus* sp. exhibited a high uptake capacity for Pb, Cd, Cu, Ni, Co, Mn, Cr and Zn and that a mixture of heavy metals, as opposed to exposure to a single metal, is more toxic to bacterial growth. *Stenotrophomonas*, *Pseudomonas*, *Bacillus*, *Micrococcus* (Yilmaz, 2004) and *Acinetobacter* sp. (Boswell, 2001), amongst others, have been shown to exhibit heavy metal-tolerance in previous studies, as well as in the current study. They could be applied in bioremediation strategies in different pollution sources, due to their high tolerance to various metals (Malik, 2004).

According to Leung et al. (2000) *Pseudomonas*, *Bacillus*, *Klebsiella*, *Aeromonas*, *Xanthomonas*, *Kocuria* and *Micrococcus* sp., amongst others were isolated from activated sludge treating industrial wastewater. The authors studied the metal resistance of *Pseudomonas pseudoalcaligenes* and *Micrococcus luteus* to heavy metals. Their results indicated that Cu uptake by *Micrococcus luteus* increased by 61% and that the *Micrococcus* sp. also showed an ability to increase Pb uptake.

Arthrobacter sp. resistance to metals could be due to novel mechanisms of the genome of the species. Research has shown that *Arthrobacter* resistance to Al and Cr could be due to the production of extracellular soluble substances (Benyehuda et al., 2003). In a previous study, where the metal tolerance of micro-organisms isolated from river water using flow cells was determined, the authors isolated PAO-12 *Microbacterium*, *Pseudomonas* sp., *Delftia* sp., *Sphingomonas* sp., *Bacillus* sp. and *Kocuria* sp. from flow cells after exposure to the varying metal concentrations (Jackson et al., 2008b). The present study showed the efficiency of an on-site bioreactor system in the removal of metals from river water through the sequestering of metals from the system by means of biofilms. The diversity and number of micro-organisms isolated from bioreactor two could be beneficial in the remediation process due to their ability to resist metal pollutants. The bioreactor system should however be optimised to increase it's efficacy.

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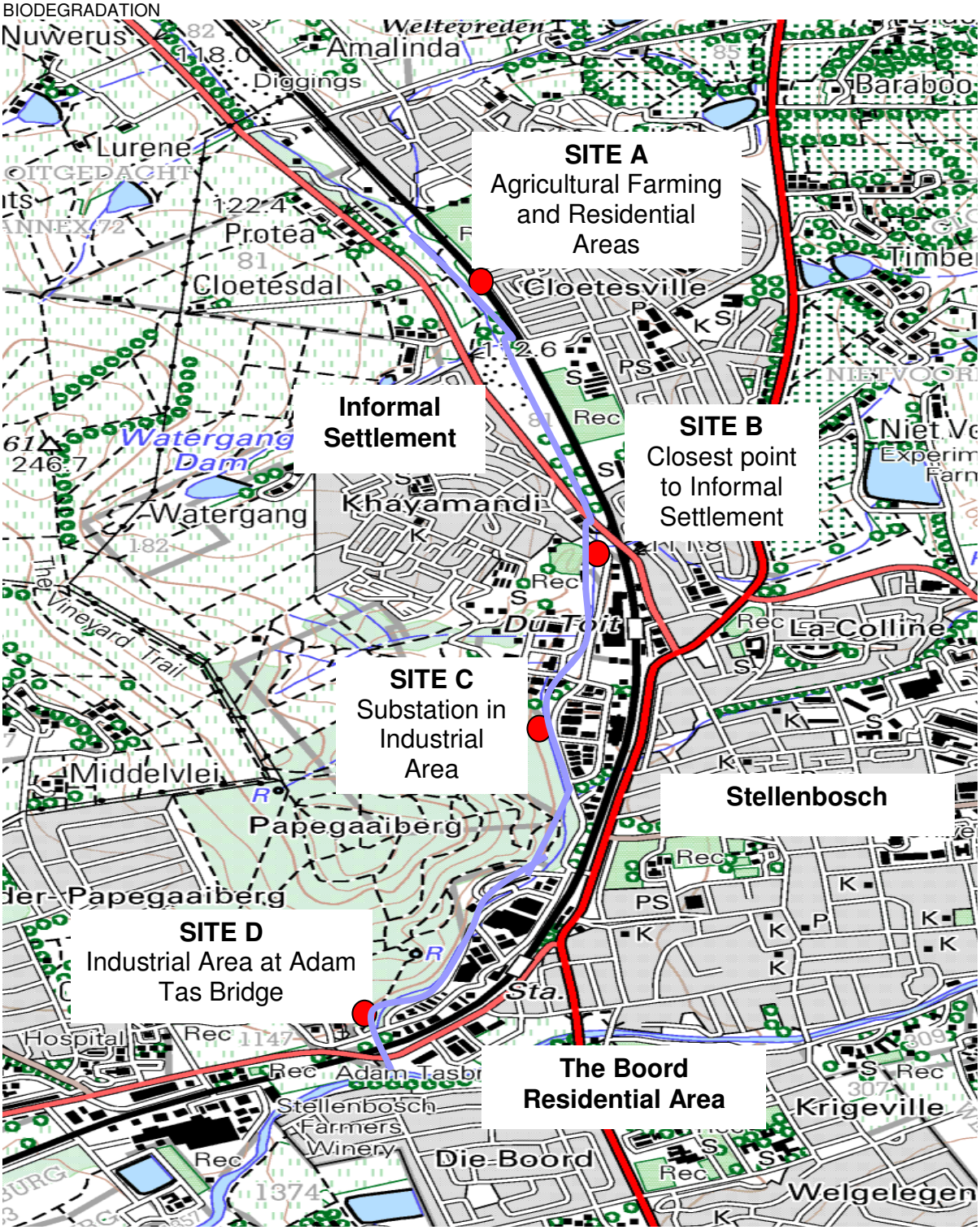

Fig. 5.1. Map of the Plankenburg River indicating the different sampling points: Site A – agricultural farming and residential areas; Site B – close to the informal settlement; Site C – Substation in the industrial area and Site D – industrial area at Adam Tas Bridge.

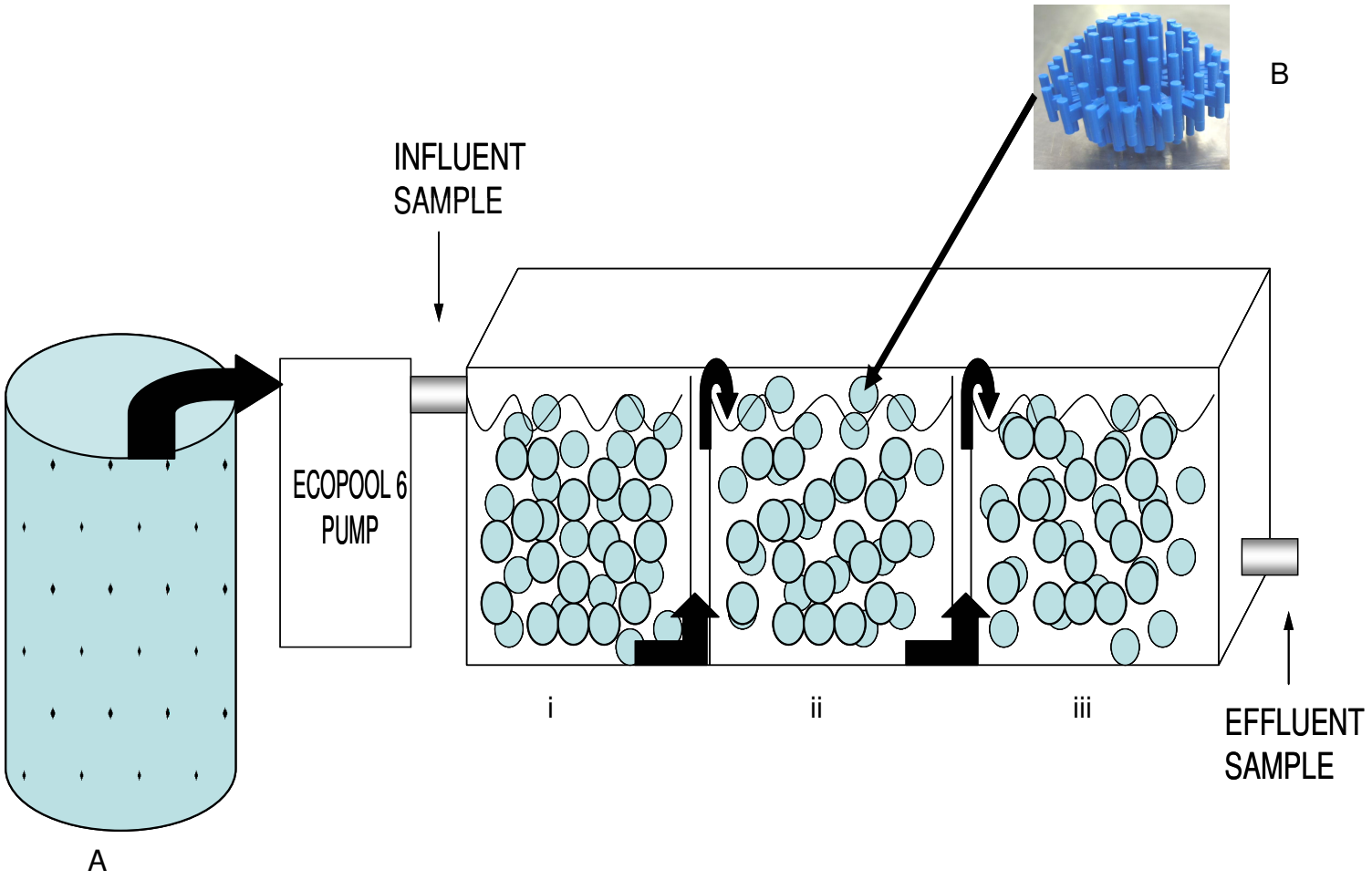

Fig. 5.2. Laboratory-scale batch system bioreactor, containing Bioballs™, which is composed of acrylonitrile, butadiene and styrene (ABS).

A B

Fig. 5.3 (A) On-site large-scale bioreactor established at Site C and (B) bioballs in bioreactor during treatment.

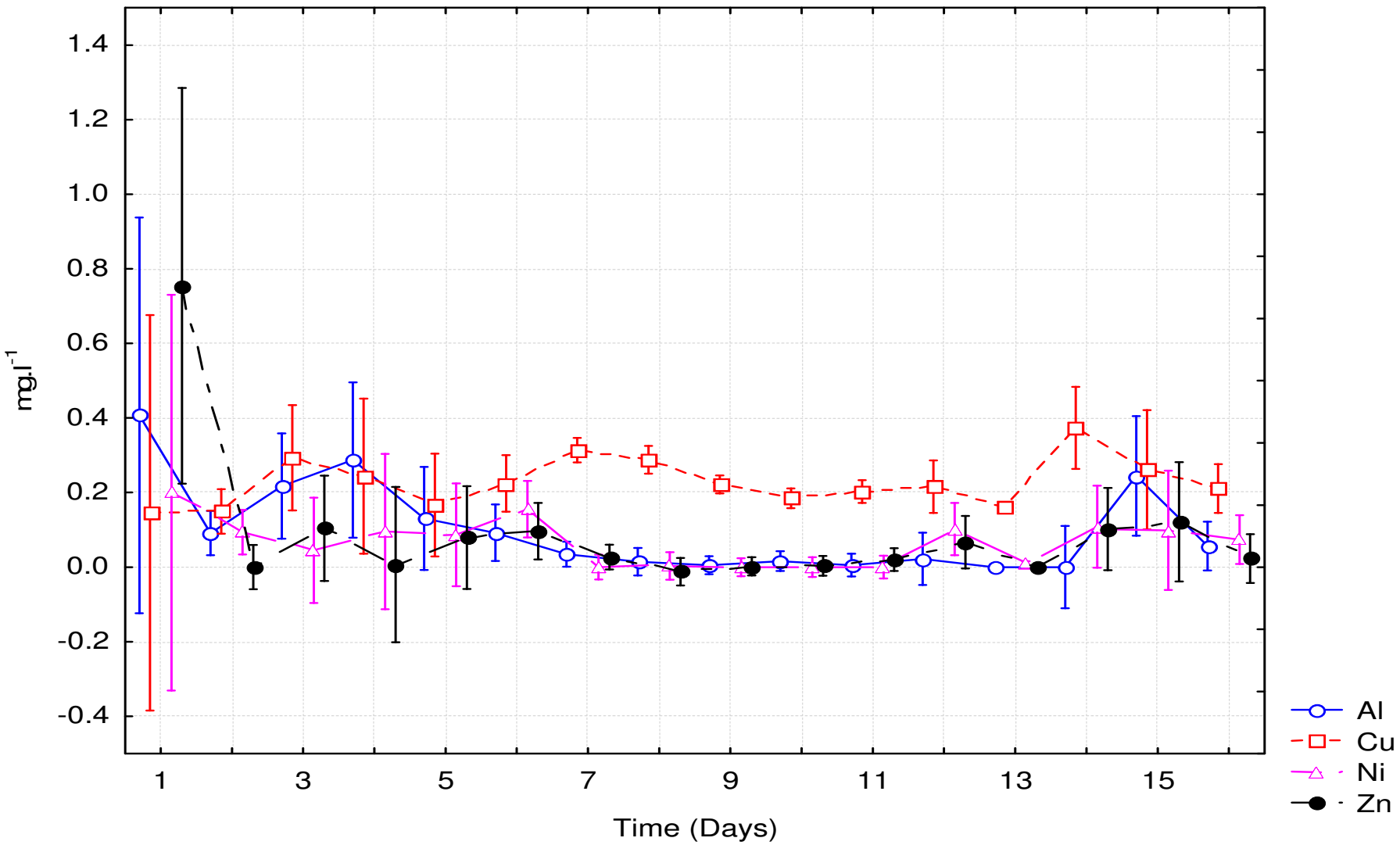

Fig. 5.4. Metal concentrations (mg.l^{-1}) recorded in water samples collected from the first laboratory-scale bioreactor (Plankenburg River).

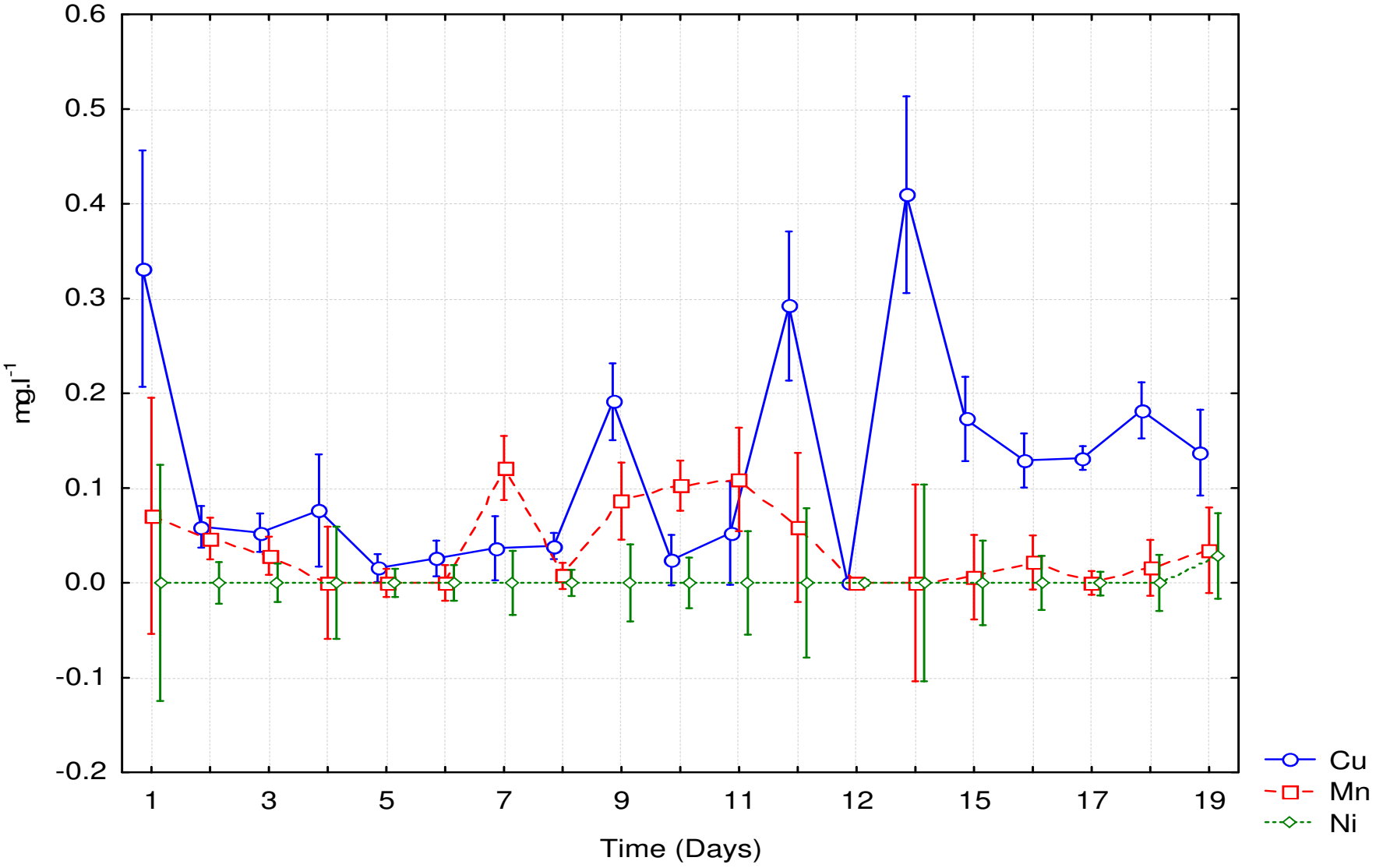

Fig. 5.5. Metal concentrations (mg.l^{-1}) (Cu, Mn and Ni) recorded in water samples collected from the second laboratory-scale bioreactor (Plankenburg River).

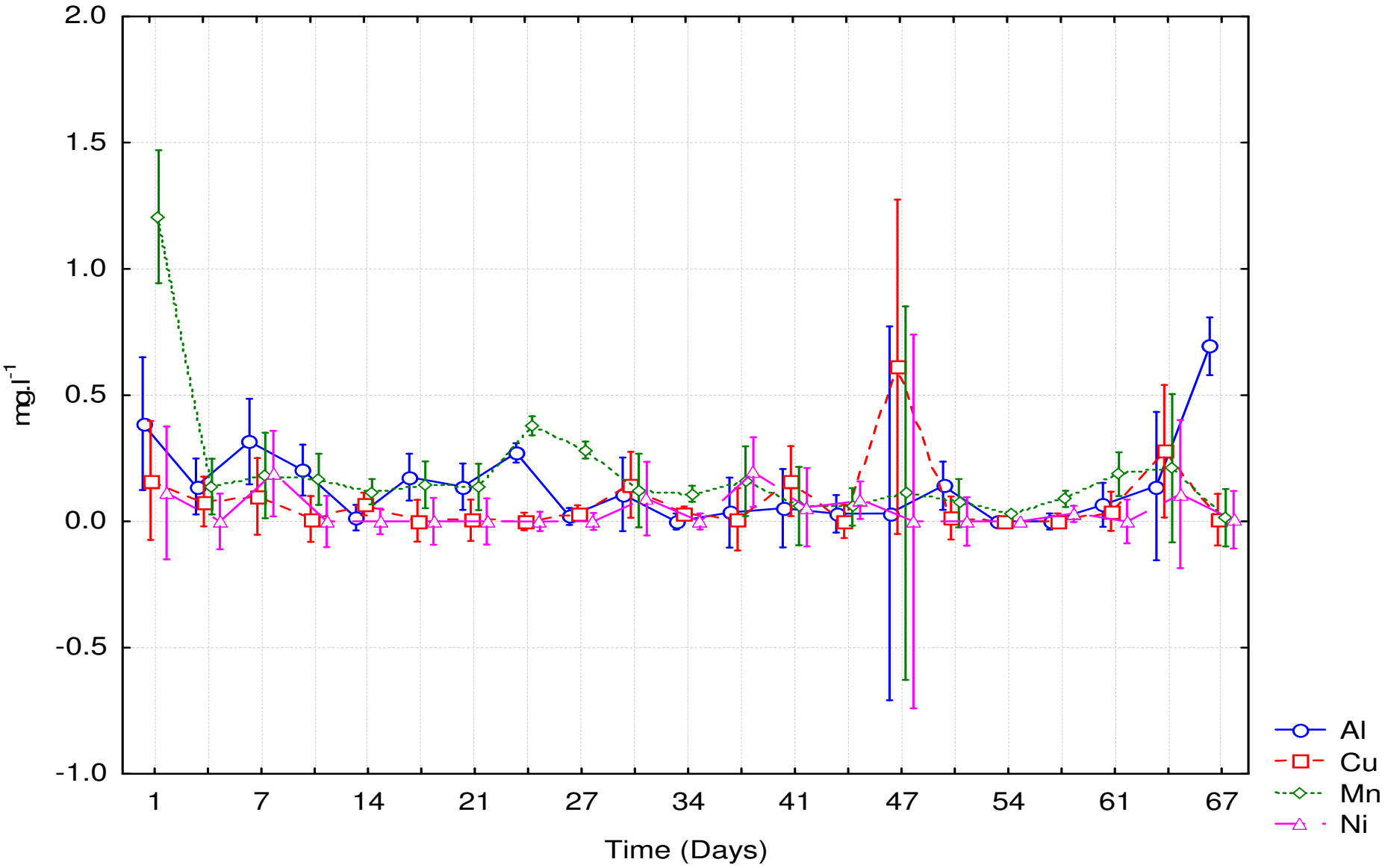

Fig. 5.6. Metal concentrations (mg.l^{-1}) (Al, Cu, Mn and Ni) recorded in water samples collected from the on-site bioreactor (Plankenburg River).

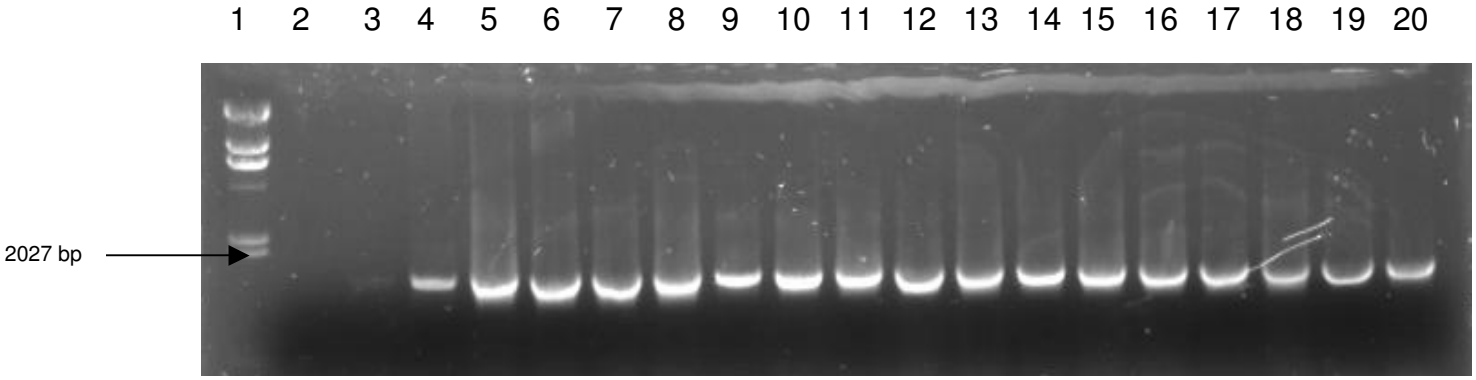

Fig. 5.7a. Representative result of agarose gel electrophoresis of organisms isolated from bioballs at the start of bioreactor three. Lane one represents the marker (Lambda DNA/HINDIII), lane two, the negative control, and lanes three to 20, represent the Purified PCR products.

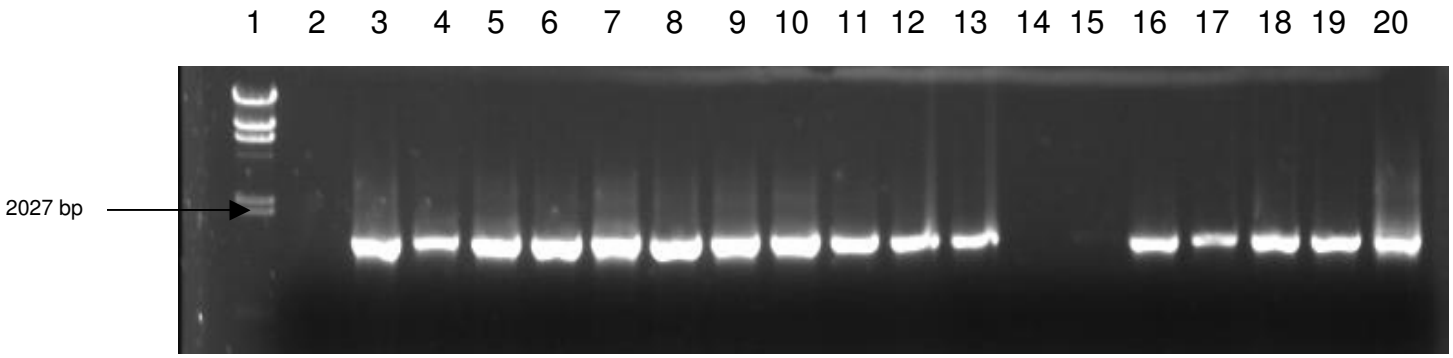

Fig. 5.7b. Representative result of agarose gel electrophoresis of organisms isolated from bioballs at the end of bioreactor three. Lane one represents the marker (Lambda DNA/HINDIII), lane two, the negative control, and lanes three to 20, represent the Purified PCR products.

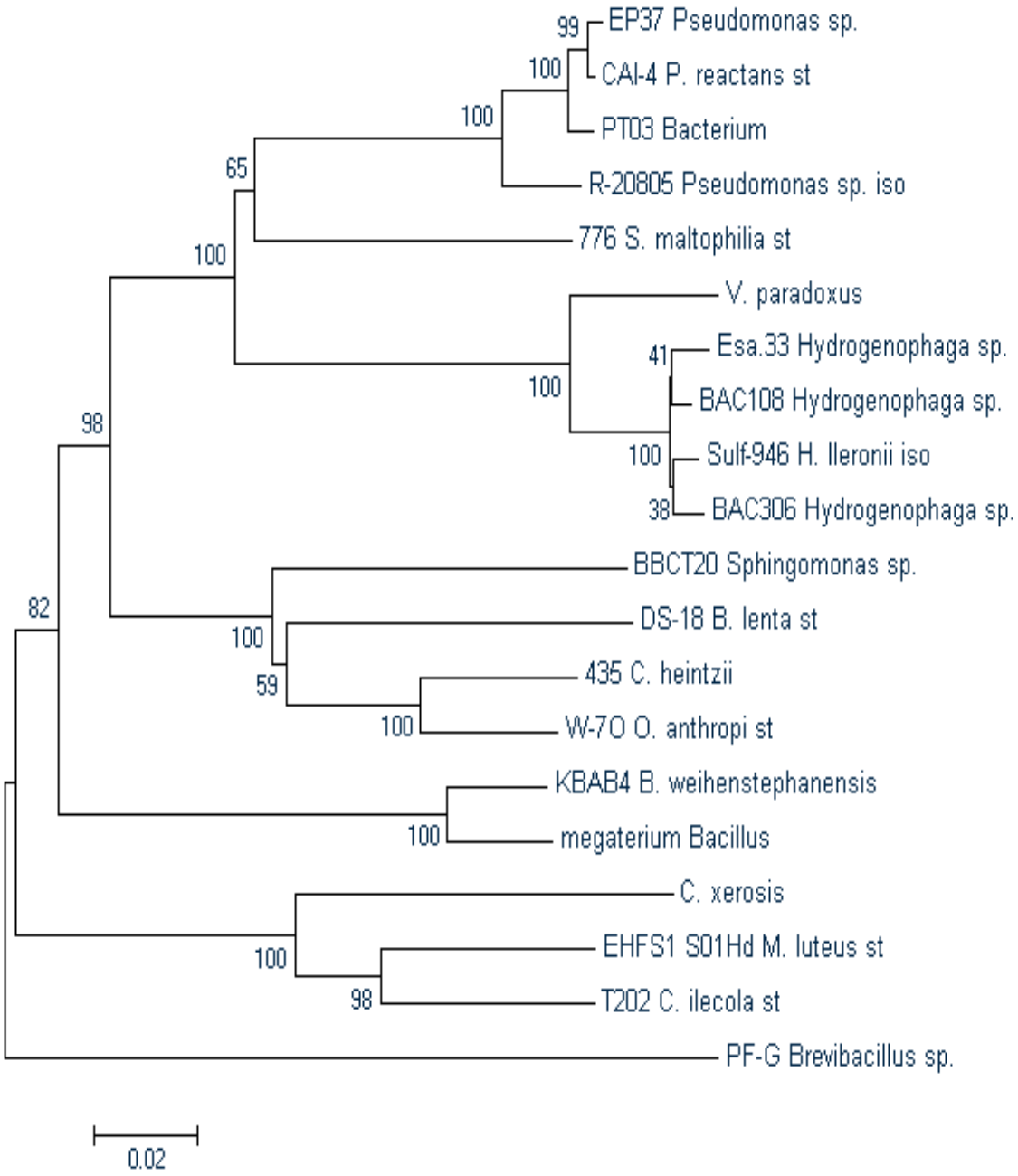

Fig. 5.8. An unrooted phylogenetic tree of organisms isolated from bioballs at the start of bioreactor three. A tree of 20 isolates was constructed using the Neighbour-joining algorithm of ClustalX. Bootstrap values are shown at the nodes.

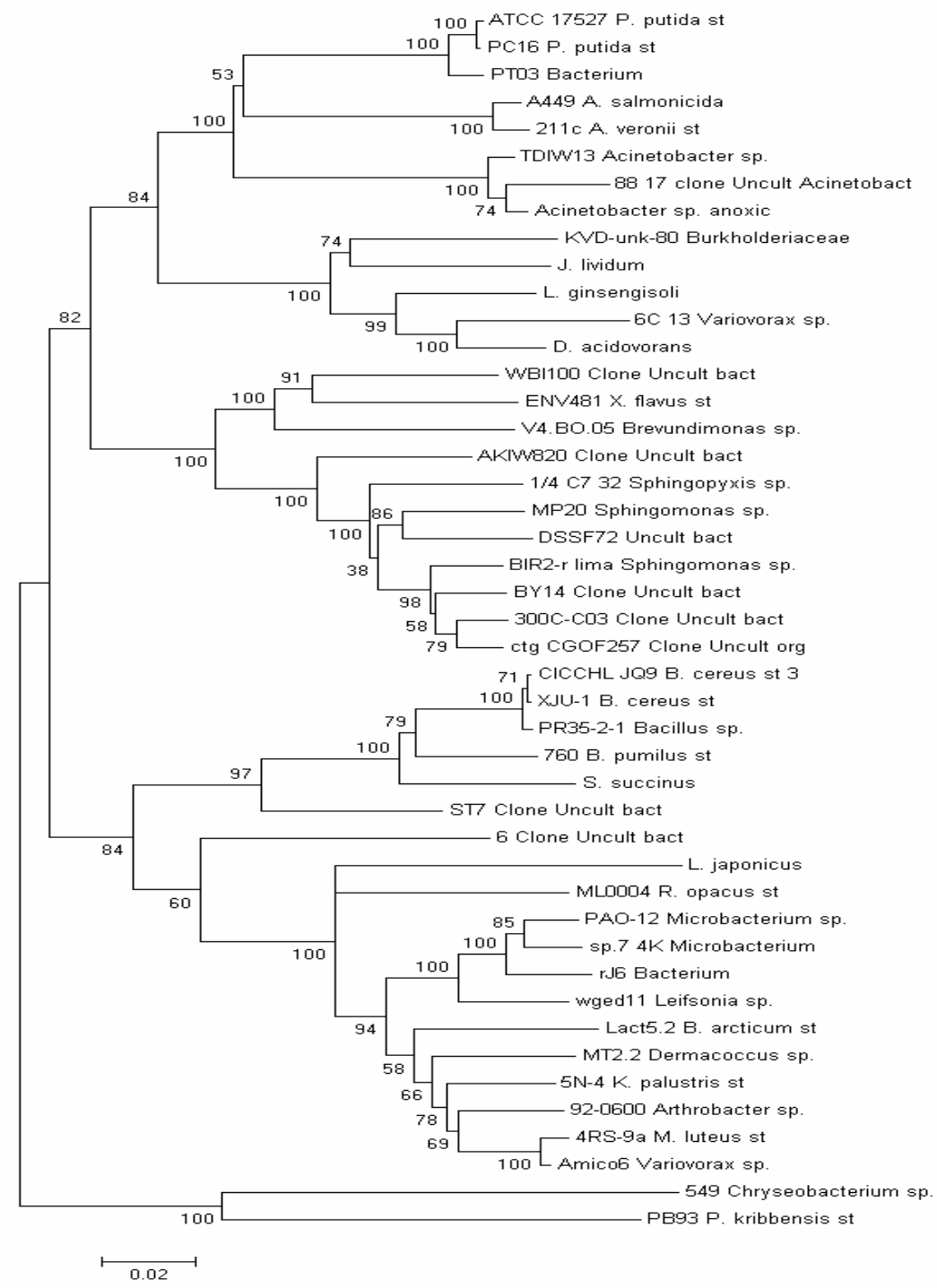

Fig. 5.9. An unrooted phylogenetic tree of organisms isolated from bioballs during the course of the three-week bioreactor experiment. A tree of 45 isolates was constructed using the Neighbour-joining algorithm of ClustalX. Bootstrap values are shown at the nodes.

Table 5.1

Table of 20 isolates, their names presented on the tree and accession numbers.

Name presented on tree	Organism	Accession #
EP37 Pseudomonas sp.	*Pseudomonas* sp. EP37	AM403728.1
CAI-4 P reactans sp.	*Pseudomonas reactans* strain CAI-4	DQ257418.1
PTO3 Bacterium	Bacterium PT03	DQ136048.2
R-20805 Pseudomonas sp. iso	*Pseudomonas* sp. isolate R-20805	AM114534.1
776 S maltophilia st.	*Stenotrophomonas maltophilia* strain 776	EU430096.1
V paradox	*Variovorax paradoxus*	AF532868.1
Esa.33 Hydrogenophaga sp.	*Hydrogenophaga* sp. Esa.33	AY569978.1
BAC108 Hydrogenophaga sp.	*Hydrogenophaga* sp. BAC108	EU130958.1
Sulf-946 H lleronii iso.	*Hydrogenophaga palleronii* isolate Sulf-946	AM922191.1
BAC306 Hydrogenophaga sp.	*Hydrogenophaga* sp. BAC306	EU130968.1
BBCT20 Sphingomonas sp.	*Sphingomonas* sp. BBCT20	DQ337548.1
DS-18 B lenta st.	*Brevundimonas lenta* strain DS-18	EF363713.1
435 C. heintzii	*Chelatobacter heintzii* 435	AF250406.1
W-70 O. anthropi	*Ochrobactrum anthropi* strain W-7	EU187487.1
KBAB4 weihenstephanensis	*Bacillus weihenstephanensis* KBAB4	CP000903.1
megabacterium Bacillus	*Bacillus megaterium*	DQ105968.1
C. xerosis	*Corynebacterium xerosis*	AF145257.1
EHFS1 SO1 Hd M. luteus st.	*Micrococcus luteus* strain EHFS1_S01Hd	EU071591.1
T202 C. ilecola st.	*Crocebacterium ilecola* strain T202	DQ826511.1
PF-G Brevibacillus sp.	*Brevibacillus* sp. PF-G	DQ207364.1

Table 5.2

Table of 45 isolates, their names presented on the tree and accession numbers.

Name presented on tree	Organism	Accession #
TDIW13 *Acinetobacter* sp.	*Acinetobacter* sp. TDIW13	EU000454.1
A449 *A. salmonicida*	*Aeromonas salmonicida* subsp. *salmonicida* A449	CP000644.1
92-0600 *Arthrobacter* sp.	*Arthrobacter* sp. 92-0600	EU086811.1
CICCHL JQ9 *B. cereus* st3	*Bacillus cereus* strain CICCHLJ Q93	EF528295.1
XJU-1*B.cereus* st	*Bacillus cereus* strain XJU-1	EF185296.1
760*B.pumilus* st	*Bacillus pumilus* strain 760	EU430090.1
PR35-2-1 *Bacillus* sp.	*Bacillus* sp. PR35-2-1	EU057855.1
PT03 Bacterium	Bacterium PT03	DQ136048.2
rJ6 Bacterium	Bacterium rJ6	AB021324.1
Lact 5.2 *B. arcticum* st	*Brachybacterium arcticum* strain Lact 5.2	AF434185.1
V4.BO.05 *Brevundimonas* sp.	*Brevundimonas* sp. V4.BO.05	AJ244704.1
KVD-unk-80 *Burkholderiaceae*	*Burkholderiaceae* bacterium KVD-unk-80	DQ490281.1
549 *Chryseobacterium* sp.	*Chryseobacterium* sp. 549	EF565935.1
MT2.2 *Dermacoccus* sp.	*Dermacoccus* sp. MT2.2	AY894329.1
J. lividum	*Janthinobacterium lividum*	Y08846.1
5N-4 *K. palustris* st.	*Kocuria palustris* strain 5N-4	EU379291.1
L. japonicus	*Luteococcus japonicus*	Z78208.1
L. ginsengisoli	*Leptothrix ginsengisoli*	AB271046.1
sp 7 4K *Microbacterium*	*Microbacterium* sp. 7_4K	EF540477.1
PAO-12 *Microbacterium* sp.	*Microbacterium* sp. PAO-12	EF514877.1
4RS-9a *M. luteus* st	*Micrococcus luteus* strain 4RS-9a	EU379286.1
ATCC 17527 *P. putida* st	*Pseudomonas putida* strain ATCC 17527	AF094743.1
PC16 *P. putida* st	*Pseudomonas putida* strain PC16	AY918067.1
ML0004 *R. opacus* st	*Rhodococcus opacus* strain ML0004	DQ474758.1
BIR2-rlima *Sphingomonas* sp.	*Sphingomonas* sp. BIR2-rlima	EF153191.1
MP20 *Sphingomonas* sp.	*Sphingomonas* sp. MP20	AY521015.2
1/4 C7 32 *Sphingopyxis* sp.	*Sphingopyxis* sp. 1/4_C7_32	EF540469.1
S. succinus	*Staphylococcus succinus*	AF004219.1
88 17 clone Uncult *Acinetobact*	Uncultured *Acinetobacter* sp. clone 88_17	AF467302.1
211c *A. veronii* st	*Aeromonas veronii* strain 211c	AY987746.1

6 clone Uncult bact	Uncultured bacterium clone 6	AY682678.1
300C-C03 clone Uncult bact	Uncultured bacterium clone 300C-C03	AY662023.1
AKIW820 clone Uncult bact	Uncultured bacterium clone AKIW820	DQ129610.1
BY14 Clone Uncult bact	Uncultured bacterium clone BY14	DQ494790.1
PB93*P. kribbensis* st	*Pedobacter kribbensis* strain PB93	EF660752.1
D. acidovorans	*Delftia acidovorans*	AB020186.1
ST7 Clone Uncult bact	Uncultured bacterium clone ST7	DQ347893.1
WBI100 Clone Uncult bact	Uncultured bacterium clone WBI100	EU024391.1
DSSF72 Uncult bact	Uncultured bacterium DSSF72	AY328694.1
6C 13 *Variovorax* sp.	*Variovorax* sp. 6C_13	AY689053.1
Acinetobacter sp. anoxic	*Acinetobacter* sp. 'anoxic'	AY055373.1
ctg CGOF257 Clone Uncult org	Uncultured organism clone ctg_CGOF257	DQ395648.1
wged11 *Leifsonia* sp.	*Leifsonia* sp. wged11	DQ473536.1
Amico6 *Variovorax* sp.	*Variovorax* sp. Amico6	AY512635.1
ENV481 *X. flavus* st	*Xanthobacter flavus* strain ENV481	EF592179.1

GENERAL CONCLUSIONS

6.1. INVESTIGATION INTO METAL CONTAMINATION IN THE BERG RIVER, WESTERN CAPE, SOUTH AFRICA

Natural watercourses, such as rivers, can be contaminated with metals and micro-organisms. When the concentrations of naturally occurring metals exceed a stipulated limit (South African Bureau of Standards, 2001; World Health Organisation, 1991), they may become toxic to the surrounding environment. Sources of metal contamination include industrial and medical waste (Dorigo *et al.*, 2004), pesticides, petroleum by-products (Mowat & Bundy, 2001), household products, as well as urban and pharmaceutical waste (Brooks *et al.*, 2003). Domestic and household sources of metal contamination generally occur as a result of corrosion of metal plumbing fittings, galvanised roofs and wire fences and metal-containing products, such as sunblocks, shampoos, amongst others (Alloway, 1995b).

Micro-organisms inhabit the natural environment in the form of planktonic organisms and sessile biofilms. These biofilms can be defined as a community of attached microbial cells organised within extracellular polymer matrices (EPS). This EPS assists in the bacterial survival by providing protection against metals, predation and environmental fluctuations, and also provides increased resistance against antimicrobial agents (Decho, 1990). Biofilms are advantageous in that they encapsulate toxic molecules, such as metals (Costerton *et al.*, 1978), by providing a substrate for them to adhere to, thereby limiting the diffusion of biocides and other toxic molecules across the EPS (De Beer *et al.*, 1994; Huang *et al.*, 1995).

The Berg River flows through many different areas, which include a wastewater works, an agricultural area and an informal settlement. It also serves as a source of water for towns, cities, and recreational users (River Health Programme, 2004). In recent years, the Berg River has experienced incidences of increased pollution. The objective of this study was to investigate the concentration of the following metals: aluminium (Al), zinc (Zn), copper (Cu), iron

(Fe), lead (Pb), nickel (Ni) and manganese (Mn), which occur within the river water, sediment and the biofilm samples collected from different sites along the Berg River (Paarl) in the Western Cape, South Africa. The figures and tables discussed are presented in article one (chapter two). As indicated on **Figure 2.1**, the samples were collected from site A (agricultural farming area), site B (Plot 8000), where storm water drainage pipes from the communities in a nearby settlement enter the river at site B, and site C (the Newton pumping station), which serves as an inlet of storm drainage water and wastewater into the river from the residential area of Newton as well as certain areas of Mbekweni.

Results obtained from the nitric acid digestion method, in conjunction with Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), revealed that the highest mean metal concentrations were recorded for Al (6 $mg.l^{-1}$), Fe (14.6 $mg.l^{-1}$) and Mn (18.8 $mg.l^{-1}$) in water samples (**Figure 2.2A**). The concentrations of Al and Fe were significantly higher than the recommended guidelines for Al (0.1 to 0.15 $mg.l^{-1}$ and 0.005 to 0.1 $mg.l^{-1}$) and Fe (0.3 $mg.l^{-1}$) in freshwater systems as stipulated by the Department of Water Affairs and Forestry (DWAF, 1996) and the Canadian Council of Ministers of the Environment (CCME, 2001). Manganese concentrations fell within the recommended quality guideline range of 1.3 $mg.l^{-1}$ (DWAF, 1996) for most of the sampling period with the exception of weeks 25, 45 and 49, where increased concentrations ranging from 3.6 $mg.l^{-1}$ to 18.8 $mg.l^{-1}$ at sites C and A (**Figure 2.2A**) respectively, were recorded. The recorded concentrations of Cu, Ni and Zn in water samples varied throughout the study period and fluctuated above and below the recommended guidelines of 0.002 $mg.l^{-1}$ to 0.012 $mg.l^{-1}$ (DWAF, 1996) and 0.002 $mg.l^{-1}$ to 0.004 $mg.l^{-1}$ (CCME, 2001), 0.025 $mg.l^{-1}$ to 0.15 $mg.l^{-1}$ (CCME, 2001) and 0.036 $mg.l^{-1}$ (DWAF, 1996) and 0.03 $mg.l^{-1}$ (CCME, 2001), respectively (**Table 2.1 & Figure 2.2B**). Lead could not be detected in any of the analysed samples.

In the sediment samples (**Figure 2.3**), the highest concentrations were recorded at 17448.8 $mg.kg^{-1}$ for Al and 26473.3 $mg.kg^{-1}$ for Fe. No recommended sediment quality guidelines for Al, Fe, Pb, Mn and Ni were available from DWAF (1996) and the CCME (2001). **Tables 2.2 & 2.3** represent

the mean concentrations for Cu, Mn, Ni, Pb and Zn in sediment samples. Manganese, Ni and Pb concentrations were significantly low throughout the study period in sediment samples, with the highest mean metal concentrations for Mn, Ni and Pb recorded at: 70 $mg.kg^{-1}$ (week 5, site C); 44 $mg.kg^{-1}$ (week 1, site B) and 23 $mg.kg^{-1}$ (week 45, site B) (**Tables 2.2 & 2.3**), respectively. The highest mean Cu concentration of 74 $mg.kg^{-1}$ (week 1, site B) (**Table 2.2**) was higher than the recommended environmental quality guideline of 35.7 $mg.kg^{-1}$ in freshwater sediment as specified by the CCME (2001). Thereafter, during weeks 5 to 49, concentrations for Cu decreased significantly ($p < 0.05$), to levels lower than the guideline (CCME, 2001). The highest mean Zn concentration of 395 $mg.kg^{-1}$ (week 1, site B) (**Table 2.3**), was higher than the recommended Canadian sediment quality guidelines of 123 $mg.kg^{-1}$ (CCME, 2001). Thereafter the mean Zn concentrations ranged from 4 $mg.kg^{-1}$to 36 $mg.kg^{-1}$ (**Table 2.3**), which fell within the accepted CCME guidelines.

The recorded results for Fe and Al in biofilm samples fluctuated throughout the entire study period. The mean concentrations recorded for Al and Fe ranged from 14.1 $mg.l^{-1}$ (week 33) to 876.8 $mg.l^{-1}$ (week 37), and from 18 $mg.l^{-1}$ (week 33) to 1017.5 $mg.l^{-1}$ (week 37), at site A, respectively (**Figure 2.4**). The highest mean metal concentrations in biofilm samples for Cu, Mn, Ni, Pb and Zn were recorded at: 2 $mg.l^{-1}$ (weeks 1 and 37, sites C and A); 71 $mg.l^{-1}$ (week 25, site A); 19 $mg.l^{-1}$ (week 17, site A); 1.6 $mg.l^{-1}$ (week 5, site C) and 8.4 $mg.l^{-1}$ (week 25, site A), respectively (**Tables 2.4 and 2.5**).

The highest concentrations of metals in water was recorded at the agricultural area (site A), where pesticides, such as Mancozeb™ and Phosguard™, amongst others, are utilised. Aluminium is a component of Phosguard™ (Seachem), and Mn composes 60% of Mancozeb™, which is a fungicide used for the treatment of plant diseases in the Western Cape. Vermeulen *et al*. (2001) reported that Mancozeb™ usage in the Western Cape amounts to 1343 x 10^3 kg/annum. The highest Al (week one) and Fe (weeks one and five) concentrations in sediment samples were recorded at site A for Al and at sites A and B (Informal settlement) for Fe. It was concluded that the high concentrations in the informal settlement could have been due to the leaching of

Al and Fe from household products and galvanised roofing and building materials into the river. The high concentrations of Al and Fe in the biofilm samples, especially during week 37, could be correlated with a corresponding increase in microbial counts in the biofilm sample (3.9 x 10^7 organisms.ml^{-1}) (Paulse *et al.*, 2008), collected during that particular sampling time period. The increase in the number of micro-organisms could have facilitated the Al and Fe accumulation in the biofilm, as it has been shown in previous studies that microorganisms have developed unique means of resistance to specific metals (Roane & Pepper, 2000), largely due to it's ability to develop a strong extracellular polymeric layer (EPS) (Mayer *et al.*, 1999).

6.2. INVESTIGATION INTO THE METAL CONTAMINATION OF THE PLANKENBURG- AND DIEP RIVERS, WESTERN CAPE, SOUTH AFRICA

Point- and non-point source pollution contributes to a decline in the quality of the water when leaching occurs into the surrounding environment (DWAF, 2004). Agricultural contamination was also shown by the Agency for Toxic Substances and Disease Registry (2000), to be due to the discharging of pesticides into rivers. In addition, pollutants such as micro-organisms, metals, oils and other toxic substances contribute to the decrease in water quality (Pegram *et al.*, 1999). High concentrations of metals usually deposit on and integrate in river sediment, which are either organic or inorganic materials, removed by erosion and transported by fluid flow to different locations (Prange & Dennison, 2000; Marchand *et al.*, 2006), where the highest metal content available for transport between sites is stored in the sediment-water interfaces (Maanan *et al.*, 2004).

The Plankenburg River flows downstream through Stellenbosch's industrial area, which includes amongst others a clothing factory, a well-known cheese factory, spray painting and mechanical workshops and yoghurt and dairy producing plants. The river also flows through an informal settlement. Farmers up- and downstream from the settlement utilise the river water for the irrigation of

vineyards, as well as other crops. In the Diep River catchment, land in the upper catchment area is dominated by agricultural activities. In the lower part of the catchment, land use is largely reserved for urban development, which includes formal and informal settlements, industrial establishments, such as spray painting, chemical and clothing manufacturers, a wastewater treatment works and an oil refinery. It also serves as a storage area for sediment-rich water during floods.

The aim of this study was to investigate the spatial and temporal variation in the metal contamination in the Plankenburg and Diep Rivers in the Western Cape, South Africa. Metal concentrations in water and sediment samples were analysed using the nitric acid digestion method followed by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Saleh et al., 2000). The two rivers selected borders industrial areas, residential areas, agricultural areas and informal settlements. Increased metal pollutants also have a detrimental effect on human health (Wright & Welbourne, 2002), where exposure is mainly due to the ingestion of food and water contaminated with metals leaching into groundwater (Piver, 1992). The figures and tables discussed are presented in article two (chapter three).

Metal concentrations in sediment and water samples were determined at different sites (**Figures 3.1 & 3.2**) along the rivers, using the nitric acid digestion technique in conjunction with Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). The sampling sites for the Plankenburg River were: site A (Agricultural Farming and Residential Areas); site B (Informal Settlement of Kayamandi); site C (Substation in Industrial Area) and site D (Industrial Area at Adam Tas Bridge) (**Figure 3.1**). The sampling sites for the Diep River were: site A (Zoarvlei Nature Reserve - Industrial as well as Residential Areas); site B (Theo Marais Sportsclub - Industrial and Residential Area); site C (Potsdam Wastewater Treatment Works) and site D (Rietvlei Boating Club and Nature Reserve) (**Figure 3.2**).

The recorded Al and Fe concentrations were mostly higher than all the other metals analysed. Metal concentrations recorded for Al and Fe in water samples collected at the Plankenburg River ranged from 0.3 $mg.l^{-1}$ (week 1, site

A) to 13.6 mg.l^{-1} (week 18, site B) and 0.3 mg.l^{-1} (week 39, site B) to 48 mg.l^{-1} (week 43, site A), respectively (**Tables 3.1, 3.2 and 3.5**). The Al concentrations mostly exceeded the recommended concentrations of 0.1 mg.l^{-1} to 0.15 mg.l^{-1} (DWAF, 1996) and 0.005 mg.l^{-1} to 0.1 mg.l^{-1} (CCME, 2001). The Fe concentrations also exceeded the guidelines of 0.3 mg.l^{-1} (CCME, 2001) and the 'World average' of 0.04 mg.l^{-1} (Martin and Windom, 1991). No Al guidelines were available for the 'World average' and no Al and Fe guidelines were available for the Australian and New Zealand Environment and Conservation Council (ANZECC, 2000). Lead (Pb) was not detected in any of the analysed samples from the Plankenburg River. The mean metal concentrations recorded for copper (Cu), manganese (Mn), nickel (Ni) and zinc (Zn) in the Plankenburg River water samples, are presented in **Tables 3.1 & 3.2**. The concentrations of Cu, Ni and Zn recorded in water samples from the Plankenburg River varied throughout the study period. The Cu concentrations ranged from 0.3 mg.l^{-1} (weeks 27 and 52, site D) to 2.2 mg.l^{-1} (week 1, site A) (**Tables 3.1, 3.2 & 3.5**). Throughout the study period, the recorded Cu concentrations were higher than the recommended concentrations of 0.002 – 0.012 mg.l^{-1}, 0.002 – 0.004 mg.l^{-1}, 0.0015 mg.l^{-1} and 0.0001 – 0.00015 mg.l^{-1} as stipulated by DWAF (1996b), the CCME (2001), Martin and Windom (1991) and ANZECC (2000), respectively (**Table 3.5**). Mean Mn concentrations ranged from below the detection limit (sites A, B, C and D) to 0.4 mg.l^{-1} (site C) (**Tables 3.1 & 3.2**) at different weeks during the sampling period, and always fell within the recommended quality guideline of 1.3 mg.l^{-1} (DWAF, 1996) for the Plankenburg River. The recorded concentrations, were however higher than the 'World average' of 0.0015 mg.l^{-1} (Martin and Windom, 1991) (**Table 3.5**). The mean metal concentrations for Ni recorded throughout the study period ranged from 0.1 mg.l^{-1} (sites B, C and D) to 0.5 mg.l^{-1} (site A) for the Plankenburg River. The recorded values generally exceeded the recommended concentrations of 0.025 mg.l^{-1} to 0.15 mg.l^{-1} (CCME, 2001), with the exception of weeks 27, 36, 43 and 52 (**Tables 3.1 & 3.2**), where the Ni concentration was recorded at 0.1 mg.l^{-1}. The concentrations for Ni were significantly higher ($p < 0.05$) than the 'World average' guideline of 0.0005 mg.l^{-1} (Martin and Windom, 1991) and the ANZECC (2000) guideline of 0.0001 –

0.0005 mg.l^{-1} (**Table 3.5**). No environmental quality guideline for Ni was available from DWAF (1996b). Throughout the entire study period, the overall concentrations for Zn in the water samples were mostly higher than the recommended concentrations of 0.03 mg.l^{-1}, 0.036 mg.l^{-1}, 0.0006 mg.l^{-1} and 0.0009 mg.l^{-1}, as stipulated by the CCME (2001), DWAF (1996b), Martin and Windom (1991) and ANZECC (2000), respectively (**Table 3.5**).

The concentrations of Al and Fe recorded in water from the Diep River, ranged from below the detection limit to 4 mg.l^{-1} (week 1, site A) and from 0.1 mg.l^{-1} (week 9, site A) to 513 mg.l^{-1} (week 27, site A), respectively (**Tables 3.3, 3.4 and 3.5**). No Al guidelines were available for the 'World average' and no Al and Fe guidelines were available for the Australian and New Zealand Environment and Conservation Council (ANZECC, 2000). No guidelines for Fe in river water samples were available from DWAF. Lead (Pb) was not detected in any of the analysed samples from the Diep Rivers. The concentrations of Cu, Ni and Zn in the Diep River water samples varied throughout the study period. The Cu concentrations ranged from 0.1 mg.l^{-1} (weeks 1 and 36, sites C and D, respectively) to 0.8 mg.l^{-1} (week 32, site D, respectively) (**Table 3.4**). Throughout the study period, the recorded concentrations for Cu were higher than the recommended concentrations of 0.002 – 0.012 mg.l^{-1}, 0.002 – 0.004 mg.l^{-1}, 0.0015 mg.l^{-1} and 0.0001 – 0.00015 mg.l^{-1} as stipulated by DWAF (1996b), the CCME (2001), Martin and Windom (1991) and ANZECC (2000), respectively (**Table 3.5**). The highest Mn concentration recorded for the Diep River was 1.3 mg.l^{-1} (week 27, site A) (**Table 3.3**), which is the maximum limit as stipulated by DWAF (1996) (**Table 3.5**). The Mn concentrations however mostly exceeded the 'World average' of 0.0015 mg.l^{-1} (Martin and Windom, 1991) (**Table 3.5**). The mean metal concentrations for Ni recorded throughout the study period ranged from below the detection limit to 0.4 mg.l^{-1} (weeks 23 and 32, sites D and C, respectively) for the Diep River. The recorded concentrations exceeded the recommended concentration of 0.025 – 0.15 mg.l^{-1} (CCME, 2001), 0.0005 mg.l^{-1} (Martin and Windom, 1991) and the Australian and New Zealand guidelines of 0.0001- 0.00015 mg.l^{-1} (ANZECC, 2000) during Weeks 5, 14, 18, 23, 27 and 32 at various sampling sites. The overall concentrations recorded throughout the

study period for Zn were mostly higher than the recommended concentrations of 0.03 $mg.l^{-1}$ (CCME, 2001), 0.036 $mg.l^{-1}$ (DWAF, 1996b), 0.0006 $mg.l^{-1}$ (Martin and Windom, 1991) and 0.0009 $mg.l^{-1}$ (ANZECC, 2000) (**Table 3.5**).

No sediment quality guidelines for Al, Cu, Fe, Mn, Ni, Pb and Zn were available from DWAF and the 'World average' (Martin and Windom, 1991) and guidelines for only Cu and Zn were available from the CCME (2001). Guidelines for Cu, Ni, Pb and Zn were available from ANZECC (2000). Results for metal concentrations in sediment samples in the Plankenburg River were as follows: Al [1609 $mg.kg^{-1}$ (week 52, site D) to 15018 $mg.kg^{-1}$ (week 1, site C)] and for Fe [3763 $mg.kg^{-1}$ (week 52, site D) to 19179 $mg.kg^{-1}$ (week 1, site C)] (**Figure 3.3**). The Mn concentrations ranged from 15.93 $mg.kg^{-1}$ (week 22, site B) to 225 $mg.kg^{-1}$ (week 5, site A) in the Plankenburg River (**Figure 3.4**). The Pb concentrations in the Plankenburg River ranged from 7.38 $mg.kg^{-1}$ (week 52, site C) (**Figure 3.5**) to 275 $mg.kg^{-1}$ (week 9, site C). The highest Pb concentration exceeded the Australian and New Zealand quality guideline of 50 $mg{\cdot}kg^{-1}$ (ANZECC, 2000). The concentrations recorded for Ni in the Plankenburg River, ranged from 0.62 $mg.kg^{-1}$ (week 1, site C) to 11.7 $mg.kg^{-1}$ (week 52, site D) (**Figure 3.5**). The highest Ni concentration was lower than the quality guideline as stipulated by ANZECC (2000). The highest Cu concentrations recorded for the Plankenburg River was recorded at 251.8 $mg.kg^{-1}$ (week 9, site C). This concentration was significantly higher ($p < 0.05$) than the recommended environmental quality guideline of 35.7 $mg{\cdot}kg^{-1}$ in freshwater sediment as stipulated by the CCME (2001) and the Australian and New Zealand quality guideline of 65 $mg.kg^{-1}$ (ANZECC, 2000). The highest Zn concentration recorded in the Plankenburg River was 269.5 $mg.kg^{-1}$ during Week 1 at Site B (**Figure 3.4**). The highest recorded concentration was significantly higher ($p < 0.05$) than the recommended environmental quality guideline of 123 $mg.kg^{-1}$ in freshwater sediment as stipulated by the CCME (2001) and 200 $mg.kg^{-1}$ as stipulated by ANZECC (2000).

No sediment quality guidelines for Al, Cu, Fe, Mn, Ni, Pb and Zn were available from DWAF and the 'World average' (Martin and Windom, 1991) and guidelines for only Cu and Zn were available from the CCME (2001). Guidelines

for Cu, Ni, Pb and Zn were available from ANZECC (2000). The results for Al and Fe in the sediment samples collected from the Diep River revealed the following: 175.5 $mg.kg^{-1}$ (week 1, site D) to 14363.8 $mg.kg^{-1}$ (week 1, site A) and from 299.3 $mg.kg^{-1}$ (week 14, site B) to 106379.5 $mg.kg^{-1}$ (week 9, site C) (**Figure 3.6**), respectively. The highest Mn concentration in the Diep River was recorded at 1353.5 $mg.kg^{-1}$ (week 1, site C). The highest mean metal concentration for Pb in the Diep River was 643.06 $mg.kg^{-1}$ (week 1, site A). The highest Pb concentration exceeded the Australian and New Zealand quality guideline of 50 $mg.kg^{-1}$ (ANZECC, 2000). For the Diep River, the highest Ni concentration was recorded at 15.81 $mg.kg^{-1}$ (week 9, site C) (**Figure 3.8**). The highest Ni concentration was lower than the quality guideline as stipulated by ANZECC (2000). The highest Cu concentrations recorded for the Diep River was recorded at 370.5 $mg.kg^{-1}$ (week 1, site B) and was significantly higher ($p < 0.05$) than the recommended environmental quality guideline of 35.7 $mg.kg^{-1}$ in freshwater sediment as stipulated by the CCME (2001) and the Australian and New Zealand quality guideline of 65 $mg.kg^{-1}$ (ANZECC, 2000). The highest Zn concentrations recorded for the Diep River was 1081.2 $mg.kg^{-1}$ and was significantly higher ($p < 0.05$) than the recommended environmental quality guideline of 123 $mg.kg^{-1}$ in freshwater sediment as stipulated by the CCME (2001) and 200 $mg.kg^{-1}$ as stipulated by ANZECC (2000).

Comparison of the overall results obtained at the various sites, showed that the point sources of pollution at the Plankenburg and Diep Rivers could not conclusively be identified as concentrations at the various sites fluctuated throughout the entire study period. The increased concentrations of Al and Fe at the various sites could be due to the fact that they are the two most abundant metals in the environment [Agency for Toxic Substances and Disease Registry (ATSDR), 1995]. The sources of Al, Mn and Fe contamination could be due to the leaching and the inadequate disposal of pesticides and fertilisers into the river (ATSDR, 2000; Jackson *et al*., 2007).

The increased concentrations of Fe, Zn and Pb in the informal settlement of the Plankenburg River, was similar to results obtained by the Nairobi River Basin Programme Phase II Pollution Monitoring Stakeholders in 2003, where the

authors attributed the high Fe and Zn concentrations to the use of Fe sheets galvanised with Zn. Similarly, the informal settlement situated along the banks of the Plankenburg River, also use the same materials in the construction of their informal housing.

Contamination at the industrial sites of both rivers could have been due to the leaching of waste products from the surrounding industrial activities. The increased concentrations recorded from the waste products leaching into the river from a stormwater drain coming from the oil refinery close to the Diep River, correlates with results obtained in studies conducted by Mwamburi (2003), where increased concentrations of Fe, Mn, Zn, Cr and Al in sediment samples, were recorded in the Kasat River (Kenya), where the direct input of waste from municipal and industrial sources could have contributed to the increased concentrations.

Davies *et al.* (2006) evaluated the accumulation of Cr, Cd and Pb in water, sediment and periwinkle (*Tympanotonus fuscatus var radula*; shell and soft tissues). The results showed that the concentrations of Cr were highest in both the sediment and water samples at all the sampling sites, where concentrations of 0.01 $mg.kg^{-1}$ were recorded. The authors found that effluent from heavily industrialised and highly populated settlements contributed to the metal accumulation at the affected sites. The authors concluded that for future use, the metal concentrations in sediment must be monitored on a regular basis. As with the results recorded in the present study, the concentrations recorded in the sediment samples exceeded the concentrations recorded in the water samples.

The recorded concentrations were compared to the baseline values obtained by Micó et al. (2007). Baseline values for heavy metals were proposed by Micó et al. (2007) to identify soil contamination in Alicante, Spain. Cadmium, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn concentrations were determined using atomic absorption spectrometry. The baseline values identified were 0.7 $mg.kg^{-1}$, 11 $mg.kg^{-1}$, 36 $mg.kg^{-1}$, 28 $mg.kg^{-1}$, 19,822 $mg.kg^{-1}$, 402 $mg.kg^{-1}$, 31 $mg.kg^{-1}$, 28 $mg.kg^{-1}$ and 83 $mg.kg^{-1}$ for Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn, respectively. The authors concluded that the baseline concentrations would be beneficial to compare the concentrations of metals for which there are no

recommended quality guidelines, such as Al, Fe and Mn. The values could also provide a basis to identify contaminated sites. The concentration of Fe in the soil recorded by Micó et al. (2007) was comparable to the concentrations of Fe recorded in the Plankenburg River, but lower than the concentration for Fe recorded in the Diep River. The Mn concentrations recorded at the Plankenburg River was lower than the Micó et al. (2007) results, but the Diep River result exceeded to baseline concentration recorded by Micó et al. (2007). The highest Cu, Pb and Zn concentrations recorded at both rivers were significantly higher ($p < 0.05$) than the baseline concentration stipulated by Micó et al. (2007), while for Ni, the recorded concentrations at both rivers were below the baseline concentrations of Micó et al. (2007). Results from the present study show that the metal concentrations in the river systems should routinely be analysed. The national guidelines should be updated or revised to accurately reflect the current state of the rivers and pollution influences.

6.3. IDENTIFICATION OF METAL-TOLERANT ORGANISMS ISOLATED FROM THE PLANKENBURG RIVER, WESTERN CAPE, SOUTH AFRICA

In water and the environment, micro-organisms exist mostly as biofilm communities attached to surfaces (Teitzel & Parsek, 2003). Microbial biofilms exhibit high affinities for contaminants due to the ability of the exopolymers produced by the biofilm to bind and sequester antimicrobial agents from the surrounding environment (Hunt, 1986). Studies performed by various researchers have highlighted the ability of certain micro-organisms to resist metal contamination (Roanne & Pepper, 2000). Research by Geesey *et al.* (1989) showed that extracellular polymeric substances (EPS) could serve as potential agents for the concentration and deposition of fine-grained minerals. Biofilms have also been shown by Roane & Pepper (2000) to be one of the most effective treatments for the removal of metals from metal-contaminated water. These authors also showed that because of the resistance build-up of the different micro-organisms within the biofilm, certain micro-organisms are more effective in

the removal of different metals from the contaminated environment (Roane & Pepper, 2000). Factors contributing to the increased resistance of micro-organisms to metal pollution include the taxonomic groups of the different micro-organisms within the biofilm matrix, as well as the age and size of the biofilm exposed to the contaminants (Wright & Welbourne, 2002). These factors accelerate and improve the immobilisation and degradation of pollutants (Singh *et al.*, 2006).

Devices developed to assist in the treatment of contaminated water bodies, such as flow cell systems, have been used to cultivate microbial biofilms *in vivo* (Caldwell *et al.*, 2002). They are multi-channelled to allow for experimental replication and simplified handling, and are made of Perspex. They can also be used to identify potential metal-tolerant micro-organisms *in situ*, through exposure to varying metal concentrations. The adverse effect of metals on micro-organisms can be evidenced by various factors, such as reductions in microbial diversity and number, and alterations in community structure (Ellis *et al.*, 2003). The composition of the micro-organisms can be determined genetically (Christensen *et al.*, 1998), through the amplification of the 16S or 23S rRNA region of the genomic DNA, using specific primers to identify organisms (Amann *et al.*, 1995). The objective of this investigation was to isolate metal-tolerant micro-organisms from a site where high metal concentrations were recorded along the Plankenburg River (Jackson *et al.*, 2008). The figures and tables discussed are presented in article three (chapter four). The micro-organisms were cultured and isolated in flow cell systems (**Figure 4.2**) after exposure to varying metal [aluminium (Al) (10 $mg.l^{-1}$, 500 $mg.l^{-1}$ and 900 $mg.l^{-1}$), iron (Fe) (10 $mg.l^{-1}$, 500 $mg.l^{-1}$ and 1000 $mg.l^{-1}$), copper (Cu) (0.5 $mg.l^{-1}$, 2.5 $mg.l^{-1}$ and 10 $mg.l^{-1}$), manganese (Mn) (1.5 $mg.l^{-1}$, 15 $mg.l^{-1}$ and 80 $mg.l^{-1}$), nickel (Ni) (0.5 $mg.l^{-1}$, 1 $mg.l^{-1}$ and 20 $mg.l^{-1}$) and zinc (Zn) (0.5 $mg.l^{-1}$, 1 $mg.l^{-1}$ and 40 $mg.l^{-1}$)] concentrations (**Table 4.1**). They were then identified using the Polymerase Chain Reaction (PCR) technique to amplify the 16S rRNA region. The phylogeny of the representative organisms in GenBank, were analysed using the Neighbour-joining algorithm in Clustal X.

The results for the determination of metal-tolerance, revealed an increase in the number of dead cells (4.47% increase) when the biofilm was exposed to 900 $mg.l^{-1}$ Al, in comparison to the untreated control (**Figures 4.3A & 4.3B**). For Cu, exposure to 10 $mg.l^{-1}$ resulted in an increase in the number of dead cells by 3.71% in comparison to the untreated control (**Figure 4.4A & 4.4B**). Exposure of the biofilms to 1000 $mg.l^{-1}$ Fe and 80 $mg.l^{-1}$ Mn showed an increase in the number of dead cells by 3.59% and 9.16%, respectively, in comparison with the untreated controls (**Figures 4.5A, B & Figures 4.6A, B**). A difference in live and dead cells exposed to the varying Ni and Zn concentrations was not evident (**Figures 4.7A, B & Figures 4.8A, B**). When exposed to the lowest concentrations of 10 $mg.l^{-1}$ (Al), 0.5 $mg.l^{-1}$ (Cu), 10 $mg.l^{-1}$ (Fe), 1.5 $mg.l^{-1}$ (Mn), 0.5 $mg.l^{-1}$ (Ni) and 0.5 $mg.l^{-1}$ (Zn), no notable differences in the obtained percentages could be detected in comparison with the untreated control, for any of the metals analysed for.

Exposure of the respective flow cell channels to the highest Al and Fe concentrations (Figures 4.3B and 4.5B) showed that the organisms tended to clump together in response to the metal exposure. The ability of extracellular polymeric substances to bind metals and pollutants also contribute to the clumping of cells (McLean *et al.*, 1990). Metals can alter the number, biochemical activity, diversity and community structure of micro-organisms in many different ways (Ellis *et al.*, 2003). Previous research performed by Teitzel & Parsek (2003) has shown that cells at the biofilm-bulk liquid interface were exposed to the highest concentrations of the pollutants. The two fluorophores used (SYTO 9 and Propidium Iodide) stains the living cells green and the dead cells red, respectively. When the cells fluoresce yellow, it means that the two images are superimposed and is impossible to distinguish live cells from dead cells (Figs. 3B to 8B). Teitzel & Parsek (2003) also reported that in minimal media with short exposure times, biofilms have a demonstrable resistance to the heavy metals Cu^{2+}, Zn^{2+} and Pb^{2+}

Phylogenetic analysis showed that a variety of organisms, which included *Bacillus* sp., *Pseudomonas* sp., *Delftia tsuruhatensis* strain A90, *Kocuria kristinae* strain 6J-5b, *Comamonas testosteroni* WDL7, *Stenotrophomonas maltophilia*

strain 776, *Staphylococcus* sp. MOLA:313, *Micrococcus* sp. TPR14, *Sphingomonas* sp. 8b-1 and *Microbacterium* sp. PAO-12, were isolated from the flow cells after exposure to the various metal concentrations. Two major clusters could also be distinguished based on their Gram-reactions (**Figure 4.10**).

In previous research it was shown that several Gram-positive and Gram-negative organisms were shown to be resistant to Pb, Hg, Cd, Cu, Co and Zn (Trajanovska *et al*., 1997), which could be correlated to the results obtained in the present study (**Table 4.4**). The major difference between the two clusters, was that the Gram-positive organisms, *Staphylococcus* MOLA:313, *Micrococcus* sp. TPR1 and *Kocuria kristinae* st. 6J-5b were resistant to Mn (**Figure 4.10 & Table 4.4**), and that the clade consisting of the *Pseudomonas* sp. all exhibited tolerance to Ni exposure. The clusters also contained more Gram-negative than Gram-positive organisms (**Figure 4.10**). Duxbury & Bicknell (1983), amongst others, suggested that Gram-negative organisms predominated in metal-polluted environments, and also showed that it was reasonable to expect a certain degree of overlap between Gram-positive and Gram-negative bacteria, as some of these species are resistant to the same metals. Isolates such as *Stenotrophomonas maltophilia* strain 776, Bacillus sp. ZH6 and *Staphylococcus* sp. MOLA:313 displayed resistance to multiple metals (Zn, Ni, Cu, Al and Fe), as well as *Pseudomonas* sp. and *Delftia tsuruhatensis* strain A90 (Cu and Ni resistance) to a lesser extent. The other isolated organisms did not exhibit multiple metal resistance (**Table 4.4**).

6.4. BIOREMEDIATION OF METAL CONTAMINATION IN THE PLANKENBURG RIVER, WESTERN CAPE, SOUTH AFRICA

Bioremediation is a process by which microbial degradation processes are used in technical and controlled treatment systems (Langwaldt & Puhakka, 2000). Bioremediation can also be applied as green technologies, due to its negligible effects on the environment, and its proven cost-efficiency (Adriaens *et al*., 2006). The attached organisms (biofilms) then aid in effectively reducing the toxicity in

the contaminated area. Bioreactors which can be applied in bioremediation strategies are basically tanks in which living organisms carry out biological reactions. Their efficiency is based on the ability of bacteria to attach to inert packing, such as granular activated carbon, at interfaces to generate high biomass (Bouwer & McCarty, 1982; Teitzel & Parsek, 2003). The reactor should also be easy to maintain and operate (Evangelho *et al*., 2001; Teitzel & Parsek, 2003), and should be able to function under aerobic and anaerobic conditions (Langwaldt & Puhakka, 2000).

Bioreactors have been utilised in many studies to remove or reduce metal concentrations in wastewater and various types of effluent. It is also essential to obtain information on the structure and distribution of micro-organisms in the contaminated area, as tolerant micro-organisms become dependant on the types of pollutants in specific areas (Marín-Guirao *et al*., 2005). Organisms that have been isolated from contaminated sites can also be identified and used in bioreactor systems to improve the removal efficiency of the contaminants.

The objective of this investigation was to assess the efficiency of a bioreactor system to remove, or decrease the concentrations of metal contaminants at a site along the Plankenburg River, Western Cape, South Africa. Potential metal-tolerant micro-organisms were also isolated from the attachment material.

The figures and tables discussed are presented in article four (chapter five). Two laboratory-scale bioreactors were developed (**Figure 5.2**) and the effect of different parameters, such as time and environmental conditions, were evaluated. The bioreactor system was optimised and a six-tank large-scale on-site bioreactor (**Figure 5.3**) was constructed at the most polluted area along the Plankenburg River, which was identified as the site located closest to the industrial area. Successful operation of the bioreactor system, could lead to the utilisation of the system in other settings, such as wastewater treatment works and effluent clean-up or removal. It is also essential to identify organisms isolated from the contaminated sites, for possible future use in bioreactor systems to improve the contaminant removal efficiency.

Metal concentrations in the laboratory-scale bioreactors were analysed by nitric acid digestion and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Aluminium (Al), nickel (Ni) and zinc (Zn) concentrations in water samples from bioreactor one decreased from 0.41 $mg.l^{-1}$ to 0.06 $mg.l^{-1}$, 0.2 $mg.l^{-1}$ to 0.07 $mg.l^{-1}$ and 75 $mg.l^{-1}$ to 0.02 $mg.l^{-1}$, respectively (**Figure 5.4**). Copper (Cu) and iron (Fe) concentrations increased from 0.15 $mg.l^{-1}$ to 0.21 $mg.l^{-1}$ (**Figure 5.4**) and 4.98 $mg.l^{-1}$ to 7.06 $mg.l^{-1}$ (result not shown), respectively.

The recorded concentrations for Al, Cu, Fe and manganese (Mn) decreased after completion of the three-week (bioreactor two) experimental procedure. These decreases were recorded at 1.55 $mg.l^{-1}$ to 0.38 $mg.l^{-1}$ (75%) for Al (results not shown), 0.33 $mg.l^{-1}$ to 0.14 $mg.l^{-1}$ (57%) for Cu, 0.07 $mg.l^{-1}$ to 0.03 $mg.l^{-1}$ (57%) for Mn (**Figure 5.5**) and 71.99 $mg.l^{-1}$ to 40.4 $mg.l^{-1}$ (44%) for Fe (results not shown). The results recorded in the biofilm samples revealed a negligible increase in the mean metal concentrations for Al, Cu, Fe and Mn from compartments i to iii. The Al, Cu, Fe and Mn concentrations in the biofilm suspension removed from the bioballs collected from compartments i to iii, increased from 2.2 $mg.l^{-1}$ to 2.9 $mg.l^{-1}$, 0.23 $mg.l^{-1}$ to 0.36 $mg.l^{-1}$, 5.43 $mg.l^{-1}$ to 6.59 $mg.l^{-1}$ and 0.08 $mg.l^{-1}$ to 0.10 $mg.l^{-1}$, respectively. Nickel and Zn concentrations from tanks i to iii decreased from 0.21 $mg.l^{-1}$ to 0.17 $mg.l^{-1}$ and 1.01 $mg.l^{-1}$ to 0.78 $mg.l^{-1}$, respectively (results not shown). Comparison of the metal concentrations in water and biofilm samples showed that the Al, Mn and Ni concentrations in the biofilm samples were higher than that of the corresponding water samples. Although the concentration of Fe in the bioballs was lower than that of the water samples, the concentration recorded in the biofilm samples increased during the course of the study period.

Results recorded for Cu, Mn, Ni and Fe decreased in the on-site bioreactor from initial (tank A) to final (tank F) concentrations and ranged from: 0.16 $mg.l^{-1}$ to 0.01 $mg.l^{-1}$, 0.12 $mg.l^{-1}$ to 0.01 $mg.l^{-1}$, 0.1 $mg.l^{-1}$ to 0.01 $mg.l^{-1}$ (**Figure 5.6**) and 4.2 $mg.l^{-1}$ to 0.5 $mg.l^{-1}$, respectively. The concentrations for Zn (0.66 $mg.l^{-1}$ to 0.8 $mg.l^{-1}$) and Al (0.42 $mg.l^{-1}$ to 0.66 $mg.l^{-1}$) (results not shown) increased from tanks A to F. The concentrations of metals recorded in

the biofilm suspension removed from bioballs collected from the on-site bioreactor (results not shown) exceeded the concentrations recorded in the corresponding water samples. The concentrations recorded in the biofilm suspension from tanks A to F for Al (47 $mg.l^{-1}$ to 9 $mg.l^{-1}$), Cu (0.8 $mg.l^{-1}$ to 0.09 $mg.l^{-1}$), Fe (83 $mg.l^{-1}$ to 52 $mg.l^{-1}$) and Zn (3 $mg.l^{-1}$ to 2 $mg.l^{-1}$) decreased throughout the study period (results not shown). The concentrations of Mn increased from 0.5 $mg.l^{-1}$ to 0.7 $mg.l^{-1}$ over the study period, while Ni concentrations stayed constant at 0.1 $mg.l^{-1}$ (results not shown). The decreases in metal concentration, between tanks A and F, indicated the efficiency of the bioballs to remove metals from the river water. This then could explain the decreased metal concentrations in the biofilm suspension collected from tank F, as there may not have been a high degree of available metals to be sequestered by the bioballs.

In a previous study by Jackson *et al.* (2007a) the authors reported a reduction in the concentrations of Al (0.75 $mg.l^{-1}$ to 0.18 $mg.l^{-1}$) and Ni (0.19 $mg.l^{-1}$ to 0.06 $mg.l^{-1}$). Aluminium concentrations were, however still higher than the recommended concentrations. In contrast, the final concentrations in the optimised bioreactor (one) were lower than the recommended concentrations, indicating that the optimised bioreactor was more efficient at removing metals from the system. The starting concentrations for laboratory-scale bioreactor two, however, was higher that the initial concentrations recorded in laboratory-scale bioreactor one, and even though the concentrations for most of the metals decreased, they still exceeded the recommended guidelines (CCME, 2001 & DWAF, 1996).

The on-site, six-tank bioreactor system was able to reduce Cu, Fe, Mn and Ni concentrations in the water samples by 88% to 94%. In a previous study by Costley & Wallis (2001) the efficiency of a rotating biological contactor bioreactor to reduce Cu, Cd and Zn from heavy metal contaminated industrial wastewater was evaluated. The system was able to reduce Cd, Cu and Zn concentrations by 30.4%, 81.1% and 49.6%, respectively. The on-site bioreactor system was able to reduce Cu, Fe, Mn and Ni concentrations in the

water samples by 88% to 94%. Overall, the removal efficiency of metals from the river water in the on-site bioreactor proved to be high.

Results from a study by Shirdam *et al.* (2006) showed that metal accumulation was two to three times higher in immobilised cells. The superiority of metal uptake by immobilised cells could have accounted for the increased concentrations of Al, Cu and Fe in biofilm samples recorded in bioreactor two and where increased Mn concentrations were recorded in bioreactor two and the on-site bioreactor.

Phylogenetic analysis (**Figures 5.8 & 5.9**) of potentially metal-tolerant organisms, such as *Pseudomonas* sp., *Delftia* sp., *Sphingomonas* sp., *Bacillus* sp. and *Kocuria* sp. (Bhadra *et al.*, 2007; Piotrowska-Seget *et al.*, 2005; Kuffner *et al.*, 2008; Selenska-Pobell *et al.*, 2006; Leung *et al.*, 2000) showed that most of the organisms isolated were similar to those found in a previous study by Jackson *et al.* (2008a), which implies that these organisms could possibly be utilised for bioremediation purposes, to increase efficiency of the bioreactors. The number of Gram-negative organisms exceeded the number of Gram-positive organisms in **Figures 5.8 & 5.9**. A study by Duxbury & Bicknell (1983) also showed the predominance of Gram-negative organisms in metal-polluted areas such as the river water. Certain organisms, such as *Hydrogenophaga* sp., *Ochrobactrum* sp., *Chelatobacter* sp., amongst others were present at the start (three days) of the reactor run, but not at the end (15 days) of the reactor run. The *Hydrogenophaga* sp. and *Ochrobactrum* sp. have been shown to be quite common in activated sludge, preceding wastewater treatment and in the case of *Ochrobactrum* sp. contributing to the fouling of membranes and contribute to biofilm development (Xia et al., 2008). The development of the biofilm results in a lack of nitrogen sources, necessary for the growth of nitrate-reducing organisms, which could have contributed to the loss of the above-mentioned bacteria in the later stages of the bioreactor run. The surviving populations present both in the beginning and at the end of the bioreactor run belonged predominantly to the genera, *Pseudomonas* and *Bacillus*, which have been shown in previous studies, to have the ability to survive under both aerobic and anaerobic conditions and utilise nitrogen as an alternative electron

acceptor if necessary (Eusébio et al., 2007). Männistö *et al.* (2001) also showed that the presence of many *Pseudomonas* isolates exhibited strong inhibition against certain Gram-positive species. Metal-tolerant organisms, *Bacillus*, *Pseudomonas*, *Micrococcus* and *Stenotrophomonas*, amongst others, could possibly be utilised for bioremediation purposes. The bioreactor system will however be optimised further to improve its efficacy.

6.5. MAJOR FINDINGS OF THE STUDY

The significant results of this study were as follows:

6.5.1.1. Aluminium (Al) and Fe were recorded at consistently higher concentrations than all the other metals analysed for in water, sediment and biofilm samples.

6.5.1.2. In both the sediment and biofilm samples, the concentrations of Al and Fe were significantly higher ($p < 0.05$) than Cu, Zn, Pb, Ni, and Mn.

6.5.1.3. The results for Cu and Zn were higher than the recommended quality guidelines in freshwater sediment (CCME, 2001).

6.5.1.4. The highest metal concentrations were obtained in the sediment and biofilm samples, yet no freshwater guidelines for metals in sediment were available from DWAF and no guidelines for metal concentrations in biofilms were available from either DWAF or the CCME.

6.5.2.1. Aluminium and Fe concentrations were higher than all the other metals analysed for in the water samples collected from the Plankenburg River, which exceeded the guidelines stipulated by DWAF and the CCME (Al and Fe) and the 'World average' (Fe).

6.5.2.2. Concentrations of Cu and Zn (with the exception of Week 1, Site B) in the Plankenburg River water samples exceeded the guidelines stipulated by the CCME, DWAF, ANZECC and the 'World average'.

6.5.2.3. Concentrations of Mn fell within the DWAF guidelines, as well as the 'World average'. No guidelines for Mn were available from the CCME.

6.5.2.4. No Pb could be detected in any of the Plankenburg and Diep River water samples.

6.5.2.5. The highest mean metal concentrations in sediment samples were recorded for Al and Fe at Site C (substation in the industrial area) in the Plankenburg River.

6.5.2.6. The highest mean metal concentrations in water samples were recorded for Al, Fe and Zn at Site A (industrial area) in the Diep River, which exceeded the guidelines stipulated by DWAF, the CCME, ANZECC and the 'World average', and for Fe and Zn, the baseline values of Micó et al. (2007).

6.5.2.7. Concentrations for Cu in water samples from the Diep River exceeded the recommended concentrations for ANZECC, DWAF, the 'World average' and the CCME, while Ni concentrations fluctuated above and below the recommended guidelines at Sites A, B, C and D.

6.5.2.8. Manganese concentrations fell below the recommended guideline during the sampling period, with the exception of Week 27, where the Mn concentration was 1.3 $mg.l^{-1}$, at Site A.

6.5.2.9. The highest mean Al concentration in sediment samples from the Diep River was recorded at Site A (industrial area) and the highest mean Fe concentration was recorded at Site C (wastewater treatment works). The highest Fe concentration was significantly higher ($p < 0.05$) than the baseline value obtained by Mico et al. (2007).

6.5.2.10. Possible sources of contamination of the Plankenburg River could be ascribed to the leaching of household waste into the river water from the informal- and formal residential settlements, as well as the leaching of industrial effluent from the industries situated close to the river.

6.5.2.11. In addition, contamination of the Plankenburg River could also have been due to the excessive use of pesticides and insecticides on farms bordering the river system and the discarding of these pesticides into the rivers.

6.5.2.12. Possible sources of contamination of the Diep River could have been the leaching of industrial waste from various industries into the

sampled sites along the banks of the river, as well as waste from the nearby oil refinery.

6.5.2.13. Metal concentration analysis should be routinely performed to ensure an accurate assessment of the current state of the rivers, and based on these results quality guidelines should be adapted accordingly.

6.5.3.1. Exposure of the flow cell channels to the highest concentrations of Al (900 $mg.l^{-1}$), Fe (1000 $mg.l^{-1}$), Cu (10 $mg.l^{-1}$) and Mn (80 $mg.l^{-1}$) increased the percentage of dead cells.

6.5.3.2. When the channels were exposed to the lowest concentrations of 10 $mg.l^{-1}$ (Al), 0.5 $mg.l^{-1}$ (Cu), 1.5 $mg.l^{-1}$ (Mn) and 0.5 $mg.l^{-1}$ (Zn), no significant differences between the live and dead cells could be distinguished.

6.5.3.3. The percentages of dead cells when exposed to the highest concentrations of Zn (40 $mg.l^{-1}$) and Ni (20 $mg.l^{-1}$) did not show any significant differences between the live and dead cells.

6.5.3.4. Phylogenetic analysis showed that the organisms isolated from the flow cell experiment were diverse and some of the isolates exhibited multiple metal resistance, while others only exhibited resistance to specific metals.

6.5.3.5. *Stenotrophomonas maltophilia* strain 776 exhibited tolerance to Zn, Ni, Cu, Al, Fe, while *Bacillus* sp. ZH6 exhibited tolerance to Zn, Ni, Al and Fe and *Staphylococcus* sp. MOLA:313 exhibited tolerance to Zn, Mn, Al and Fe exposure. *Pseudomonas* sp. and *Delftia tsuruhatensis* strain A90 were resistant to Cu and Ni exposure.

6.5.3.6. *Comamonas testosteroni* WDL7, *Microbacterium* sp. PAO-12 and *Sphingomonas* sp. 8b-1 exhibited tolerance to Cu, Ni and Zn, respectively, while *Kocuria kristinae* strain 6J-5b and *Micrococcus* sp. TPR14 exhibited tolerance to Mn.

6.5.3.7. The major metal-resistant organisms, *Bacillus* sp. and *Pseudomonas* sp., both displayed resistance to Ni. *Bacillus* sp., however exhibited

resistance to Zn, Al and Fe, while *Pseudomonas* sp. also exhibited resistance to Cu.

6.5.4.1. The final concentrations for Al, Ni and Zn (bioreactor one) and Mn (bioreactor two), decreased to below their recommended concentrations in water samples, as stipulated by DWAF (1996) and the CCME (2001).

6.5.4.2. Although a decrease in the final concentrations recorded in the water samples collected from bioreactor two was observed for most of the metals analysed, these concentrations were, however still higher than the recommended concentrations (CCME, 2001; DWAF, 1996), as a result of the high initial concentrations recorded.

6.5.4.3. In the on-site, six-tank bioreactor system, the concentrations for Fe, Cu, Mn and Ni decreased, but still exceeded the recommended concentrations (DWAF, 1996; CCME, 2001).

6.5.4.4. The concentrations recorded in the biofilm suspensions removed from the bioballs collected from bioreactor two and the on-site bioreactor, revealed concentrations higher than those recorded in the corresponding water samples for all the metals analysed, except Fe.

6.5.4.5. The bioballs were thus shown to be efficient for biofilm attachment and subsequent metal accumulation.

6.5.4.6. The species diversity of the organisms isolated from the bioreactor experiment after three days (initial) differed from the organisms isolated after 15 days (final).

6.5.4.7. Phylogenetic analysis showed that the Gram-negative and the Gram-positive species clustered together.

6.5.4.8. *Hydrogenophaga* sp., *Ochrobactrum* sp, *Corynebacterium* sp., *Chelatobater* sp. and *Brevundimonas* sp. were present in tree one (three days of biofilm growth), but absent in tree two (15 days of biofilm growth).

6.5.4.9. The surviving populations present both in the beginning and at the end of the bioreactor experiment belonged predominantly to the genera, *Pseudomonas* and *Bacillus*.

6.5.4.10. *Microbacterium* PAO-12, *Pseudomonas* sp., *Delftia* sp., *Sphingomonas* sp., *Bacillus* sp. and *Kocuria* sp. were isolated, as in a previous study performed by the same authors, where metal-tolerant organisms were isolated (Jackson et al., 2008b).

6.5.4.11. Metal-tolerant organisms, *Bacillus*, *Pseudomonas*, *Micrococcus* and *Stenotrophomonas*, amongst others, could possibly be utilised for bioremediation purposes. The bioreactor system will however be optimised further to improve its efficacy.

6.6. RECOMMENDATIONS

Without adequate control measures to monitor pollution input/influx into the rivers from the various anthropogenic sources, the surrounding ecosystems could be further contaminated. The existing guidelines were drawn up in 1996 and 2001 for DWAF and the CCME, respectively, and from the results obtained in the study, it is suggested that the guidelines should be updated on a regular basis to incorporate the changing environmental conditions. Future research also includes the setting up of parameters or guidelines for acceptable metal concentrations in South African river water and sediment, as well as biofilms, as metals accumulate in these attached organisms. No recommended quality guidelines for metals in biofilms could be found. Also, especially for the informal settlements situated along the banks of the Plankenburg- and Berg Rivers, educational campaigns should be implemented, to inform the inhabitants of proper sanitation behaviour and the municipal responsibility of waste disposal.

The efficiency of the bioreactor to reduce metal concentrations in the Plankenburg River shows potential for future studies, where the bioreactor will be optimised further for use in other settings, such as in wastewater works, winery effluents and industrial wastes, amongst others. The increased metal

concentrations recorded in the biofilm suspensions sonicated from the bioballs over the metal concentrations recorded in the corresponding water samples, is a clear indication as to the efficacy of the bioballs utilised to reduce metal concentrations in the river water. The numerous microbial species isolated from both the flow cells and the bioballs demonstrate the species diversity accumulating within the biofilm samples. Upon identification of the afore-mentioned organisms, the particular resistance mechanisms with which these isolates protects themselves from predation and chemical attack must be further elucidated, as well as their specificity identification, i.e., the particular organism's ability to resist, or to proliferate in the presence of a particular metal contaminant. The toxicity of metals should also be investigated to further understand which metals compliment each other and which metals prevent the modes of action of others. Previous research has also shown that most metals are resistant to not only one, but many different organisms, presumably due to the presence of a cluster of genes, which confer not only metal-resistance, but antibiotic resistance as well. The genes conferring resistance must be studied further to possibly improve their removal capacity. These specific modified organisms can then be introduced into a particular environment to which they are genetically predisposed. The individual species isolated from the laboratory-scale bioreactor system, should also be further investigated to determine which organisms are the most effective in reducing metal concentrations.

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APPENDIX A

The article published in Water Science and Technology was aimed at developing a bioreactor system to reduce or remove metal pollutants from river water.

The application of bioremediation: Reduction of metal concentrations in river water and COD in distillery effluent.

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Abstract
The major aim of this study was to evaluate and develop artificial bioremediation systems to reduce or remove metal pollutants from contaminated river water and to decrease the chemical oxygen demand (COD) in distillery effluent. Metals were extracted using the nitric acid digestion method, and the concentrations determined using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES). A decrease in metal concentrations was observed for most of the metals analysed in the river water after being pumped through the bioreactor system for approximately two weeks, e.g. Al concentration decreased from 0.75 $mg.l^{-1}$ to 0.18 $mg.l^{-1}$ and for Ni, from 0.19 $mg.l^{-1}$ to 0 $mg.l^{-1}$. In addition, the COD counts decreased from 2255 $mg.l^{-1}$ to a final value of < 150$mg.l^{-1}$ in the distillery effluent. It could thus be concluded that the bioreactor system decreased the COD and metal concentrations in the distillery effluent as well as the river water, respectively. A bioreactor has been constructed on-site at a wine cellar to reduce COD and will be constructed at a site along the Plankenbrug River.

Keywords Bioremediation; distillery effluent; inductively coupled plasma atomic emission spectrometry; metals; pollution; rivers

INTRODUCTION

Bioremediation is defined as treatment technology that uses living organisms (plants, microbes, etc.) to reduce the concentration or toxicity of contaminants in soil, water and wastewater (Evangelho *et al*., 2001). Numerous applications of biofilms in bioremediation exists as these microbial communities are inexpensive labourers capable of cleaning up pollutants such as, metal contaminants, chlorinated solvents, crude oil, sewage water, etc. (Langwaldt and Puhakka, 2000), with the ideal bioremediation system being time-efficient, as well as cost-effective (Liu *et al*., 2001).

The trickling filter, which is one of the most commonly used fixed-film bioreactors, consists of a solid substratum to which microorganisms (biofilm) attach. The ideal substratum material should be highly durable, have a low cost, should not clog easily and should have a high surface area for maximum microbial adsorption (Metcalf and Eddy, 1991; Evangelho *et al.*, 2001).

The current research project focuses on the development and the application of a bioremediation system to reduce or remove metal pollutants from contaminated river water and to decrease the chemical oxygen demand (COD) in distillery effluent.

METHODS

Sampling

Distillery effluent from a settling pond, and river water from a site located near the informal settlement of Khayamandi (Stellenbosch), was evaluated in two bioreactor systems. A volume of 200 L of distillery effluent and river water respectively, was fed through the bioreactors using an Ecopool 6 pump for a period of approximately two weeks. Initial samples were collected and subsequent effluent samples collected every day from the outlet system.

Development of Trickling filter bioreactor

The distillery effluent was pumped through a continuous reactor system with the dimensions 30 cm x 30 cm x 100 cm at a flow rate of 1000 L/hour at room temperature with a retention time of 2 minutes. Polyvinylchloride (PVC) sheeting was used as the attachment surface within the reactor. Based on these results, a bioreactor system (Figure 1), with the dimensions of 35 cm x 30 cm x 100 cm, a flow rate of 1000 L/h and a retention time of 3 minutes, was constructed for the removal of metals from river water. Different materials, such as, Bioballs™ and Alphagrog™, were used to create an increased surface area for maximum metal removal efficiency.

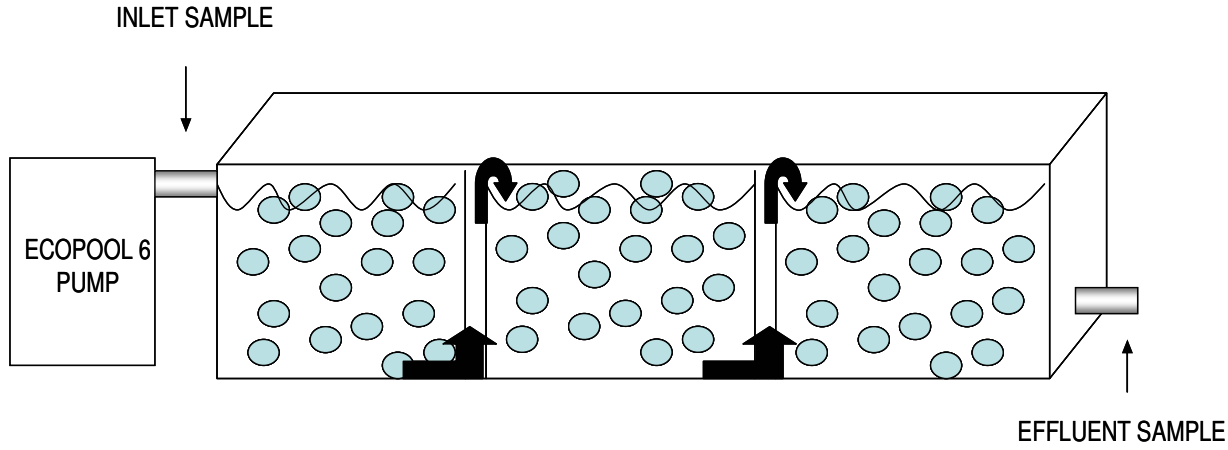

Figure 1. Horizontal trickling filter bioreactor system.

Metal concentration and Chemical oxygen demand (COD) determination

The nitric acid digestion method was used to analyse for Aluminium (Al), Copper (Cu), Nickel (Ni) Manganese (Mn) and Iron (Fe) in the effluent samples of the reactor with the concentrations measured using ICP-AES (Saleh *et al*, 2000). For comparison, a blank (control) of 10 ml 55% nitric acid was analysed along with the collected samples. Chemical Oxygen Demand (COD) measurements on the

distillery effluent samples (vertical reactor) were performed using the COD Cell Test Kit, Method photometric (15000 mg/L).

RESULTS AND DISCUSSION

Mean metal concentration in river water:

The metal concentrations at four sampling sites along the Plankenbrug River, Stellenbosch were analysed for a period of one year. Based on these results, river water was collected from the site with the highest metal concentrations.

Metal concentrations recorded from the bioreactor

The recorded concentrations in the effluent samples after being fed through the bioreactor system for a period of two weeks were lower than in the initial samples (Figure 2). The decrease in metal concentrations were as follows: Al, 0.75 $mg.l^{-1}$ to 0.18 $mg.l^{-1}$, Cu, from 0.10 $mg.l^{-1}$ to 0.06 $mg.l^{-1}$, Mn, from 0.10 $mg.l^{-1}$ to 0 $mg.l^{-1}$, Ni, from 0.19 $mg.l^{-1}$ to 0.06 $mg.l^{-1}$, and Fe from 4.9 $mg.l^{-1}$ to 4.8 $mg.l^{-1}$ (results for Fe not shown). The bioreactor effluent concentrations for Al, Cu and Fe were however still significantly higher than the recommended safe concentrations as stipulated by the Department of Water Affairs and Forestry (DWAF) and the Canadian Council of Ministers of the Environment (CCME) in their guidelines published in 1996 and 2001, respectively.

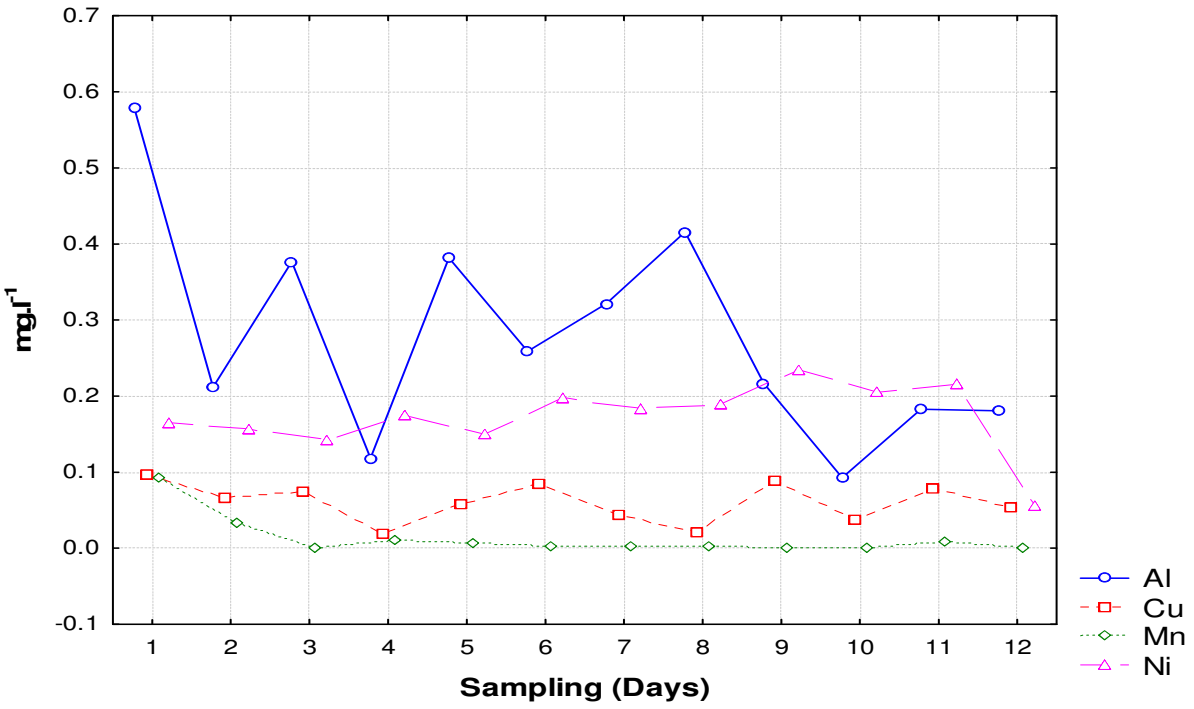

Figure 2. Mean Metal concentrations ($mg.l^{-1}$) in effluent samples over the three sampling times

COD results using a trickling filter bioreactor

Results obtained from the COD Cell Test Kit, Method photometric (15000 mg/L) for distillery effluent, revealed a significant ($p < 0.05$) decrease in COD concentration. Chemical oxygen demand concentrations decreased from an initial value of 2255 mg/L to a final value of < 150mg/L as shown by the graphical representation in Figure 3. A severe limitation of this method is that the COD could only be measured up to a concentration of 15000 mg/L and the exact initial concentration could thus not be determined.

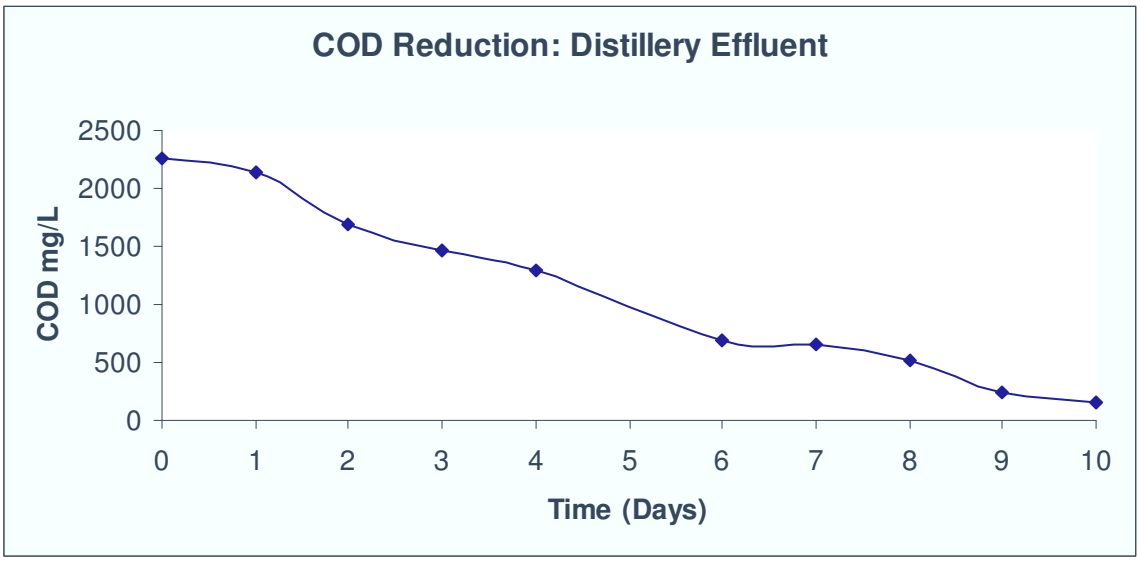

Figure 3. Graphical representation of reduction in COD (mg/l^{-1}) for samples obtained from the 3rd settling dam.

CONCLUSIONS

The bioreactor system used for the remediation of the river water sample, proved to be effective in decreasing the metal concentrations. In addition, results show a significant decrease ($p < 0.05$) in the COD concentrations of the distillery effluent pumped through the bioreactor. This system is currently being evaluated on a large-scale for winery effluent and will be constructed on-site at the most contaminated location along the Plankenbrug River.

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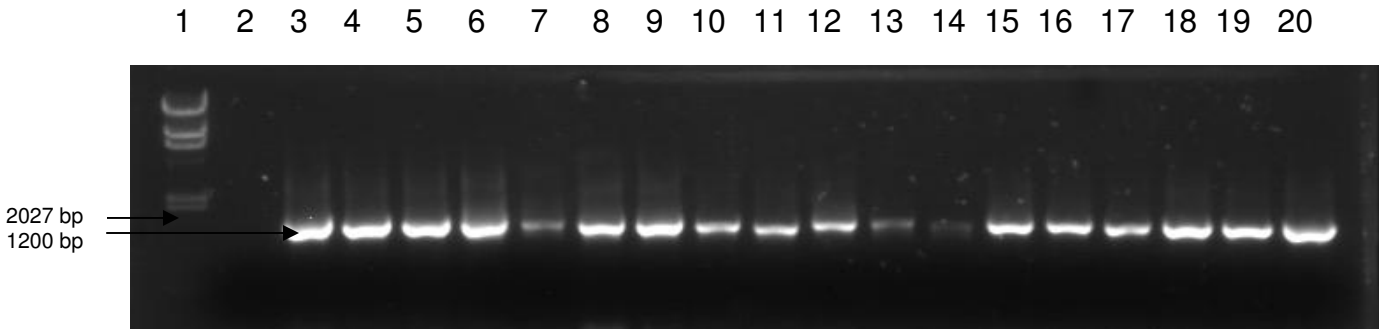
APPENDIX B1

The figures below represent the agarose gel electrophoresis results of amplified PCR products of the organisms isolated from the multi-channelled flow cells after exposure to varying metal [aluminium (Al), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and zinc (Zn)] concentrations.



LANE 1 – Marker (Lambda DNA/HIND III)

LANE 2 – Negative Control

LANE 3 – V1

LANE 4 – V2

LANE 5 – V3

LANE 6 – V4

LANE 7 – V5

LANE 8 – V6

LANE 9 – V7

LANE 10 – V8

LANE 11 – V9

LANE 12 – V10

LANE 13 – V11

LANE 14 – V12

LANE 15 – V13

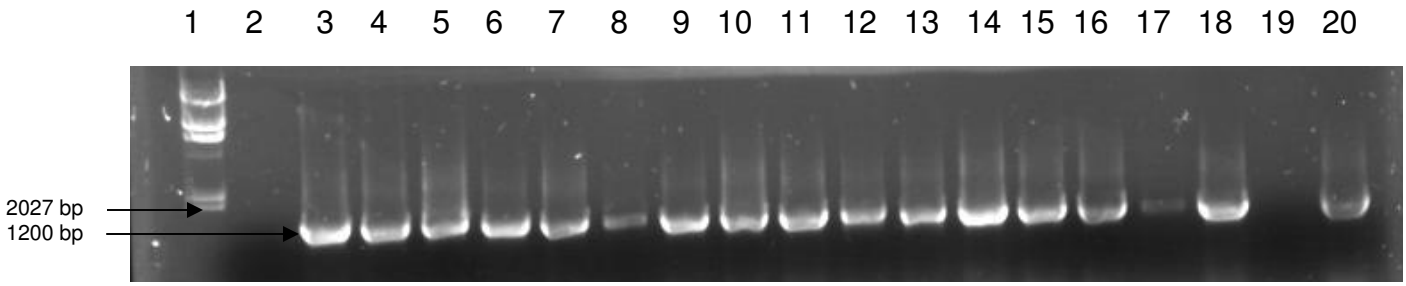
LANE 16 – V14

LANE 17 – V15

LANE 18 – V16

LANE 19 – V17

LANE 20 – V18



LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – Negative Control
LANE 3 – V19
LANE 4 – V20
LANE 5 – V21
LANE 6 – V22
LANE 7 – V23
LANE 8 – V24
LANE 9 – V25
LANE 10 – V26
LANE 11 – V27
LANE 12 – V28
LANE 13 – V29
LANE 14 – V30
LANE 15 – V31
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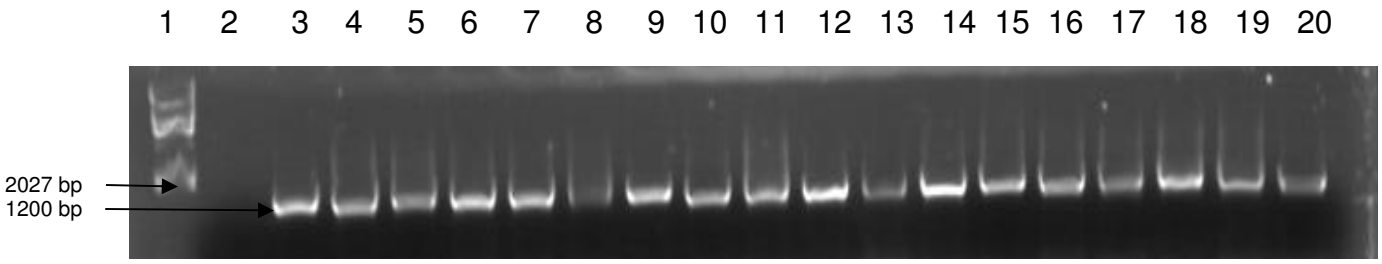
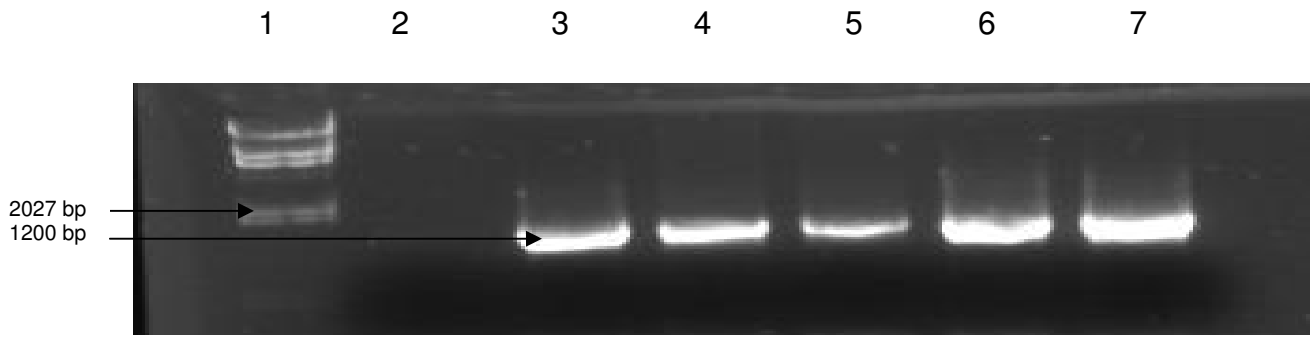
LANE 18 – V34
LANE 19 – V35
LANE 20 – V36



LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – Negative Control
LANE 3 – V37
LANE 4 – V38
LANE 5 – V39
LANE 6 – V40
LANE 7 – V41
LANE 8 – V42
LANE 9 – V43
LANE 10 – V44
LANE 11 – V45
LANE 12 – V46
LANE 13 – V47
LANE 14 – V48
LANE 15 – V49
LANE 16 – V50
LANE 17 – V51
LANE 18 – V52
LANE 19 – V53
LANE 20 – V54



LANE 1 – Marker (Lambda DNA/HIND III)

LANE 2 – Negative Control

LANE 3 – V55

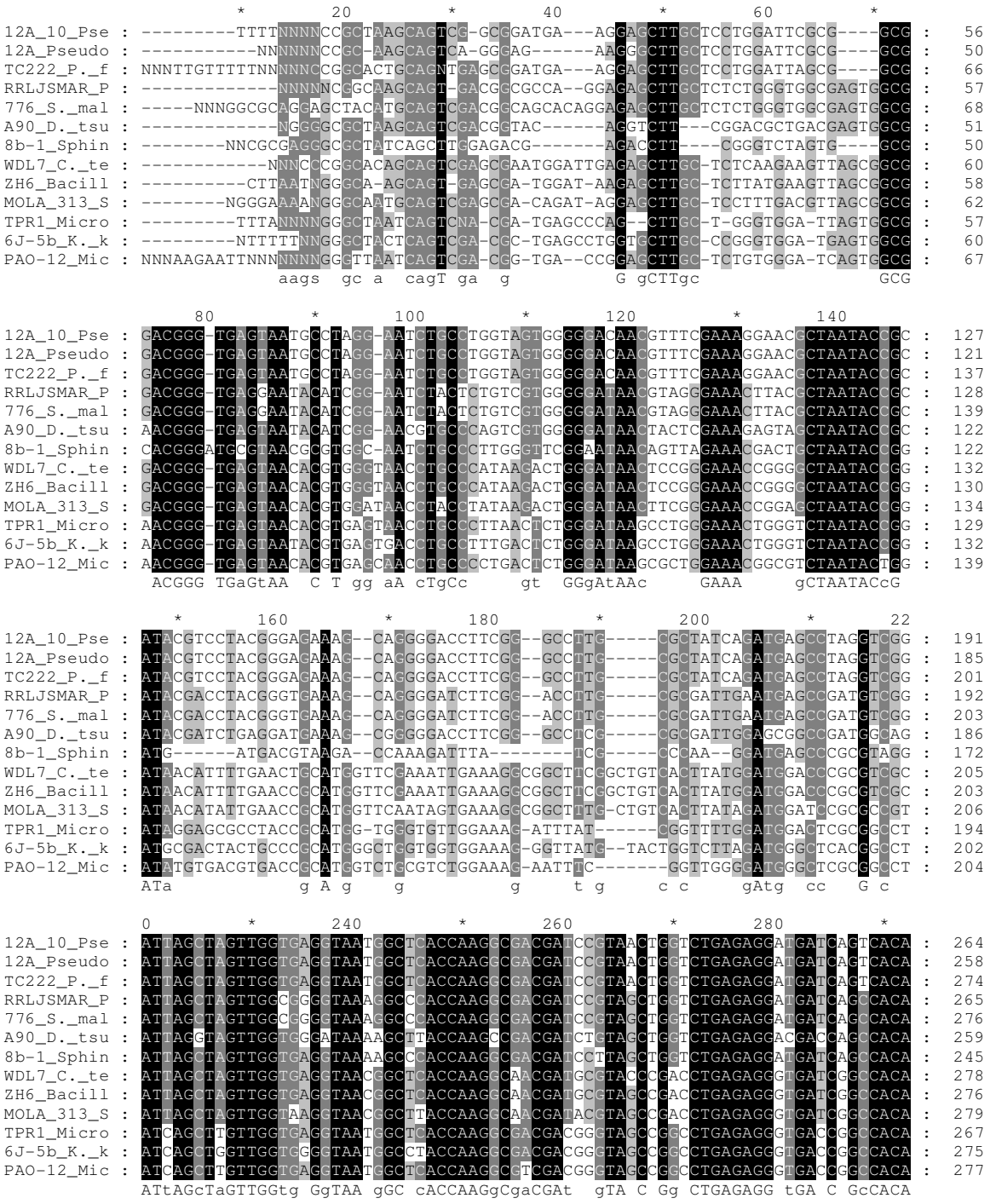
LANE 4 – V56

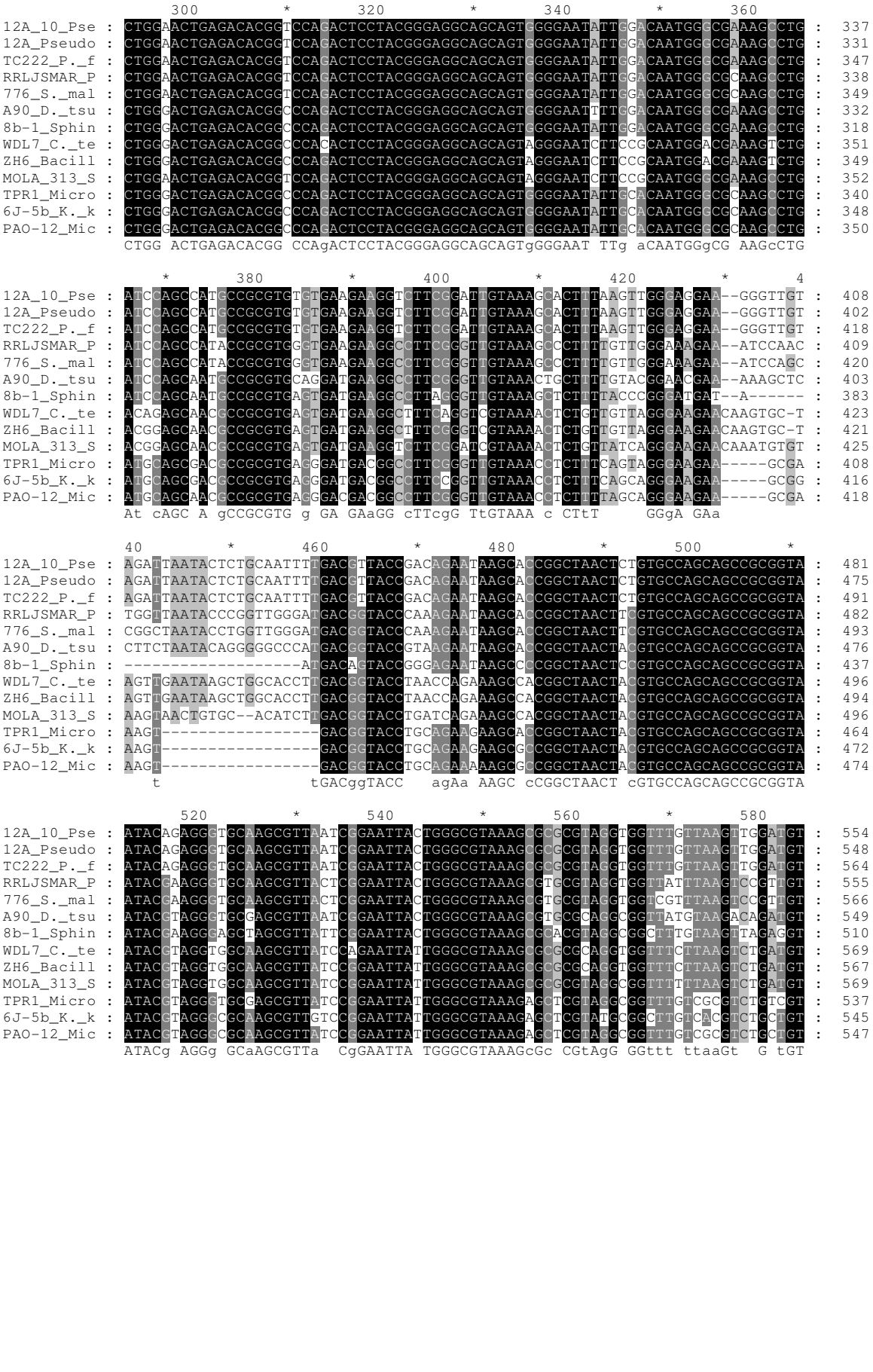
LANE 5 – V57

LANE 6 – V58

LANE 7 – V59

APPENDIX B2

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12A_Pseudo : CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG : 331
TC222_P._f : CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG : 347
RRLJSMAR_P : CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG : 338
776_S._mal : CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG : 349
A90_D._tsu : CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTG : 332
8b-1_Sphin : CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG : 318
WDL7_C._te : CTGGGACTGAGACACGGCCCACACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG : 351
ZH6_Bacill : CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG : 349
MOLA_313_S : CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTG : 352
TPR1_Micro : CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG : 340
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RRLJSMAR_P : AICCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAA--ATCCAAC : 409
776_S._mal : AICCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAA--ATCCAGC : 420
A90_D._tsu : AICCAGCAATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAA--AAAGCTC : 403
8b-1_Sphin : AICCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTACCCGGGATGAT--A------ : 383
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ZH6_Bacill : ACGGAGCAACGCCGCGTGACTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGC-T : 421
MOLA_313_S : ACGGAGCAACGCCGCGTGACTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCAGGGAAGAACAAATGTGT : 425
TPR1_Micro : AIGCAGCGACGCCGCGTGACGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAA-----GCGA : 408
6J-5b_K._k : AIGCAGCGACGCCGCGTGACGGATGACGGCCTTCCGGTTGTAAACCTCTTTCAGCAGGGAAGAA-----GCGG : 416
PAO-12_Mic : AIGCAGCAACGCCGCGTGACGGACGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAA-----GCGA : 418
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TC222_P._f : AGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA : 491
RRLJSMAR_P : TGGTTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTA : 482
776_S._mal : CGGCTAATACCTGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTA : 493
A90_D._tsu : CTTCTAATACAGGGGGCCCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTA : 476
8b-1_Sphin : --------------------ATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTA : 437
WDL7_C._te : AGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA : 496
ZH6_Bacill : AGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA : 494
MOLA_313_S : AAGTAACTGTGC--ACATCTTGACGGTACCTGATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA : 496
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6J-5b_K._k : AAGT----------------GACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTA : 472
PAO-12_Mic : AAGT----------------GACGCTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTA : 474
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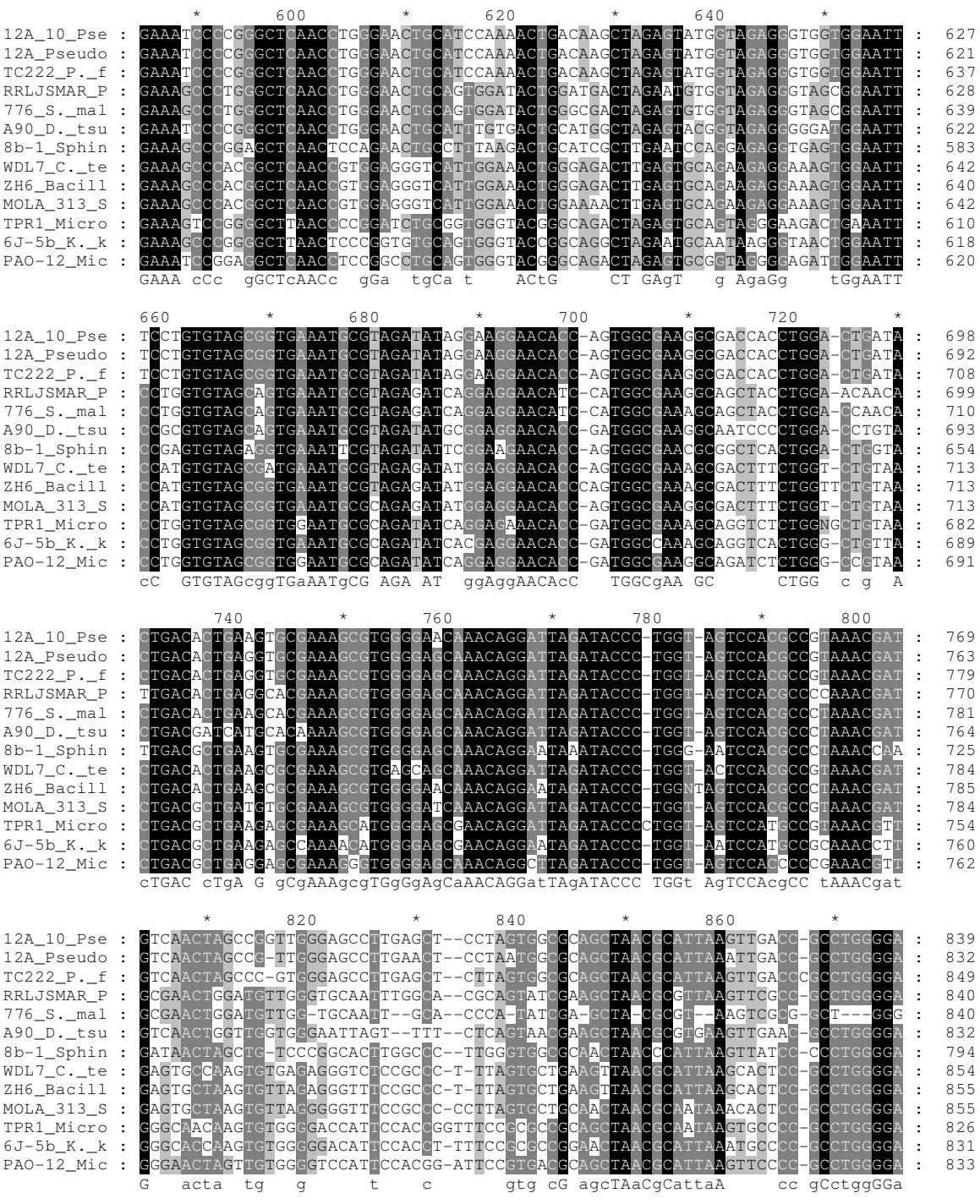
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A90_D._tsu
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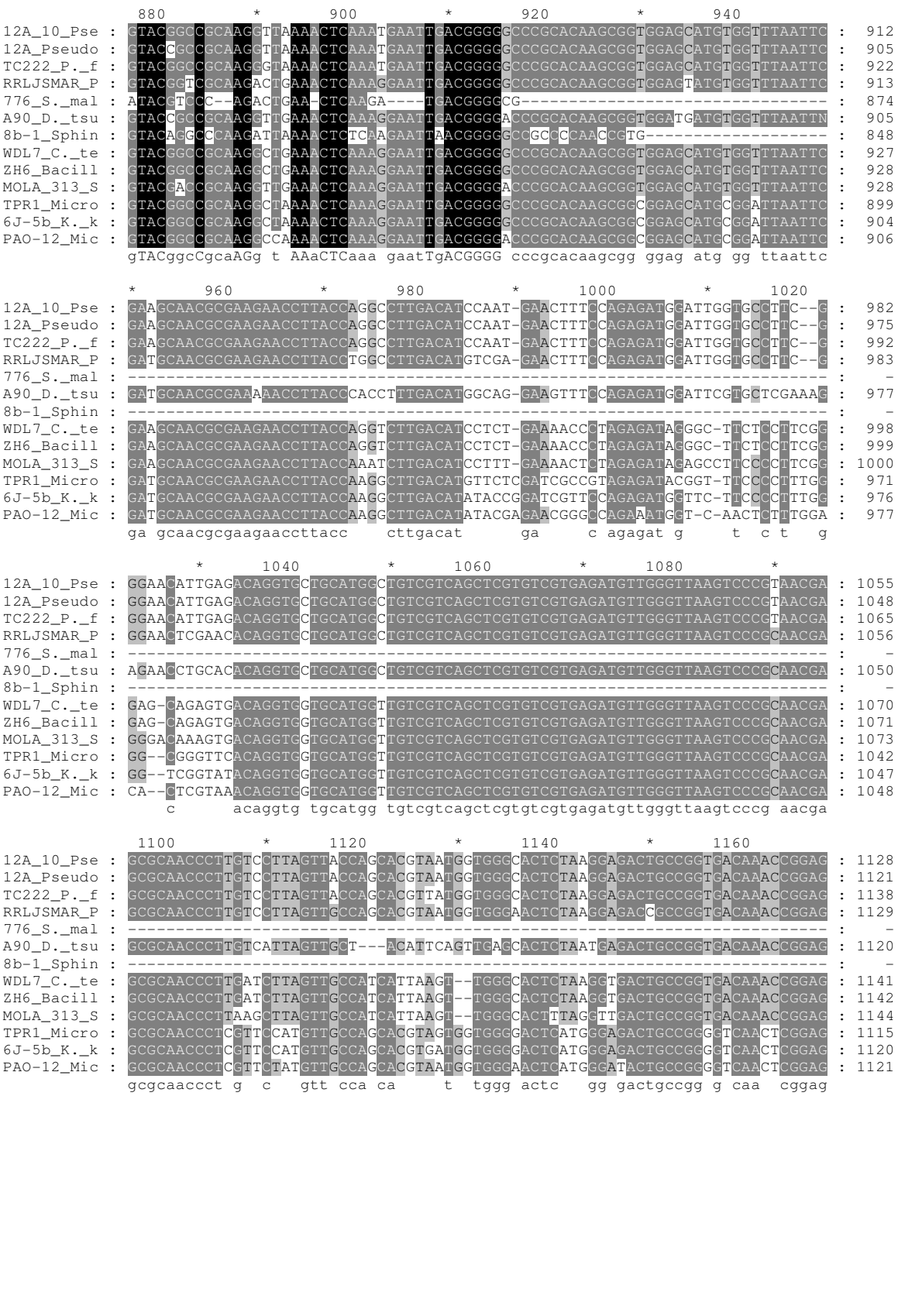
TPR1_Micro
6J-5b_K._k
PAO-12_Mic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776_S._mal : ATACGTCCC--AGACTGAA-CTCAAGA----TGACGGGGCG---------- : 874
A90_D._tsu : GTACCGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGTTTAATTN : 905
8b-1_Sphin : GTACAGGCCCAAGATTAAAACTCTCAAGAATTAACGGGGGCCGCCCCAACCGTG---------- : 848
WDL7_C._te : GTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC : 927
ZH6_Bacill : GTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC : 928
MOLA_313_S : GTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTC : 928
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* 960 * 980 * 1000 * 1020
12A_10_Pse : GAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAAT-GAACTTTCCAGAGATGGATTGGTGCCTTC--G : 982
12A_Pseudo : GAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAAT-GAACTTTCCAGAGATGGATTGGTGCCTTC--G : 975
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RRLJSMAR_P : GATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCGA-GAACTTTCCAGAGATGGATTGGTGCCTTC--G : 983
776_S._mal : ---------- : -
A90_D._tsu : GATGCAACGCGAAAAACCTTACCCACCTTGACATGGCAG-GAAGTTTCCAGAGATGGATTCGTGCTCGAAAG : 977
8b-1_Sphin : ---------- : -
WDL7_C._te : GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCT-GAAAACCCTAGAGATAGGGC-TTCTCCTTCGG : 998
ZH6_Bacill : GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCT-GAAAACCCTAGAGATAGGGC-TTCTCCTTCGG : 999
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6J-5b_K._k : GATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGATCGTTCCAGAGATGGTTC-TTCCCCTTTGG : 976
PAO-12_Mic : GATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAGAACGGGCCAGAAATGGT-C-AACTCTTTGGA : 977
ga gcaacgcgaagaaccttacc cttgacat ga c agagat g t c t g
* 1040 * 1060 * 1080 *
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12A_Pseudo : GGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGA : 1048
TC222_P._f : GGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGA : 1065
RRLJSMAR_P : GGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1056
776_S._mal : ---------- : -
A90_D._tsu : AGAACCTGCACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1050
8b-1_Sphin : ---------- : -
WDL7_C._te : GAG-CAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1070
ZH6_Bacill : GAG-CAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1071
MOLA_313_S : GGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1073
TPR1_Micro : GG--CGGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1042
6J-5b_K._k : GG--TCGGTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1047
PAO-12_Mic : CA--CTCGTAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA : 1048
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1100 * 1120 * 1140 * 1160
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12A_Pseudo : GCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAG : 1121
TC222_P._f : GCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAG : 1138
RRLJSMAR_P : GCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACGCCGGTGACAAACCGGAG : 1129
776_S._mal : ---------- : -
A90_D._tsu : GCGCAACCCTTGTCATTAGTTGCT---ACATTCAGTTGAGCACTCTAATGAGACTGCCGGTGACAAACCGGAG : 1120
8b-1_Sphin : ---------- : -
WDL7_C._te : GCGCAACCCTTGATCTTAGTTGCCATCATTAAGT--TGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG : 1141
ZH6_Bacill : GCGCAACCCTTGATCTTAGTTGCCATCATTAAGT--TGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG : 1142
MOLA_313_S : GCGCAACCCTTAAGCTTAGTTGCCATCATTAAGT--TGGGCACTTTAGGTTGACTGCCGGTGACAAACCGGAG : 1144
TPR1_Micro : GCGCAACCCTCGTTCCATGTTGCCAGCACGTAGTGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAG : 1115
6J-5b_K._k : GCGCAACCCTCGTTCCATGTTGCCAGCACGTGATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAG : 1120
PAO-12_Mic : GCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTCAACTCGGAG : 1121
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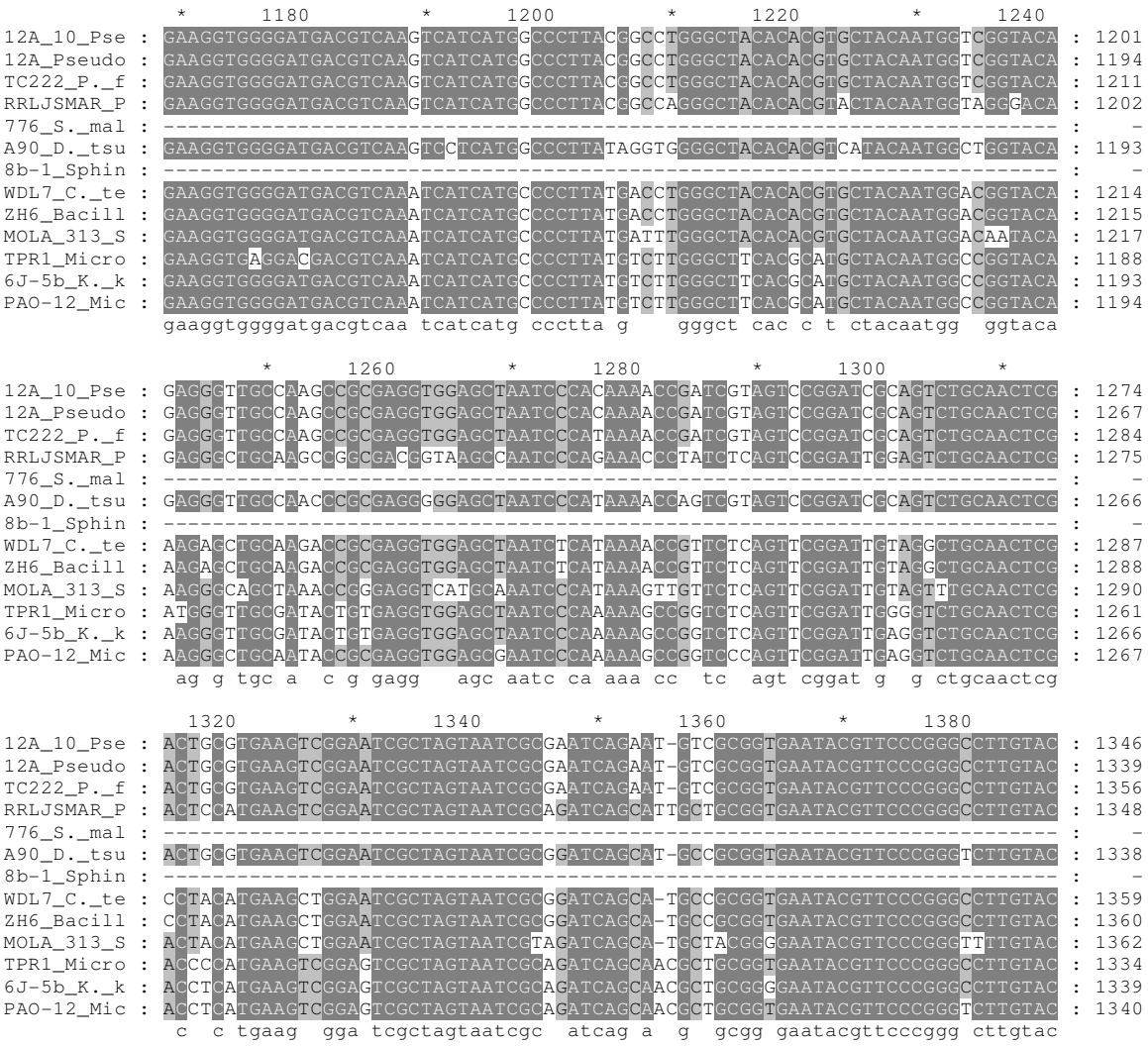
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TC222_P._f : GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACA : 1211
RRLJSMAR_P : GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTACAATGGTAGGGACA : 1202
776_S._mal : ---------- : -
A90_D._tsu : GAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCTGGTACA : 1193
8b-1_Sphin : ---------- : -
WDL7_C._te : GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACA : 1214
ZH6_Bacill : GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACA : 1215
MOLA_313_S : GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACA : 1217
TPR1_Micro : GAAGGTGAGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGGCATGCTACAATGGCCGGTACA : 1188
6J-5b_K._k : GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGGCATGCTACAATGGCCGGTACA : 1193
PAO-12_Mic : GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGGCATGCTACAATGGCCGGTACA : 1194
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* 1260 * 1280 * 1300 *
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TC222_P._f : GAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCG : 1284
RRLJSMAR_P : GAGGGCTGCAAGCCGGCGACGGTAAGGCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCG : 1275
776_S._mal : ---------- : -
A90_D._tsu : GAGGGTTGCCAACCGCGAGGGGGAGCTAATCCCATAAAACCAGTCGTAGTCCGGATCGCAGTCTGCAACTCG : 1266
8b-1_Sphin : ---------- : -
WDL7_C._te : AAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCG : 1287
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MOLA_313_S : AAGGGCAGCTAAACCGGGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTTTGCAACTCG : 1290
TPR1_Micro : ATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCG : 1261
6J-5b_K._k : AAGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCG : 1266
PAO-12_Mic : AAGGGCTGCAATACCGCGAGGTGGAGGGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCG : 1267
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1320 * 1340 * 1360 * 1380
12A_10_Pse : ACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAAT-GTCGCGGTGAATACGTTCCCGGGCCTTGTAC : 1346
12A_Pseudo : ACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAAT-GTCGCGGTGAATACGTTCCCGGGCCTTGTAC : 1339
TC222_P._f : ACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAAT-GTCGCGGTGAATACGTTCCCGGGCCTTGTAC : 1356
RRLJSMAR_P : ACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTAC : 1348
776_S._mal : ---------- : -
A90_D._tsu : ACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCAT-GCCGCGGTGAATACGTTCCCGGGTCTTGTAC : 1338
8b-1_Sphin : ---------- : -
WDL7_C._te : CCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGTTCCCGGGCCTTGTAC : 1359
ZH6_Bacill : CCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGTTCCCGGGCCTTGTAC : 1360
MOLA_313_S : ACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCA-TGCTACGGGGAATACGTTCCCGGGTTTGTAC : 1362
TPR1_Micro : ACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTAC : 1334
6J-5b_K._k : ACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGGGAATACGTTCCCGGGCCTTGTAC : 1339
PAO-12_Mic : ACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGTCTTGTAC : 1340
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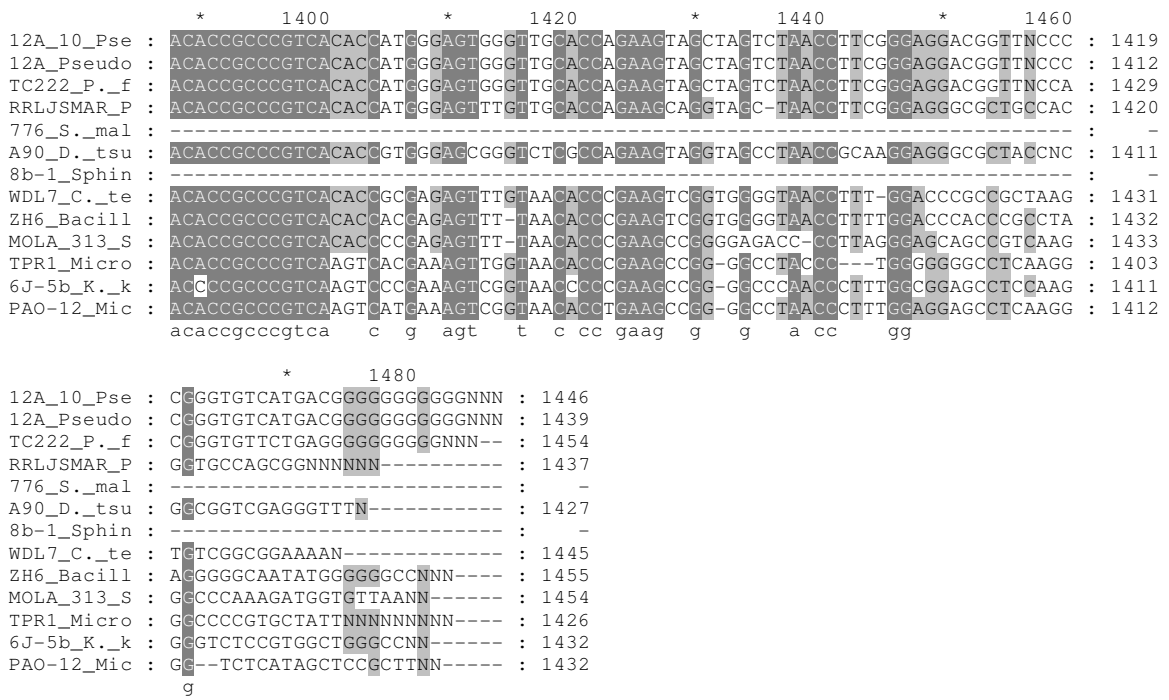
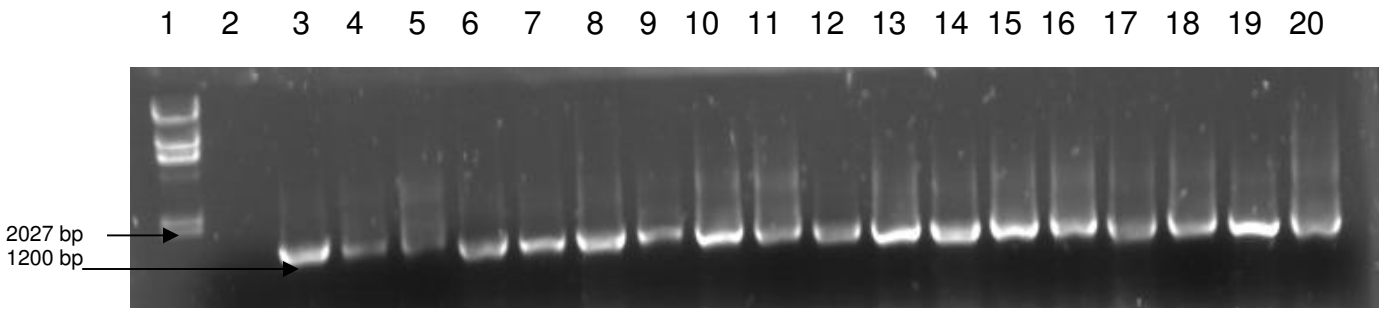


Alignment of the amino acid sequences of 13 isolates obtained after exposure to metal. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. The conserved regions are indicated with Roman numerals. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.

APPENDIX C1

The figures below represent the agarose gel electrophoresis results of purified PCR products of the organisms isolated from the biofilm samples (obtained from the bioballs) collected from the three compartments of the three-week, laboratory-scale bioreactor system, three days after start-up. The code for the organisms names are BI – Bioreactor Initial (after three days).



LANE 1 – Marker (Lambda DNA/HIND III)

LANE 2 – Negative Control

LANE 3 – BI1

LANE 4 – BI2

LANE 5 – BI3

LANE 6 – BI4

LANE 7 – BI5

LANE 8 – BI6

LANE 9 – BI7

LANE 10 – BI8

LANE 11 – BI9

LANE12 – BI10

LANE 13 – BI11

LANE 14 – BI12

LANE 15 – BI13

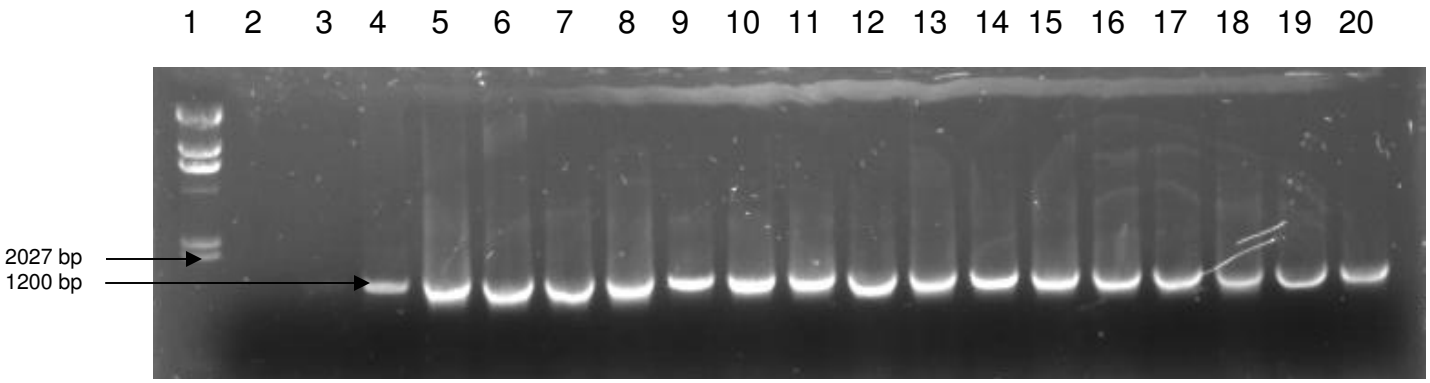
LANE 16 – BI14

LANE 17 – BI15

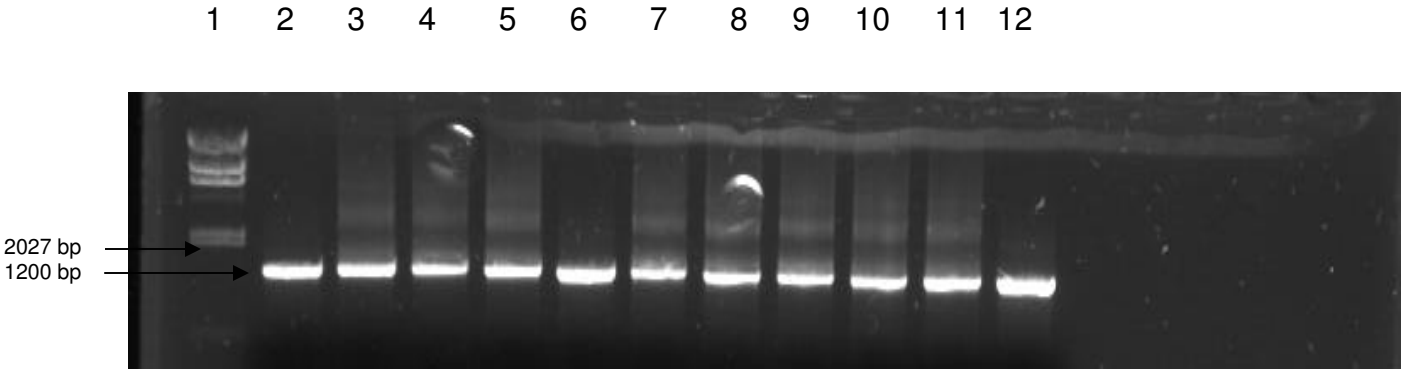
LANE 18 – BI16

LANE 19 – BI17

LANE 20 – BI18

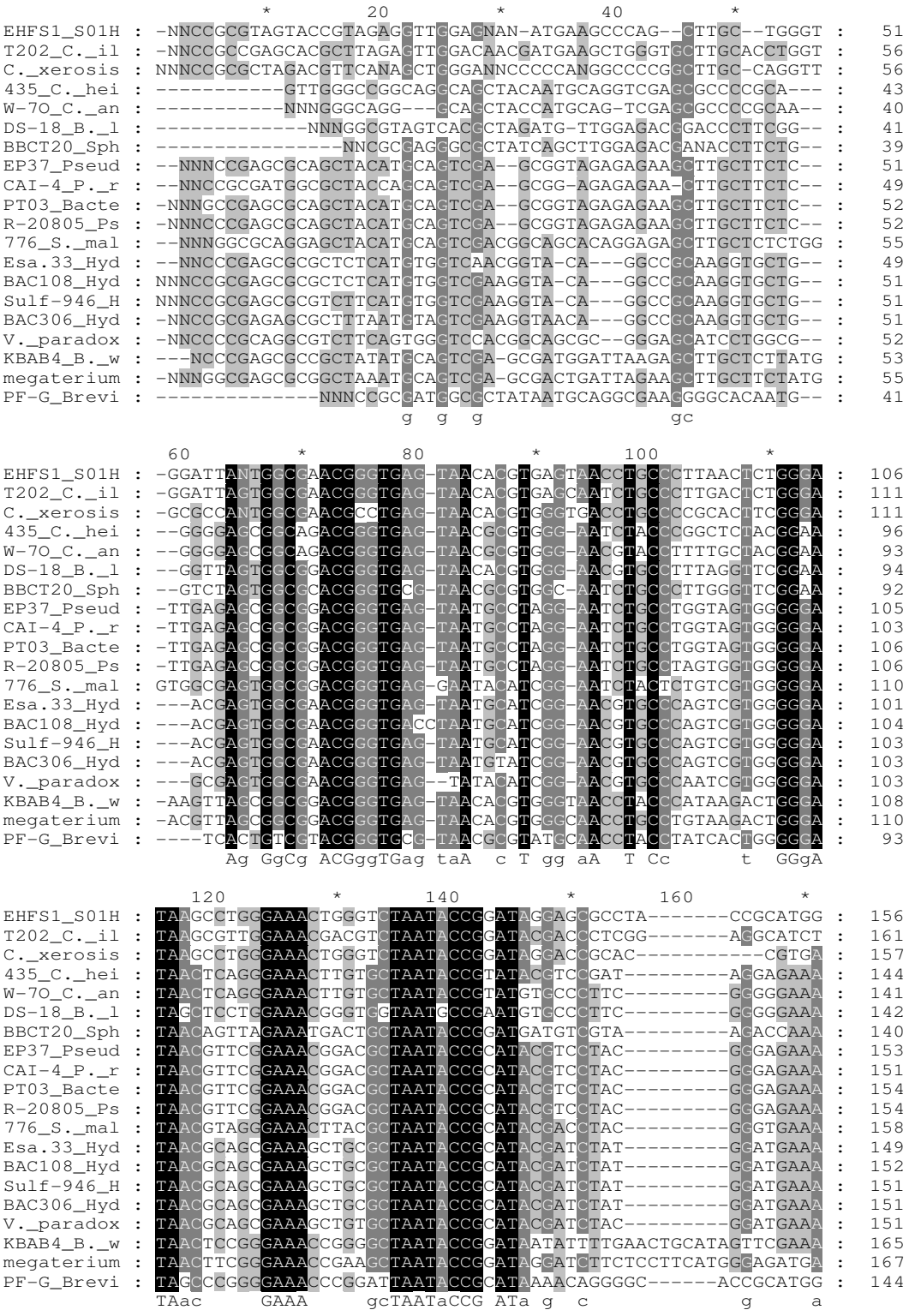


LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – Negative Control
LANE 3 – EMPTY
LANE 4 – BI19
LANE 5 – BI20
LANE 6 – BI21
LANE 7 – BI22
LANE 8 – BI23
LANE 9 – BI24
LANE 10 – BI25
LANE 11 – BI26
LANE 12 – BI27
LANE 13 – BI28
LANE 14 – BI29
LANE 15 – BI30
LANE 16 – BI31
LANE 17 – BI32
LANE 18 – BI33
LANE 19 – BI34
LANE 20 – BI35



LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – BI36
LANE 3 – BI37
LANE 4 – BI38
LANE 5 – BI39
LANE 6 – BI40
LANE 7 – BI41
LANE 8 – BI42
LANE 9 – BI43
LANE 10 – BI44
LANE 11 – BI45
LANE 12 – BI46

APPENDIX C2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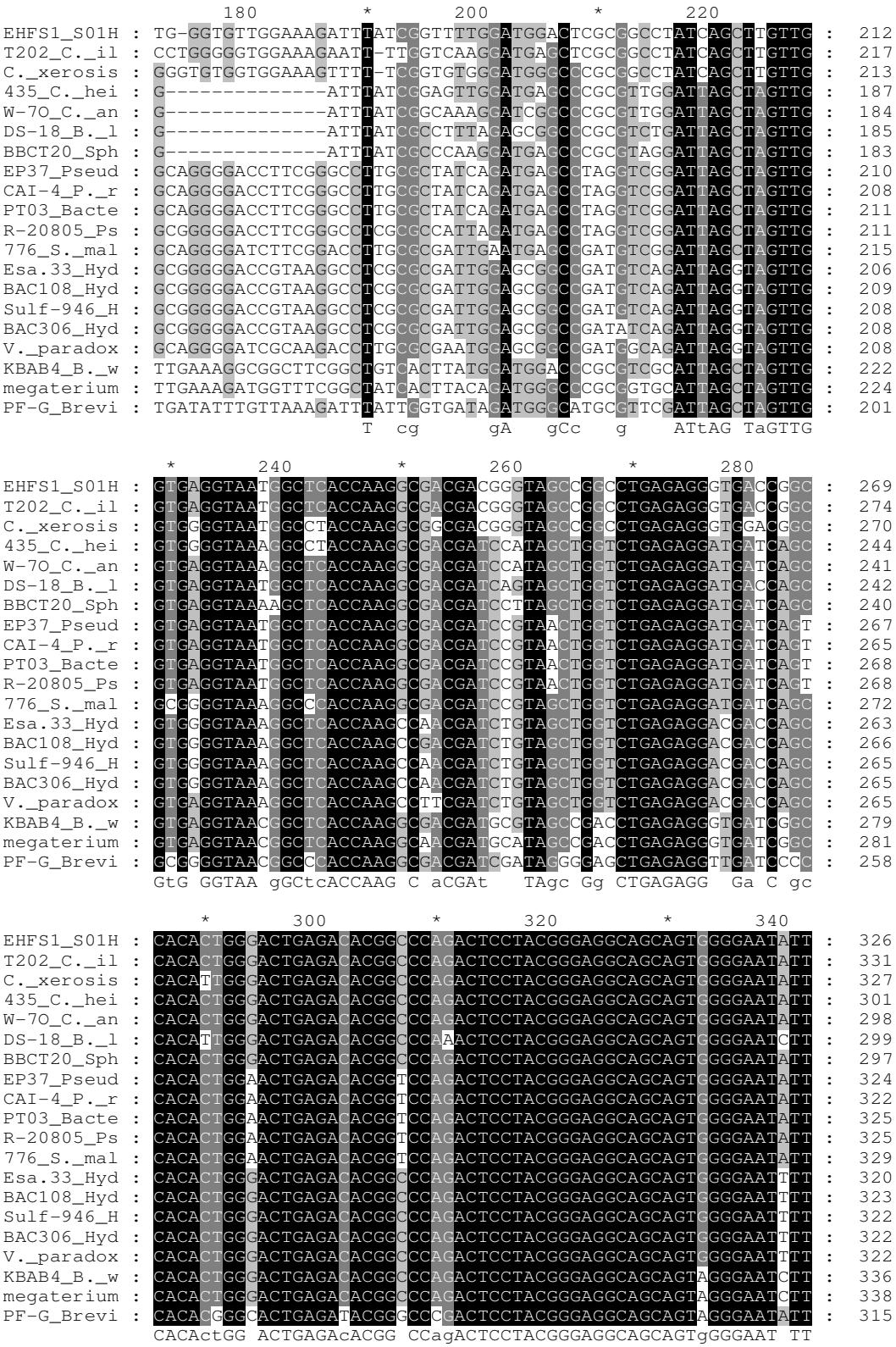
T202_C._il
C._xerosis
435_C._hei
W-7O_C._an
DS-18_B._l
BBCT20_Sph
EP37_Pseud
CAI-4_P._r
PT03_Bacte
R-20805_Ps
776_S._mal
Esa.33_Hyd
BAC108_Hyd
Sulf-946_H
BAC306_Hyd
V._paradox
KBAB4_B._w
megaterium
PF-G_Brevi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T202_C._il : CCTGGGGGTGGAAAGAATT-TTGGTCAAGGATGAGCTCGCGGCCTATCAGCTTGTTG : 217
C._xerosis : GGGTGTGGTGGAAAGTTTT-TCGGTGTGGGATGGGCCCGCGGCCTATCAGCTTGTTG : 213
435_C._hei : G--------------ATTTATCGGAGTTGGATGAGCCCGCGTTGGATTAGCTAGTTG : 187
W-7O_C._an : G--------------ATTTATCGGCAAAGGATCGGCCCGCGTTGGATTAGCTAGTTG : 184
DS-18_B._l : G--------------ATTTATCGCCTTTAGAGCGGCCCGCGTCTGATTAGCTAGTTG : 185
BBCT20_Sph : G--------------ATTTATCGCCCAAGGATGAGCCCGCGTAGGATTAGCTAGTTG : 183
EP37_Pseud : GCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTG : 210
CAI-4_P._r : GCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTG : 208
PT03_Bacte : GCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTG : 211
R-20805_Ps : GCGGGGGACCTTCGGGCCTCGCGCCATTAGATGAGCCTAGGTCGGATTAGCTAGTTG : 211
776_S._mal : GCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTG : 215
Esa.33_Hyd : GCGGGGGACCGTAAGGCCTCGCGCGATTGGAGCGGCCGATGTCAGATTAGGTAGTTG : 206
BAC108_Hyd : GCGGGGGACCGTAAGGCCTCGCGCGATTGGAGCGGCCGATGTCAGATTAGGTAGTTG : 209
Sulf-946_H : GCGGGGGACCGTAAGGCCTCGCGCGATTGGAGCGGCCGATGTCAGATTAGGTAGTTG : 208
BAC306_Hyd : GCGGGGGACCGTAAGGCCTCGCGCGATTGGAGCGGCCGATATCAGATTAGGTAGTTG : 208
V._paradox : GCAGGGGATCGCAAGACCTTGCGCGAATGGAGCGGCCGATGGCAGATTAGGTAGTTG : 208
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PF-G_Brevi : TGATATTTGTTAAAGATTTATTGGTGATAGATGGGCATGCGTTCGATTAGCTAGTTG : 201
T cg gA gCc g ATtAG TaGTTG
* 240 * 260 * 280
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T202_C._il : GTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGC : 274
C._xerosis : GTGGGGTAATGGCCTACCAAGGCGGCGACGGGTAGCCGGCCTGAGAGGGTGGACGGC : 270
435_C._hei : GTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGC : 244
W-7O_C._an : GTGAGGTAAAGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGC : 241
DS-18_B._l : GTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGACCAGC : 242
BBCT20_Sph : GTGAGGTAAAAGCTCACCAAGGCGACGATCCTTAGCTGGTCTGAGAGGATGATCAGC : 240
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776_S._mal : GCGGGGTAAAGGCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGC : 272
Esa.33_Hyd : GTGGGGTAAAGGCTCACCAAGCCAACGATCTGTAGCTGGTCTGAGAGGACGACCAGC : 263
BAC108_Hyd : GTGGGGTAAAGGCTCACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGC : 266
Sulf-946_H : GTGGGGTAAAGGCTCACCAAGCCAACGATCTGTAGCTGGTCTGAGAGGACGACCAGC : 265
BAC306_Hyd : GTGGGGTAAAGGCTCACCAAGCCAACGATCTGTAGCTGGTCTGAGAGGACGACCAGC : 265
V._paradox : GTGAGGTAAAGGCTCACCAAGCCTTCGATCTGTAGCTGGTCTGAGAGGACGACCAGC : 265
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C._xerosis : CACATTGGCACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT : 327
435_C._hei : CACACTGGCACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT : 301
W-7O_C._an : CACACTGGCACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT : 298
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BAC306_Hyd : CACACTGGCACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTT : 322
V._paradox : CACACTGGCACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTT : 322
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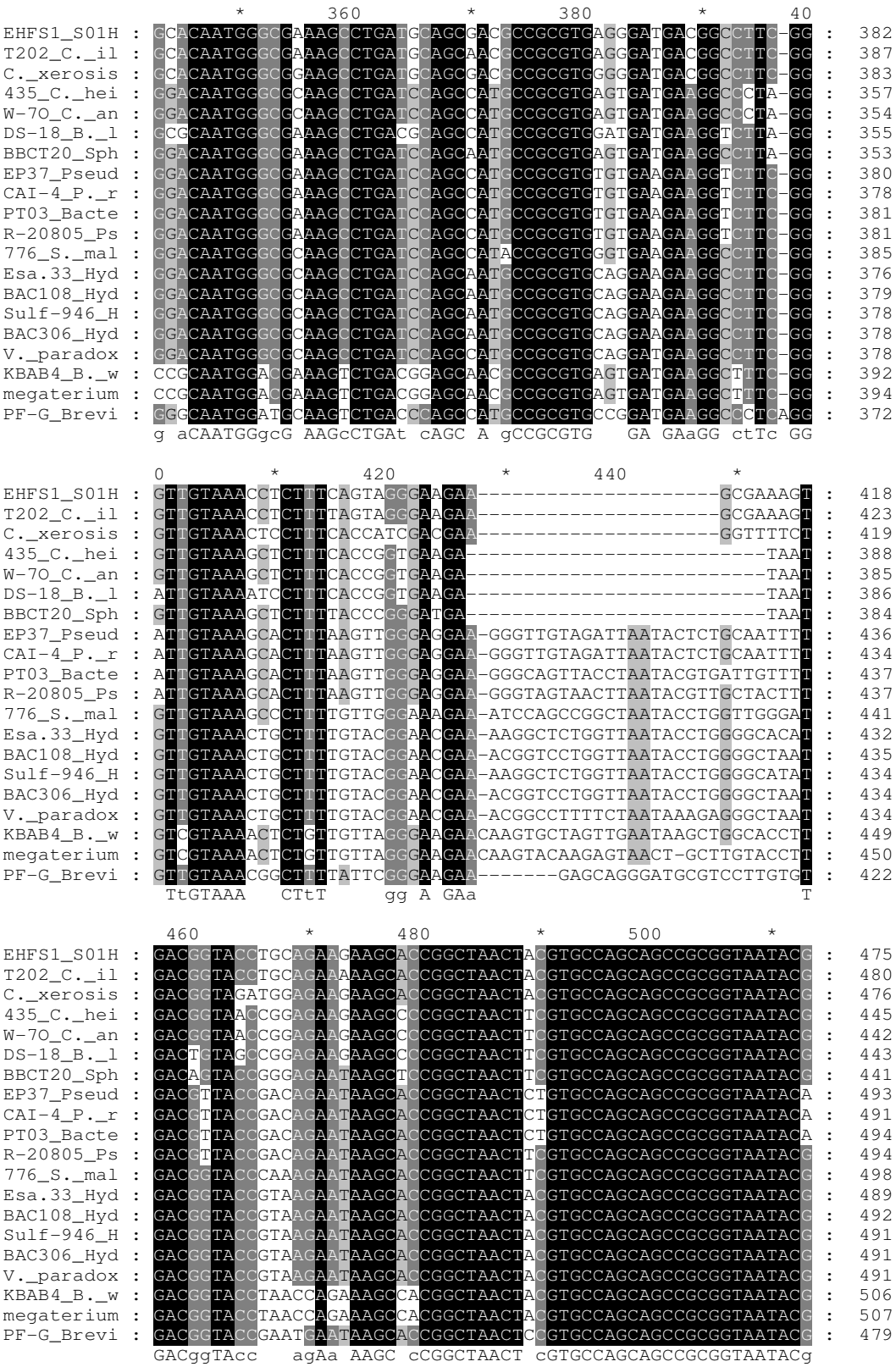
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C._xerosis
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CAI-4_P._r
PT03_Bacte
R-20805_Ps
776_S._mal
Esa.33_Hyd
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Sulf-946_H
BAC306_Hyd
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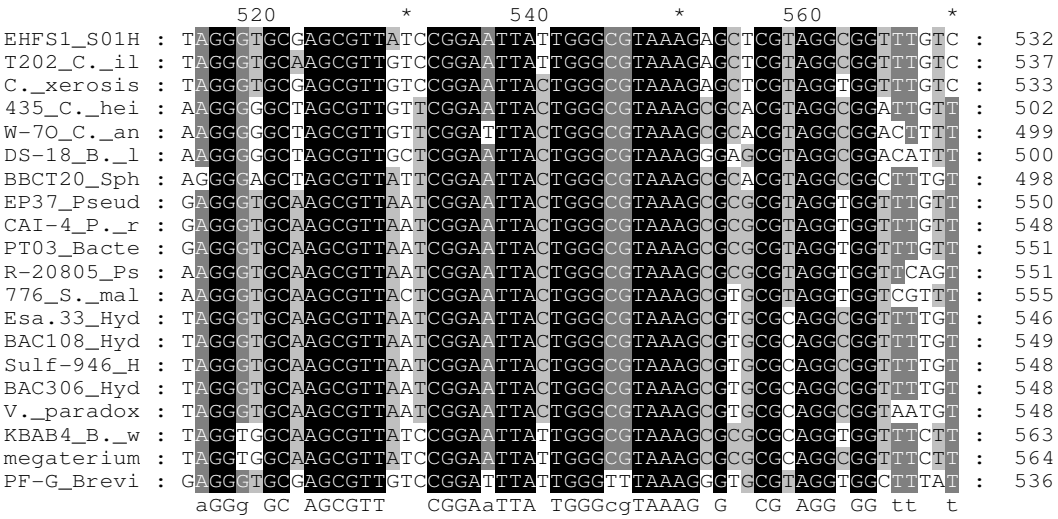
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W-7O_C._an : AAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGACTTTT : 499
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BAC108_Hyd : TAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGT : 549
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580 * 600 * 620
EHFS1_S01H : GCGTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGCA-GA : 588
T202_C._il : GCGTCTGCTGTGAAATCTGGGGGCTCAACCCCCAGCCTGCAGTGGGTACGGCA-GA : 593
C._xerosis : GCGTCGTCTGTGAAATTCCGGGGCTTAACTCCGGGCGTGCAGGCGATACGGCATAA : 590
435_C._hei : AAGTTAGGGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTAATACTGGCA-AT : 558
W-7O_C._an : AAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGAA-GT : 555
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BBCT20_Sph : AAGTTACAGGTGAAAGCCGGGGCTCAACTCCGGAATTGCCTTTAAGACTGCAT-CG : 554
EP37_Pseud : AAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACT-GA : 606
CAI-4_P._r : AAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACT-GA : 604
PT03_Bacte : AAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGAAC-GA : 607
R-20805_Ps : AAGTTGGAAGTGAAATCCCCGGGCTCAACCTGGGAACTGCTTTCAAAACTGCTG-AG : 607
776_S._mal : AAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGC-GA : 611
Esa.33_Hyd : AAGACAGGCGTGAAATCCCCGGGCTTAACCTGGGAATTGCGTTTGTGACTGCAA-GG : 602
BAC108_Hyd : AAGACAGGCGTGAAATCCCCGGGCTTAACCTGGGAATTGCGCTTGTGACTGCAA-GG : 605
Sulf-946_H : AAGACAGGCGTGAAATCCCCGGGCTCAACCTGGGAATTGCGCTTGTGACTGCAA-GG : 604
BAC306_Hyd : AAGACAGGCGTGAAATCCCCGGGCTTAACCTGGGAATGGCGCTTGTGACTGCAA-AG : 604
V._paradox : AAGACAGTTGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGTGACTGCAT-TG : 604
KBAB4_B._w : AAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGA-GA : 619
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T202_C._il : CTAGAGTGCGCTACGGCAGA-TTGGAATTCCTGGTGTA-GCGGTGGAA-TGCGCAGA : 647
C._xerosis : CTTGAGTACTGTACGGCAGA-CTGGAATTCCTGGTGTA-GCGGTGAAAATGCGCAGA : 645
435_C._hei : CAAGAGTCCGAGAGAGCTGA-GTGGAATTCCGAGTGTA-GAGGTGAAA-TTCGTAGA : 612
W-7O_C._an : CTTGAGTATGCTAGAGCTGA-GTGGAATTCCGAGTGTA-GAGGTGAAA-TTCGTAGA : 609
DS-18_B._l : CTTGAGTGTGAGAGAGCTAT-GTGGAACTCCGAGTGTA-GAGGTGAAA-TTCGTAGA : 610
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PT03_Bacte : ATAGAGTATGCAAGAGGGTG-GTGGAATTTCCTGTGTA-GCGGTGAAA-TGCGTAGA : 661
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776_S._mal : CTAGAGTGTGCTAGAGGGTA-GCGGAATTCCTGGTGTA-GCAGTGAAA-TGCGTAGA : 665
Esa.33_Hyd : CTGGAGTGCGCCACAGGGGG-ATGGAATTCCGCGTGTA-GCAGTGAAA-TGCGTAGA : 656
BAC108_Hyd : CTGGAATGCGCCAGAGGGGG-ATGGAATTCCGCGTGTA-GCAGTGAAA-TGCGTAGA : 659
Sulf-946_H : CTGGAGTGCGCCAGAGGGGG-ATGGAATTCCGCGTGTA-GCAGTGAAA-TGCGTAGA : 658
BAC306_Hyd : CTGGAGTGCGCCAGAGGGGG-ATGGAATTCCGCGTGTAAGCAGTGAAA-GGCGTAGA : 659
V._paradox : CTGGAGTACGCCAGAGGGGGGATGAAATCCCGCCTGTAAGCAGTGAAA-TGCGTAAA : 660
KBAB4_B._w : CTTGAGTGCACAAGAGGAAA-GTGGAATTCCATGTGTA-GCGGTGAAA-TGCGTAGA : 673
megaterium : CTTGAGTGCACAAGAGCAAAA-CCGGAATTCCACGTGTA-GCGGTGAAA-TGCGTAGA : 674
PF-G_Brevi : CTTGAAATAATTGGAGGCTG-CCGGAATGGATGGTGTA-GCGGTGAAA-TGCATAGA : 646
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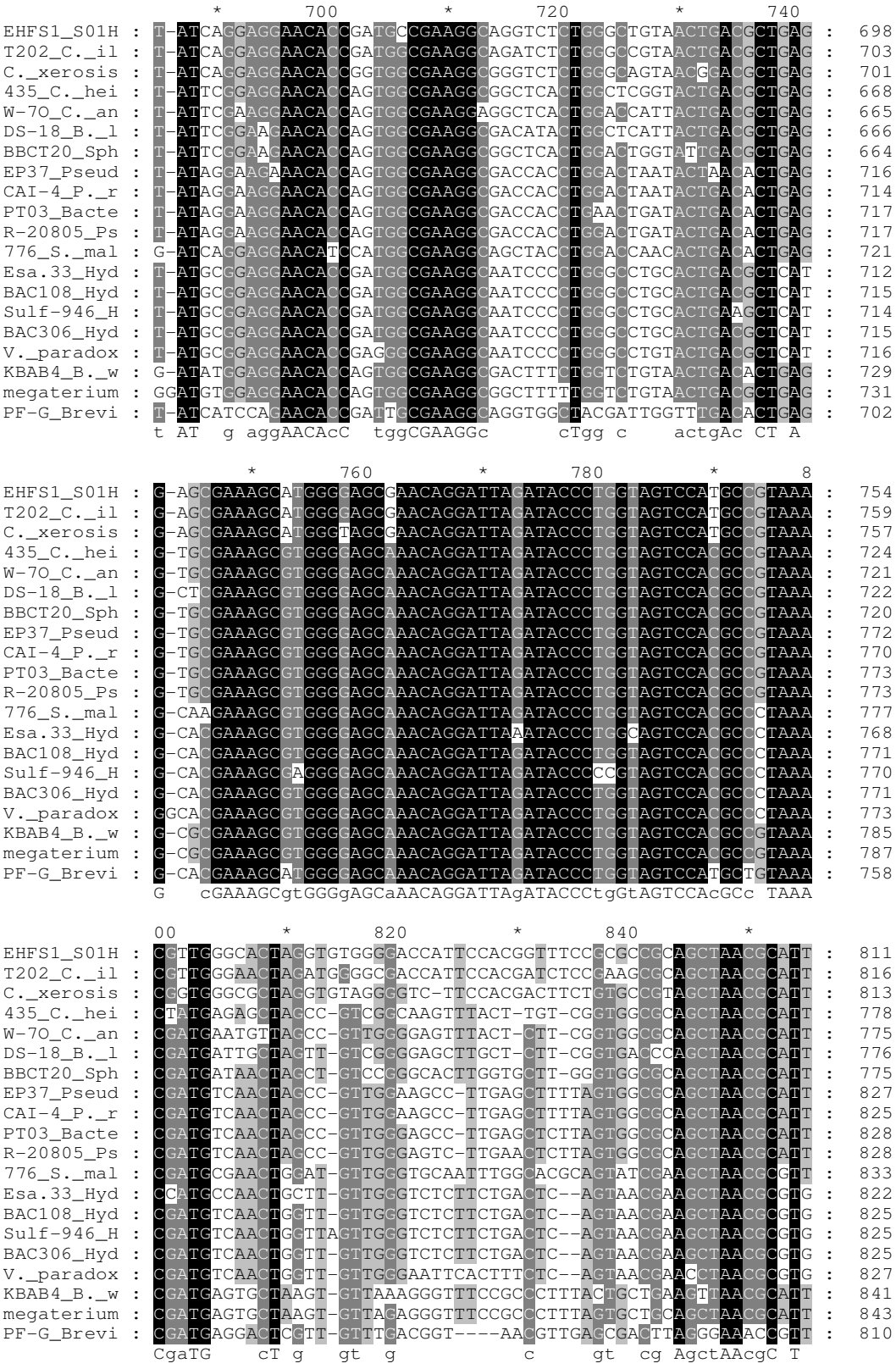
EHFS1_S01H
T202_C._il
C._xerosis
435_C._hei
W-7O_C._an
DS-18_B._l
BBCT20_Sph
EP37_Pseud
CAI-4_P._r
PT03_Bacte
R-20805_Ps
776_S._mal
Esa.33_Hyd
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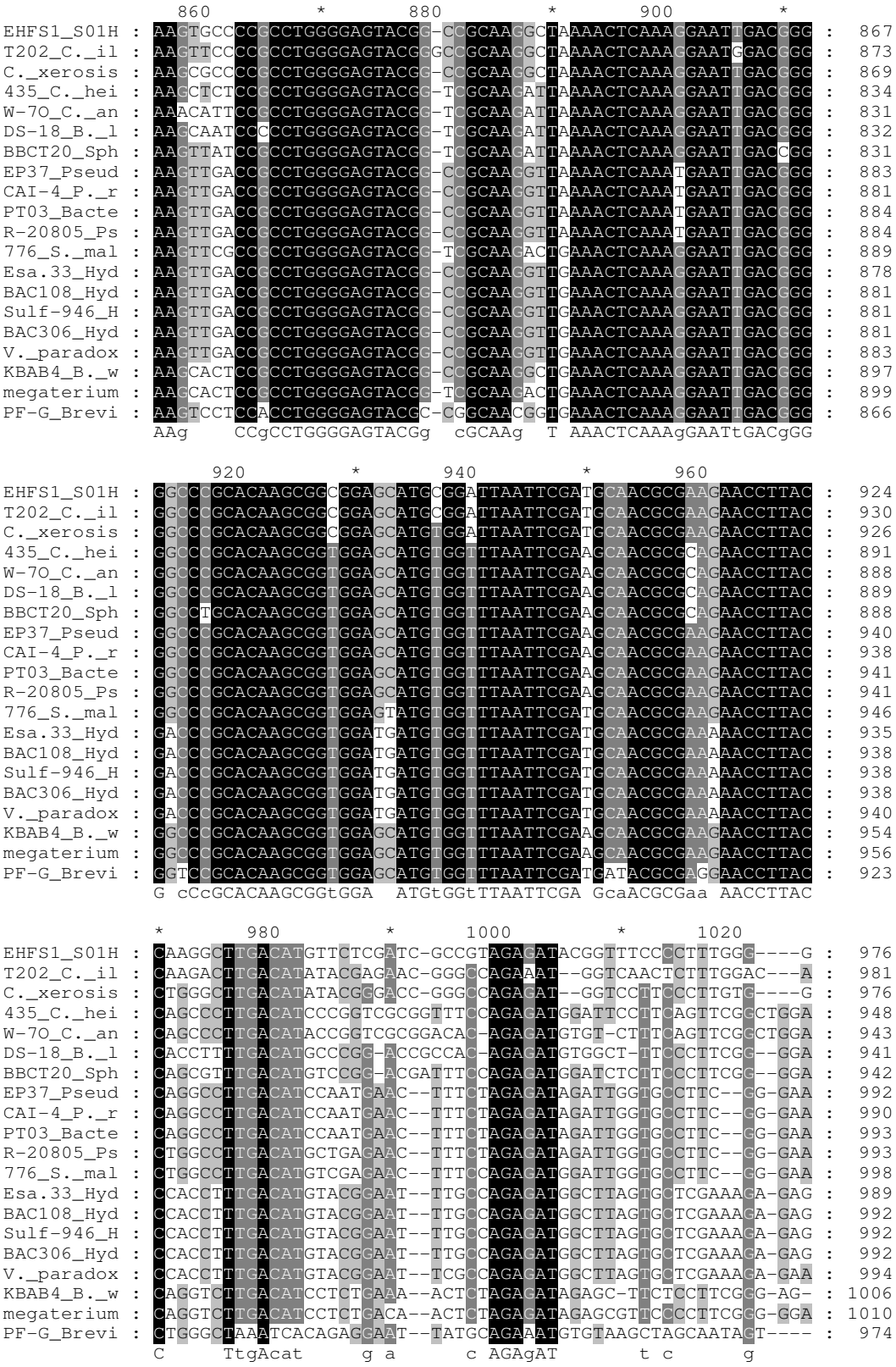
BAC306_Hyd
V._paradox
KBAB4_B._w
megaterium
PF-G_Brevi


860 * 880 * 900 *
EHFS1_S01H : AAGTGCCCCGCCTGGGGAGTACGG-CGGCAAGGCTAAAACTCAAAGGAATTGACGGG : 867
T202_C._il : AAGTTCCCCGCCTGGGGAGTACGGGCGGCAAGGCTAAAACTCAAAGGAATGGACGGG : 873
C._xerosis : AAGCGCCCCGCCTGGGGAGTACGG-CGGCAAGGCTAAAACTCAAAGGAATTGACGGG : 869
435_C._hei : AAGCTCTCCGCCTGGGGAGTACGG-TCGCAAGATTAAAACTCAAAGGAATTGACGGG : 834
W-7O_C._an : AAACATTCCGCCTGGGGAGTACGG-TCGCAAGATTAAAACTCAAAGGAATTGACGGG : 831
DS-18_B._l : AAGCAATCCCCCTGGGGAGTACGG-TCGCAAGATTAAAACTCAAAGGAATTGACGGG : 832
BBCT20_Sph : AAGTTATCCGCCTGGGGAGTACGG-TCGCAAGATTAAAACTCAAAGGAATTGACCGG : 831
EP37_Pseud : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTAAAACTCAAATGAATTGACGGG : 883
CAI-4_P._r : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTAAAACTCAAATGAATTGACGGG : 881
PT03_Bacte : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTAAAACTCAAATGAATTGACGGG : 884
R-20805_Ps : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTAAAACTCAAATGAATTGACGGG : 884
776_S._mal : AAGTTCGCCGCCTGGGGAGTACGG-TCGCAAGACTGAAACTCAAAGGAATTGACGGG : 889
Esa.33_Hyd : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTGAAACTCAAAGGAATTGACGGG : 878
BAC108_Hyd : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTGAAACTCAAAGGAATTGACGGG : 881
Sulf-946_H : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTGAAACTCAAAGGAATTGACGGG : 881
BAC306_Hyd : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTGAAACTCAAAGGAATTGACGGG : 881
V._paradox : AAGTTGACCGCCTGGGGAGTACGG-CGGCAAGGTTGAAACTCAAAGGAATTGACGGG : 883
KBAB4_B._w : AAGCACTCCGCCTGGGGAGTACGG-CGGCAAGGCTGAAACTCAAAGGAATTGACGGG : 897
megaterium : AAGCACTCCGCCTGGGGAGTACGG-TCGCAAGACTGAAACTCAAAGGAATTGACGGG : 899
PF-G_Brevi : AAGTCCTCCACCTGGGGAGTACGC-CGGCAACGGTGAAACTCAAAGGAATTGACGGG : 866
AAg CCgCCTGGGGAGTACGg cGCAAg T AAACTCAAAgGAATtGACgGG
920 * 940 * 960
EHFS1_S01H : GGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTAC : 924
T202_C._il : GGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTAC : 930
C._xerosis : GGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTAC : 926
435_C._hei : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTAC : 891
W-7O_C._an : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTAC : 888
DS-18_B._l : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTAC : 889
BBCT20_Sph : GGCCTGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTAC : 888
EP37_Pseud : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC : 940
CAI-4_P._r : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC : 938
PT03_Bacte : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC : 941
R-20805_Ps : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC : 941
776_S._mal : GGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC : 946
Esa.33_Hyd : GACCCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTAC : 935
BAC108_Hyd : GACCCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTAC : 938
Sulf-946_H : GACCCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTAC : 938
BAC306_Hyd : GACCCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTAC : 938
V._paradox : GACCCGCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTAC : 940
KBAB4_B._w : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC : 954
megaterium : GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC : 956
PF-G_Brevi : GGTCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTAC : 923
G cCcGCACAAGCGGtGGA ATGtGGtTTAATTCGA GcaACGCGaa AACCTTAC
* 980 * 1000 * 1020
EHFS1_S01H : CAAGGCTTGACATGTTCTCGATC-GCCGTAGAGATACGGTTTCCCCTTTGGC----G : 976
T202_C._il : CAAGACTTGACATATACGAGAAC-GGGCCAGAAAT--GGTCAACTCTTTGGAC---A : 981
C._xerosis : CTGGGCTTGACATATACGGGACC-GGGCCAGAGAT--GGTCCTTCCCTTGTC----G : 976
435_C._hei : CAGCCCTTGACATCCCGGTCGCGGTTTCCAGAGATGGATTCCTTCAGTTCGCCTGGA : 948
W-7O_C._an : CAGCCCTTGACATACCGGTCGCGGACAC-AGAGATGTGT-CTTTCAGTTCGCCTGGA : 943
DS-18_B._l : CACCTTTTGACATGCCCGC-ACCGCCAC--AGAGATGTGGCT-TTCCTTCGC--GGA : 941
BBCT20_Sph : CAGCGTTTGACATGTCCGC-ACGATTTCCAGAGATGGATCTCTTCCCTTCGC--GGA : 942
EP37_Pseud : CAGGCCTTGACATCCAATCAAC--TTTCTAGAGATAGATTGGTGCCTTC--CG-GAA : 992
CAI-4_P._r : CAGGCCTTGACATCCAATCAAC--TTTCTAGAGATAGATTGGTGCCTTC--CG-GAA : 990
PT03_Bacte : CAGGCCTTGACATCCAATCAAC--TTTCTAGAGATAGATTGGTGCCTTC--CG-GAA : 993
R-20805_Ps : CTGGCCTTGACATGCTGACAAC--TTTCTAGAGATAGATTGGTGCCTTC--CG-GAA : 993
776_S._mal : CTGGCCTTGACATGTCGACAAC--TTTCCAGAGATGGATTGGTGCCTTC--CG-GAA : 998
Esa.33_Hyd : CCACCTTTGACATGTACGCAAT--TTGCCAGAGATGGCTTAGTGCTCGAAACA-GAG : 989
BAC108_Hyd : CCACCTTTGACATGTACGCAAT--TTGCCAGAGATGGCTTAGTGCTCGAAACA-GAG : 992
Sulf-946_H : CCACCTTTGACATGTACGCAAT--TTGCCAGAGATGGCTTAGTGCTCGAAACA-GAG : 992
BAC306_Hyd : CCACCTTTGACATGTACGCAAT--TTGCCAGAGATGGCTTAGTGCTCGAAACA-GAG : 992
V._paradox : CCACCTTTGACATGTACGCAAT--TCGCCAGAGATGGCTTAGTGCTCGAAACA-GAA : 994
KBAB4_B._w : CAGGTCTTGACATCCTCTCAAA--ACTCTAGAGATAGAGC-TTCTCCTTCGCG-AG- : 1006
megaterium : CAGGTCTTGACATCCTCTCACA--ACTCTAGAGATAGAGCGTTCCCCTTCGCG-GGA : 1010
PF-G_Brevi : CTGGGCTAAATCACAGAGCAAT--TATGCAGAAATGTGTAAGCTAGCAATACT----- : 974
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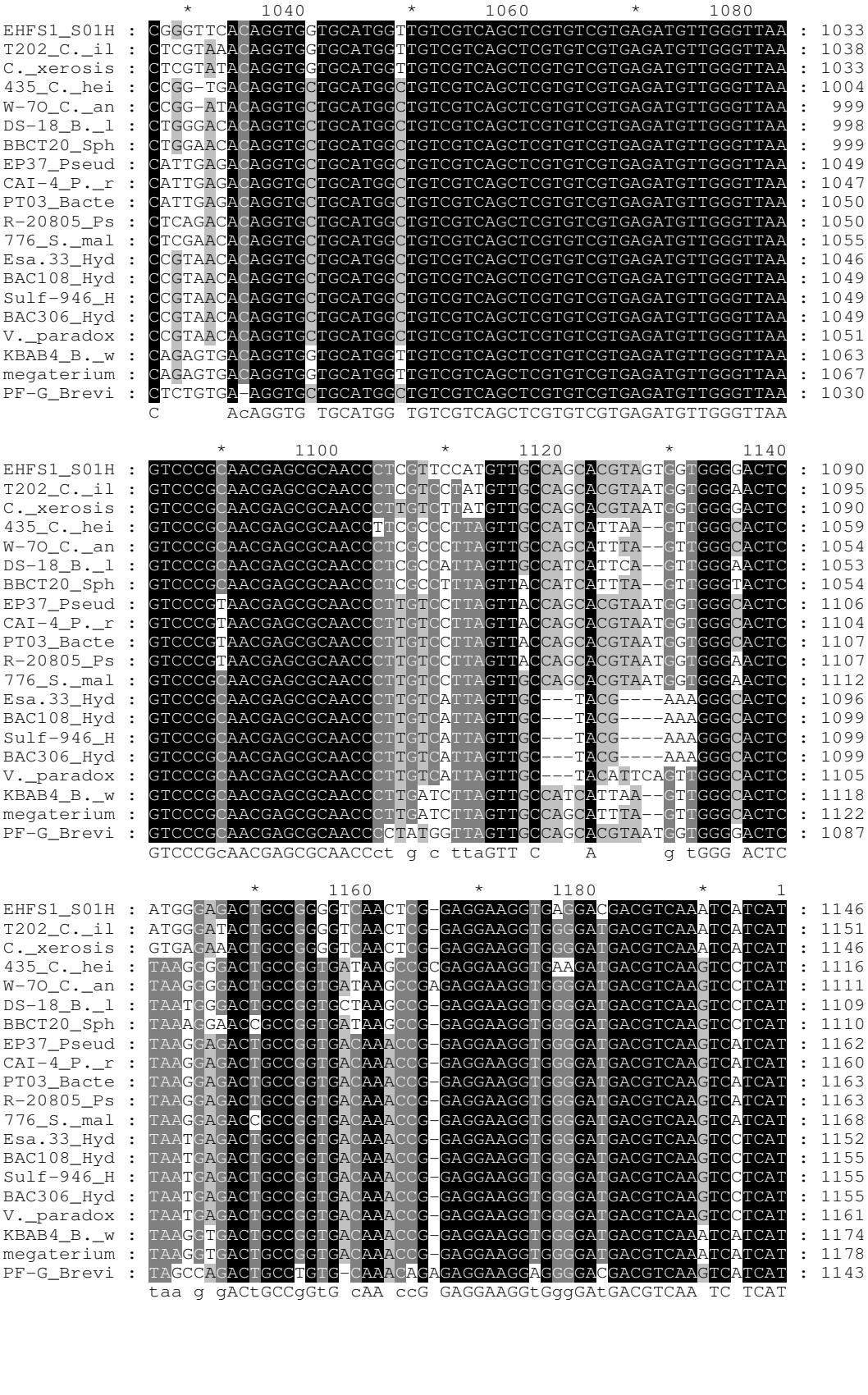
* 1040 * 1060 * 1080
EHFS1_S01H : CGGGTTCACAGGTGCTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1033
T202_C._il : CTCGTAAACAGGTGCTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1038
C._xerosis : CTCGTATACAGGTGCTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1033
435_C._hei : CCGG-TGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1004
W-7O_C._an : CCGG-ATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 999
DS-18_B._l : CTGGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 998
BBCT20_Sph : CTGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 999
EP37_Pseud : CATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1049
CAI-4_P._r : CATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1047
PT03_Bacte : CATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1050
R-20805_Ps : CTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1050
776_S._mal : CTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1055
Esa.33_Hyd : CCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1046
BAC108_Hyd : CCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1049
Sulf-946_H : CCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1049
BAC306_Hyd : CCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1049
V._paradox : CCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1051
KBAB4_B._w : CAGAGTGACAGGTGCTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1063
megaterium : CAGAGTGACAGGTGCTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1067
PF-G_Brevi : CTCTGTGA-AGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA : 1030
C AcAGGTG TGCATGG TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
* 1100 * 1120 * 1140
EHFS1_S01H : GTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAGTGGTGGGGACTC : 1090
T202_C._il : GTCCCGCAACGAGCGCAACCCTCGTCCTATGTTGCCAGCACGTAATGGTGGGAACTC : 1095
C._xerosis : GTCCCGCAACGAGCGCAACCCTTGTCTTATGTTGCCAGCACGTAATGGTGGGGACTC : 1090
435_C._hei : GTCCCGCAACGAGCGCAACCTTCGCCCTTAGTTGCCATCATTAA--GTTGGGCACTC : 1059
W-7O_C._an : GTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTTA--GTTGGGCACTC : 1054
DS-18_B._l : GTCCCGCAACGAGCGCAACCCTCGCCATTAGTTGCCATCATTCA--GTTGGGAACTC : 1053
BBCT20_Sph : GTCCCGCAACGAGCGCAACCCTCGCCTTTAGTTACCATCATTTA--GTTGGGTACTC : 1054
EP37_Pseud : GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTC : 1106
CAI-4_P._r : GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTC : 1104
PT03_Bacte : GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTC : 1107
R-20805_Ps : GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGAACTC : 1107
776_S._mal : GTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTC : 1112
Esa.33_Hyd : GTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGC---TACG----AAAGGGCACTC : 1096
BAC108_Hyd : GTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGC---TACG----AAAGGGCACTC : 1099
Sulf-946_H : GTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGC---TACG----AAAGGGCACTC : 1099
BAC306_Hyd : GTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGC---TACG----AAAGGGCACTC : 1099
V._paradox : GTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGC---TACATTCAGTTGGGCACTC : 1105
KBAB4_B._w : GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAA--GTTGGGCACTC : 1118
megaterium : GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTA--GTTGGGCACTC : 1122
PF-G_Brevi : GTCCCGCAACGAGCGCAACCCCTATGGTTAGTTGCCAGCACGTAATGGTGGGGACTC : 1087
GTCCCGcAACGAGCGCAACCct g c ttaGTT C A g tGGG ACTC
* 1160 * 1180 * 1
EHFS1_S01H : ATGGCAGACTGCCCGGCTCAACTCG-GAGGAAGGTGAGGACGACGTCAAATCATCAT : 1146
T202_C._il : ATGGCATACTGCCCGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCAT : 1151
C._xerosis : GTGAGAAACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCAT : 1146
435_C._hei : TAAGGGGACTGCCGGTCATAAGCCGCGAGGAAGGTGAAGATGACGTCAAGTCCTCAT : 1116
W-7O_C._an : TAAGGGGACTGCCGGTCATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1111
DS-18_B._l : TAATGGGACTGCCGGTCCTAAGCCGG-GAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1109
BBCT20_Sph : TAAAGGAACCGCCGGTCATAAGCCGG-GAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1110
EP37_Pseud : TAAGCAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCAT : 1162
CAI-4_P._r : TAAGGAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCAT : 1160
PT03_Bacte : TAAGCAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCAT : 1163
R-20805_Ps : TAAGCAGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCAT : 1163
776_S._mal : TAAGGAGACCGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCAT : 1168
Esa.33_Hyd : TAATGAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1152
BAC108_Hyd : TAATGAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1155
Sulf-946_H : TAATGAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1155
BAC306_Hyd : TAATGAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1155
V._paradox : TAATGAGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCAT : 1161
KBAB4_B._w : TAAGGTGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCAT : 1174
megaterium : TAAGGTGACTGCCGGTCACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCAT : 1178
PF-G_Brevi : TAGCCAGACTGCCTGTG-CAAACAGAGAGGAAGGAGGGGACGACGTCAAGTCATCAT : 1143
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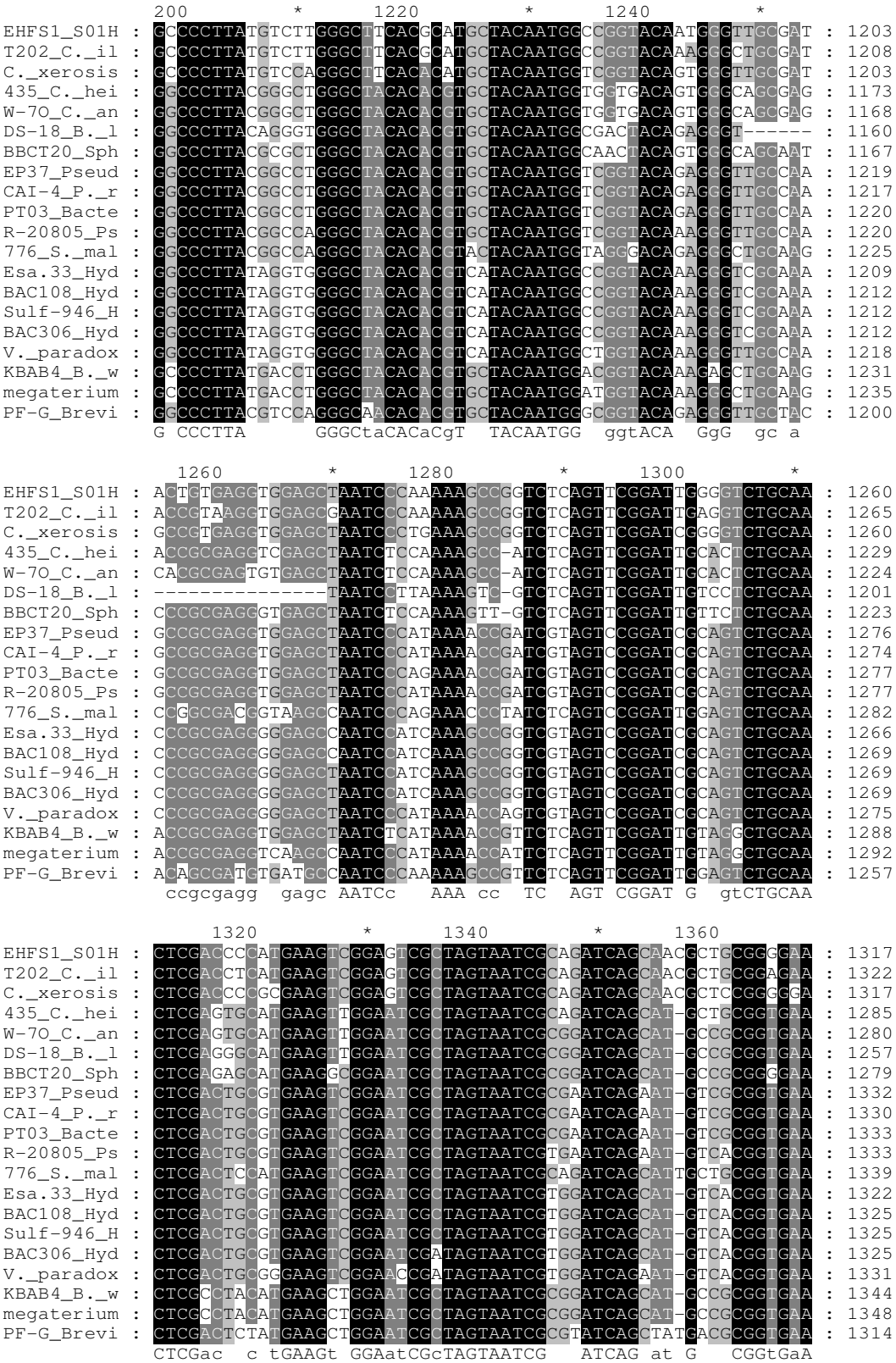
200 * 1220 * 1240 *
EHFS1_S01H : GCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGAT : 1203
T202_C._il : GCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAGGGCTGCGAT : 1208
C._xerosis : GCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGTCGGTACAGTGGGTTGCGAT : 1203
435_C._hei : GGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGGGAG : 1173
W-7O_C._an : GGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGGGAG : 1168
DS-18_B._l : GGCCCTTACAGGGTGGGCTACACACGTGCTACAATGGCGACTACAGAGGGT------ : 1160
BBCT20_Sph : GGCCCTTACGCGCTGGGCTACACACGTGCTACAATGGCAACTACAGTGGGCAGGAAT : 1167
EP37_Pseud : GGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAA : 1219
CAI-4_P._r : GGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAA : 1217
PT03_Bacte : GGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAA : 1220
R-20805_Ps : GGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAGAGGGTTGCCAA : 1220
776_S._mal : GGCCCTTACGGCCAGGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAG : 1225
Esa.33_Hyd : GGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCCGGTACAAGGGTCGCAAA : 1209
BAC108_Hyd : GGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCCGGTACAAGGGTCGCAAA : 1212
Sulf-946_H : GGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCCGGTACAAGGGTCGCAAA : 1212
BAC306_Hyd : GGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCCGGTACAAGGGTCGCAAA : 1212
V._paradox : GGCCCTTATAGGTGGGGCTACACACGTCATACAATGGCTGGTACAAGGGTTGCCAA : 1218
KBAB4_B._w : GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAG : 1231
megaterium : GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAG : 1235
PF-G_Brevi : GGCCCTTACGTCCAGGGCAACACACGTGCTACAATGGGCGGTACAGAGGGTTGGTAC : 1200
G CCCTTA GGGCtaCACaCgT TACAATGG ggtACA GgG gc a
1260 * 1280 * 1300 *
EHFS1_S01H : ACTGTGAGGTGGAGGTAATCCCAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAA : 1260
T202_C._il : ACCGTAAGGTGGAGGGAATCCCAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAA : 1265
C._xerosis : GCCGTGAGGTGGAGGTAATCCCTGAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAA : 1260
435_C._hei : ACCGCGAGGTCGAGGTAATCTCCAAAAGCC-ATCTCAGTTCGGATTGCACTCTGCAA : 1229
W-7O_C._an : CACGCGAGTGTGAGGTAATCTCCAAAAGCC-ATCTCAGTTCGGATTGCACTCTGCAA : 1224
DS-18_B._l : ---------------TAATCCTTAAAAGTC-GTCTCAGTTCGGATTGTCCTCTGCAA : 1201
BBCT20_Sph : CCCGCGAGGGTGAGGTAATCTCCAAAAGTT-GTCTCAGTTCGGATTGTTCTCTGCAA : 1223
EP37_Pseud : GCCGCGAGGTGGAGGTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAA : 1276
CAI-4_P._r : GCCGCGAGGTGGAGGTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAA : 1274
PT03_Bacte : GCCGCGAGGTGGAGGTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAA : 1277
R-20805_Ps : GCCGCGAGGTGGAGGTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAA : 1277
776_S._mal : CCGGCGACGGTAAGGCAATCCCAGAAACCCTATCTCAGTCCGGATTGGAGTCTGCAA : 1282
Esa.33_Hyd : CCCGCGAGGGGAGGCAATCCATCAAAGCCGGTCGTAGTCCGGATCGCAGTCTGCAA : 1266
BAC108_Hyd : CCCGCGAGGGGAGGCAATCCATCAAAGCCGGTCGTAGTCCGGATCGCAGTCTGCAA : 1269
Sulf-946_H : CCCGCGAGGGGAGGTAATCCATCAAAGCCGGTCGTAGTCCGGATCGCAGTCTGCAA : 1269
BAC306_Hyd : CCCGCGAGGGGAGGTAATCCATCAAAGCCGGTCGTAGTCCGGATCGCAGTCTGCAA : 1269
V._paradox : CCCGCGAGGGGAGGTAATCCCATAAAACCAGTCGTAGTCCGGATCGCAGTCTGCAA : 1275
KBAB4_B._w : ACCGCGAGGTGGAGGTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGCTGCAA : 1288
megaterium : ACCGCGAGGTCAAGGCAATCCCATAAAACCATTCTCAGTTCGGATTGTACGCTGCAA : 1292
PF-G_Brevi : ACAGCGATGTGATGGCAATCCCAAAAGCCGTTCTCAGTTCGGATTGGAGTCTGCAA : 1257
ccgcgagg gagc AATCc AAA cc TC AGT CGGAT G gtCTGCAA
1320 * 1340 * 1360
EHFS1_S01H : CTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAA : 1317
T202_C._il : CTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGAGAA : 1322
C._xerosis : CTCGACCCCGCGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTCCGGGGGA : 1317
435_C._hei : CTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCAT-GCTGCGGTGAA : 1285
W-7O_C._an : CTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT-GCCGCGGTGAA : 1280
DS-18_B._l : CTCGAGGGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCAT-GCCGCGGTGAA : 1257
BBCT20_Sph : CTCGAGAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCAT-GCCGCGGGGAA : 1279
EP37_Pseud : CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAAT-GTCGCGGTGAA : 1332
CAI-4_P._r : CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAAT-GTCGCGGTGAA : 1330
PT03_Bacte : CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAAT-GTCGCGGTGAA : 1333
R-20805_Ps : CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAAT-GTCACGGTGAA : 1333
776_S._mal : CTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAA : 1339
Esa.33_Hyd : CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGCAT-GTCACGGTGAA : 1322
BAC108_Hyd : CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGCAT-GTCACGGTGAA : 1325
Sulf-946_H : CTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGCAT-GTCACGGTGAA : 1325
BAC306_Hyd : CTCGACTGCGTGAAGTCGGAATCGATAGTAATCGTGGATCAGCAT-GTCACGGTGAA : 1325
V._paradox : CTCGACTGCGGGAAGTCGGAAGCGATAGTAATCGTGGATCAGAAT-GTCACGGTGAA : 1331
KBAB4_B._w : CTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT-GCCGCGGTGAA : 1344
megaterium : CTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT-GCCGCGGTGAA : 1348
PF-G_Brevi : CTCGACTCTATGAAGCTGGAATCGCTAGTAATCGCGTATCAGCTATGACGCGGTGAA : 1314
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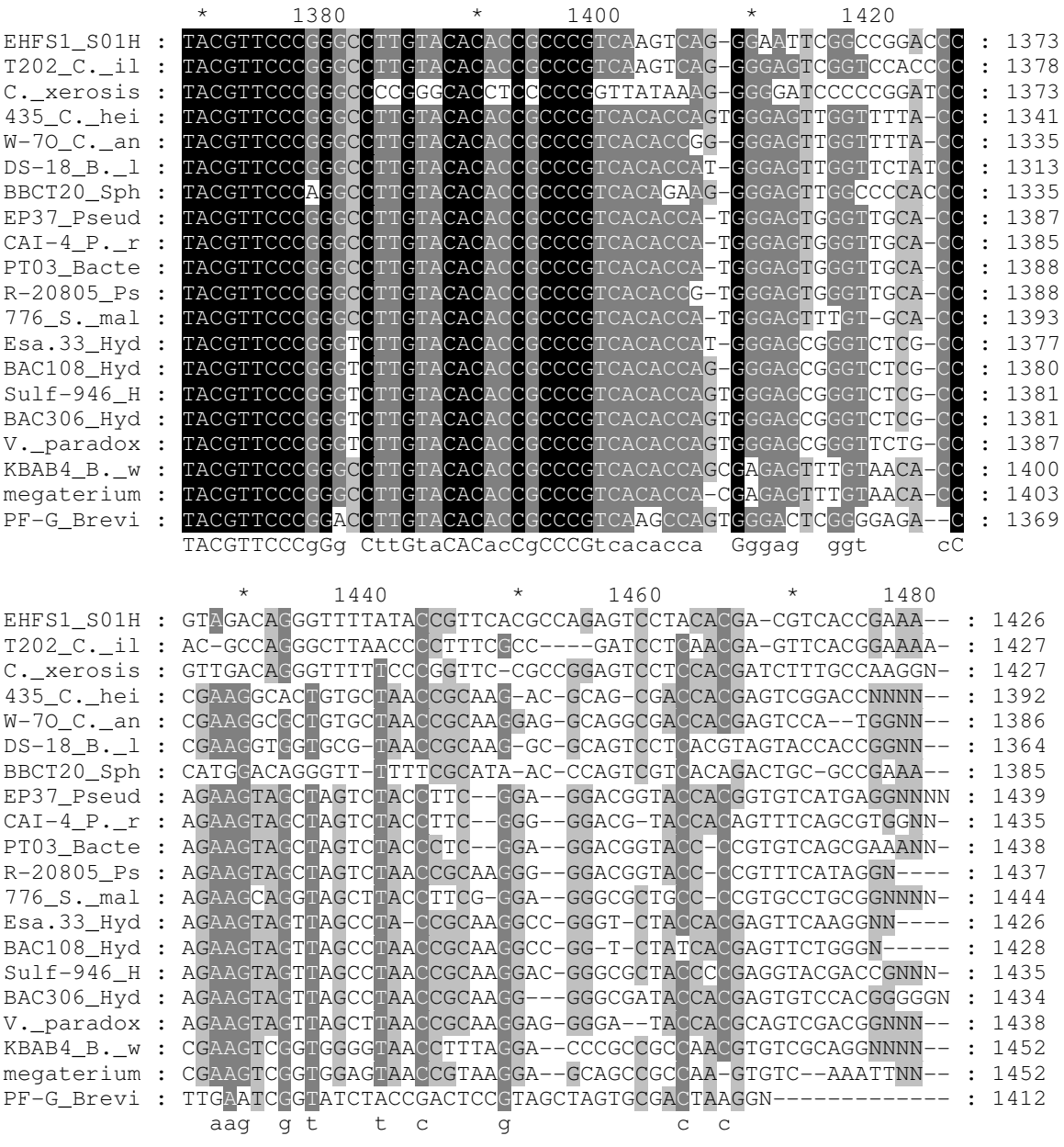
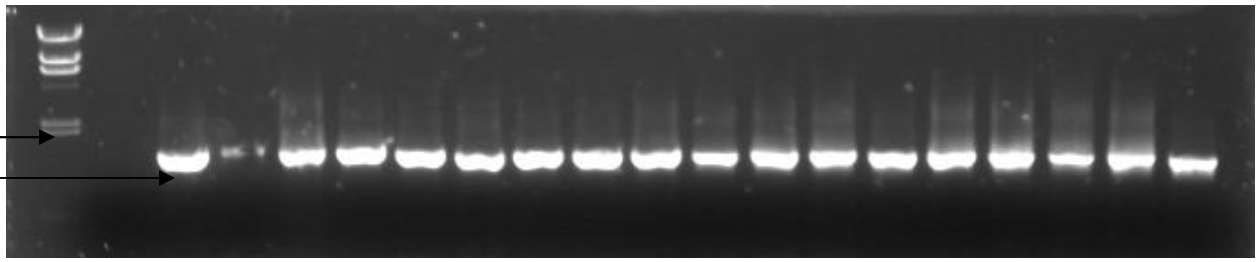


Alignment of the amino acid sequences of isolates obtained from bioballs three days into the setup of the bioreactor. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. The conserved regions are indicated with Roman numerals. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.

APPENDIX C3

The figures below represent the agarose gel electrophoresis results of purified PCR products of the organisms isolated from the biofilm samples (obtained from the bioballs) collected from the three compartments of the three-week, laboratory-scale bioreactor system, fifteen days after start-up. The code for the organisms names are BF – Bioreactor Final (after 15 days).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



LANE 1 – Marker (Lambda DNA/HIND III)

LANE 2 – Negative Control

LANE 3 – BF1

LANE 4 – BF2

LANE 5 – BF3

LANE 6 – BF4

LANE 7 – BF5

LANE 8 – BF6

LANE 9 – BF7

LANE 10 – BF8

LANE 11 – BF9

LANE 12 – BF10

LANE 13 – BF11

LANE 14 – BF12

LANE 15 – BF13

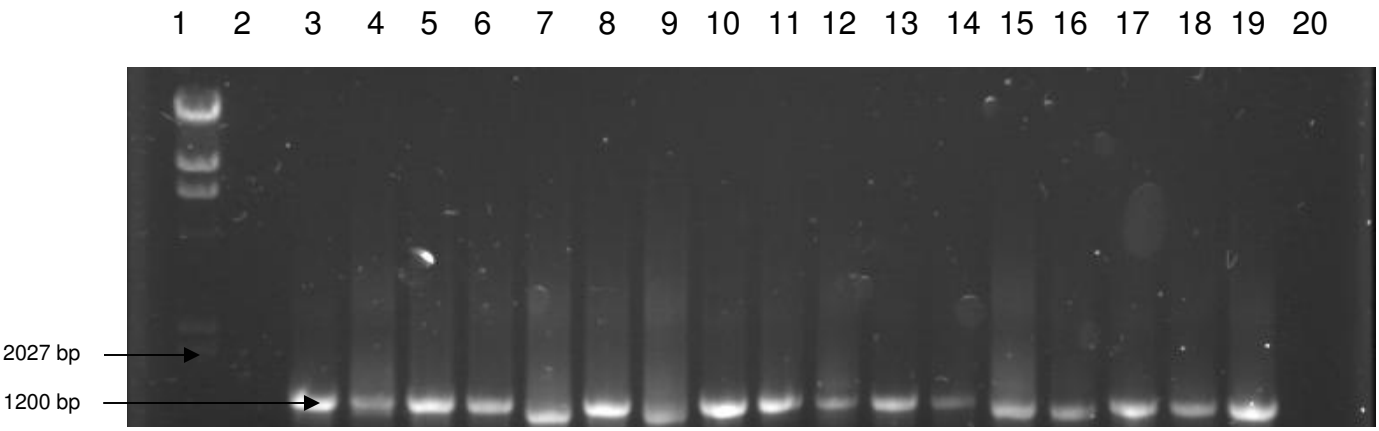
LANE 16 – BF14

LANE 17 – BF15

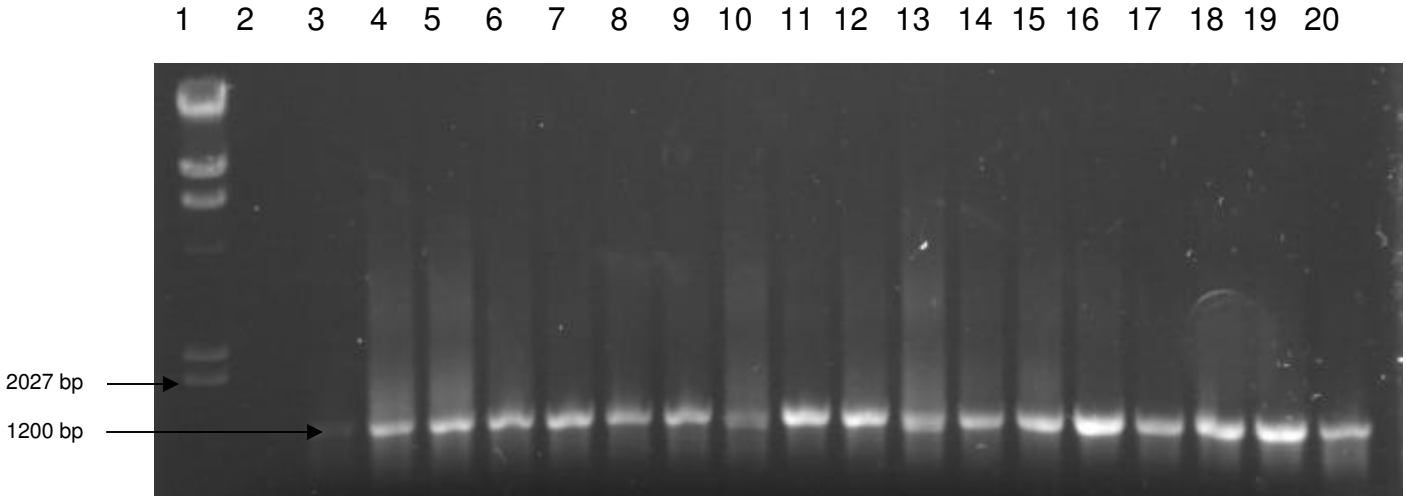
LANE 18 – BF16

LANE 19 – BF17

LANE 20 – BF18



LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – Negative Control
LANE 3 – BF19
LANE 4 – BF20
LANE 5 – BF21
LANE 6 – BF22
LANE 7 – BF23
LANE 8 – BF24
LANE 9 – BF25
LANE 10 – BF26
LANE 11 – BF27
LANE 12 – BF28
LANE 13 – BF29
LANE 14 – BF30
LANE 15 – BF31
LANE 16 – BF32
LANE 17 – BF33
LANE 18 – BF34
LANE 19 – BF35
LANE 20 – EMPTY



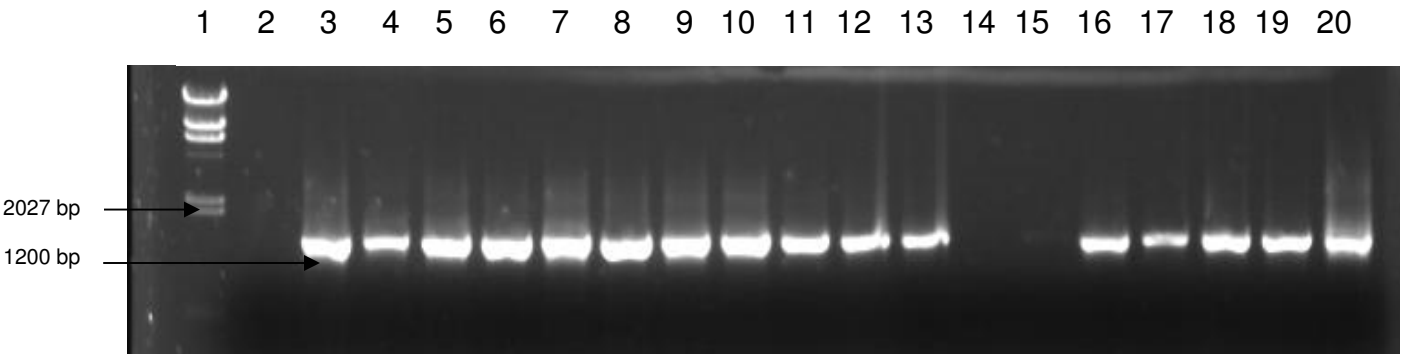
LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – Negative Control
LANE 3 – BF36
LANE 4 – BF37
LANE 5 – BF38
LANE 6 – BF39
LANE 7 – BF40
LANE 8 – BF41
LANE 9 –BF42

LANE 10 – BF43 LANE 11 – BF44 LANE 12 – BF45

LANE 13 – BF46 LANE 14 – BF47 LANE 15 – BF48

LANE 16 – BF49 LANE 17 – BF50 LANE 18 – BF51

LANE 19 – BF52 LANE 20 – BF53



LANE 1 – Marker (Lambda DNA/HIND III) LANE 2 – Negative Control LANE 3 – BF54

LANE 4 – BF55 LANE 5 – BF56 LANE 6 – BF57

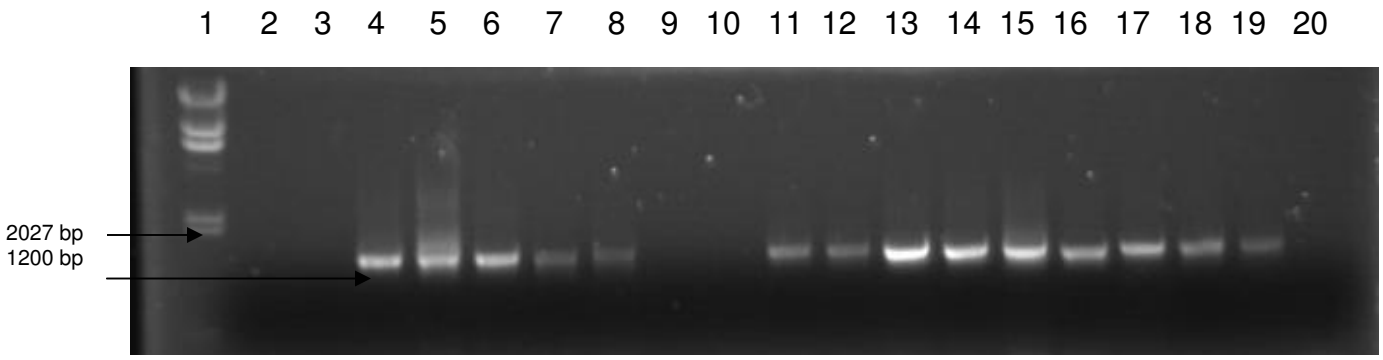
LANE 7 – BF58 LANE 8 – BF59 LANE 9 – BF60

LANE 10 – BF61 LANE 11 – BF62 LANE 12 – BF63

LANE 13 – BF64 LANE 14 –EMPTY LANE 15 – EMPTY

LANE 16 – BF65 LANE 17 – BF66 LANE 18 – BF67

LANE 19 – BF68 LANE 20 – BF69



LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – Negative Control
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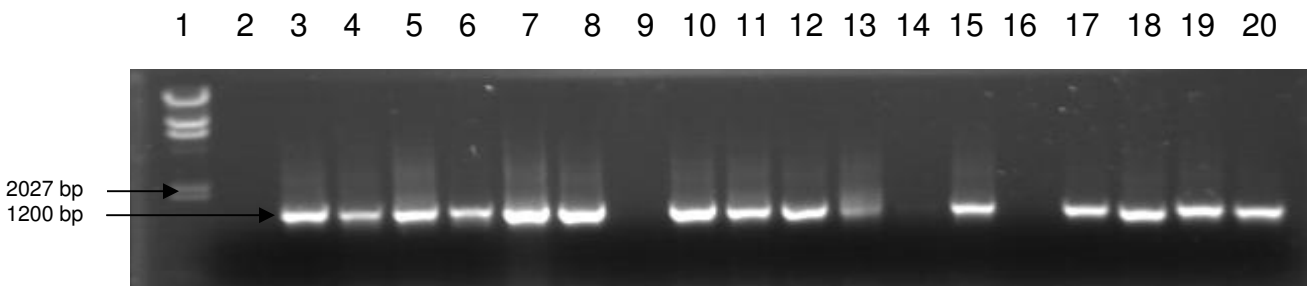
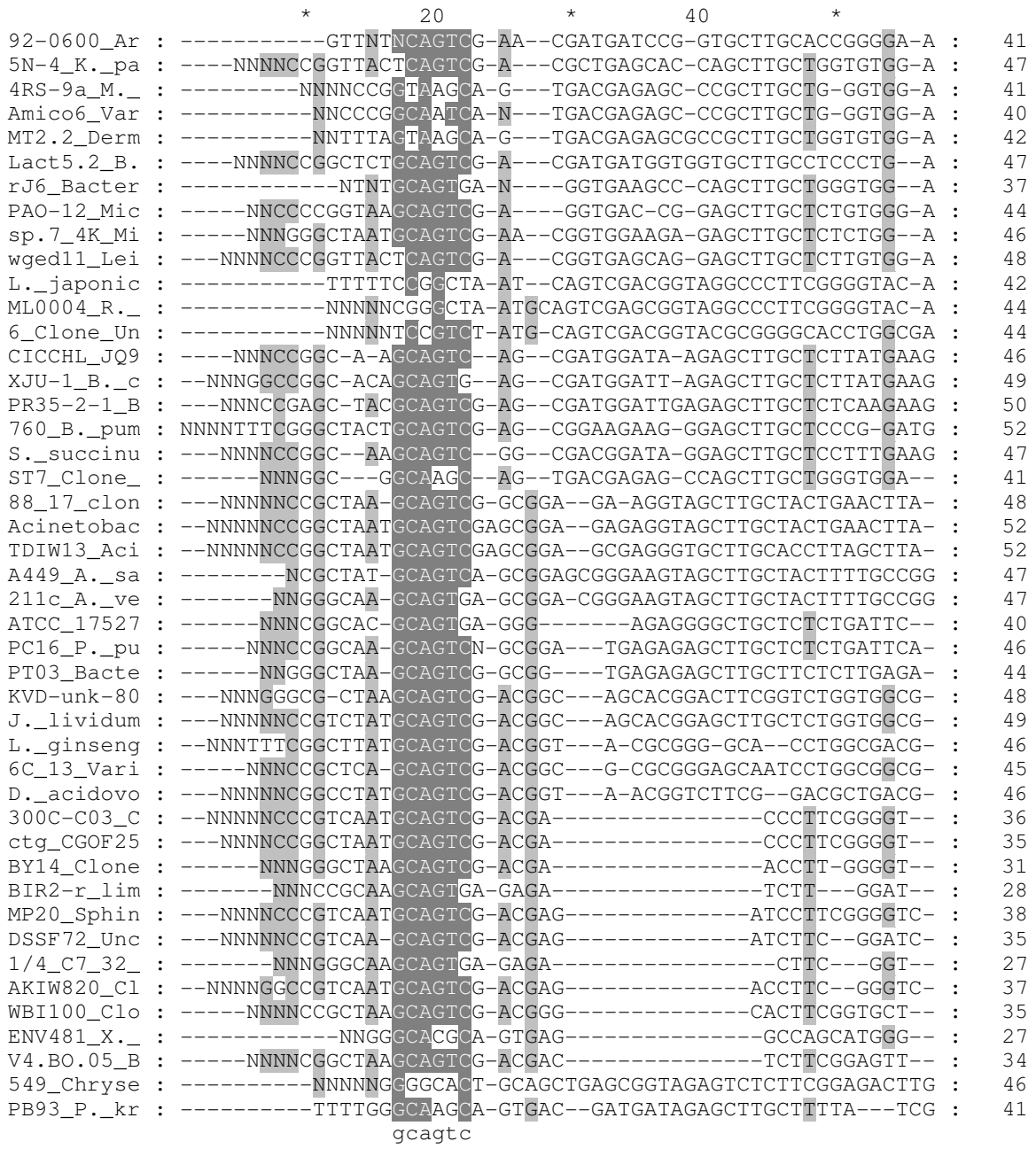
LANE 4 – BF70
LANE 5 – BF71
LANE 6 – BF72
LANE 7 – BF73
LANE 8 – BF74
LANE 9 – EMPTY
LANE 10 – EMPTY
LANE 11 – BF75
LANE 12 – BF76
LANE 13 – BF77
LANE 14 –BF78
LANE 15 – BF79
LANE 16 – BF80
LANE 17 – BF81
LANE 18 – BF82
LANE 19 – BF83
LANE 20 – EMPTY



LANE 1 – Marker (Lambda DNA/HIND III)
LANE 2 – Negative Control
LANE 3 – BF84
LANE 4 – BF85
LANE 5 – BF86
LANE 6 – BF87
LANE 7 – BF88
LANE 8 – BF89
LANE 9 – EMPTY
LANE 10 – BF90
LANE 11 – BF91
LANE 12 – BF92
LANE 13 – BF93
LANE 14 – EMPTY
LANE 15 – BF94
LANE 16 – EMPTY
LANE 17 – BF95
LANE 18 – BF96
LANE 19 – BF97
LANE 20 – BF98

APPENDIX C4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4RS-9a_M._ : ---------NNNNCCGGTAAGCA-G---TGACGAGAGC-CCGCTTGCTG-GGTGG-A : 41
Amico6_Var : ----------NNCCCGGCAATCA-N---TGACGAGAGC-CCGCTTGCTG-GGTGG-A : 40
MT2.2_Derm : ----------NNTTTAGTAAGCA-G---TGACGAGAGCGCCGCTTGCTGGTGTGG-A : 42
Lact5.2_B. : ----NNNNCCGGCTCTGCAGTCG-A---CGATGATGGTGGTGCTTGCCTCCCTG--A : 47
rJ6_Bacter : ----------NTNTGCAGTGA-N----GGTGAAGCC-CAGCTTGCTGGGTGG--A : 37
PAO-12_Mic : -----NNCCCCGGTAAGCAGTCG-A----GGTGAC-CG-GAGCTTGCTCTGTGGG-A : 44
sp.7_4K_Mi : -----NNNGGGCTAATGCAGTCG-AA--CGGTGGAAGA-GAGCTTGCTCTCTGG--A : 46
wged11_Lei : ---NNNNCCCGGTTACTCAGTCG-A---CGGTGAGCAG-GAGCTTGCTCTTGTGG-A : 48
L._japonic : ----------TTTTTCGGCTA-AT--CAGTCGACGGTAGGCCCTTCGGGGTAC-A : 42
ML0004_R._ : ----------NNNNNCGGCTA-ATGCAGTCGAGCGGTAGGCCCTTCGGGGTAC-A : 44
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S._succinu : ---NNNNCCGGC--AAGCAGTC--GG--CGACGGATA-GGAGCTTGCTCCTTTGAAG : 47
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J._lividum : ---NNNNNCCGTCTATGCAGTCG-ACGGC---AGCACGGAGCTTGCTCTGGTGGCG- : 49
L._ginseng : --NNNTTTCGGCTTATGCAGTCG-ACGGT---A-CGCGGG-GCA--CCTGGCGACG- : 46
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D._acidovo : ---NNNNNCGGCCTATGCAGTCG-ACGGT---A-ACGGTCTTCG--GACGCTGACG- : 46
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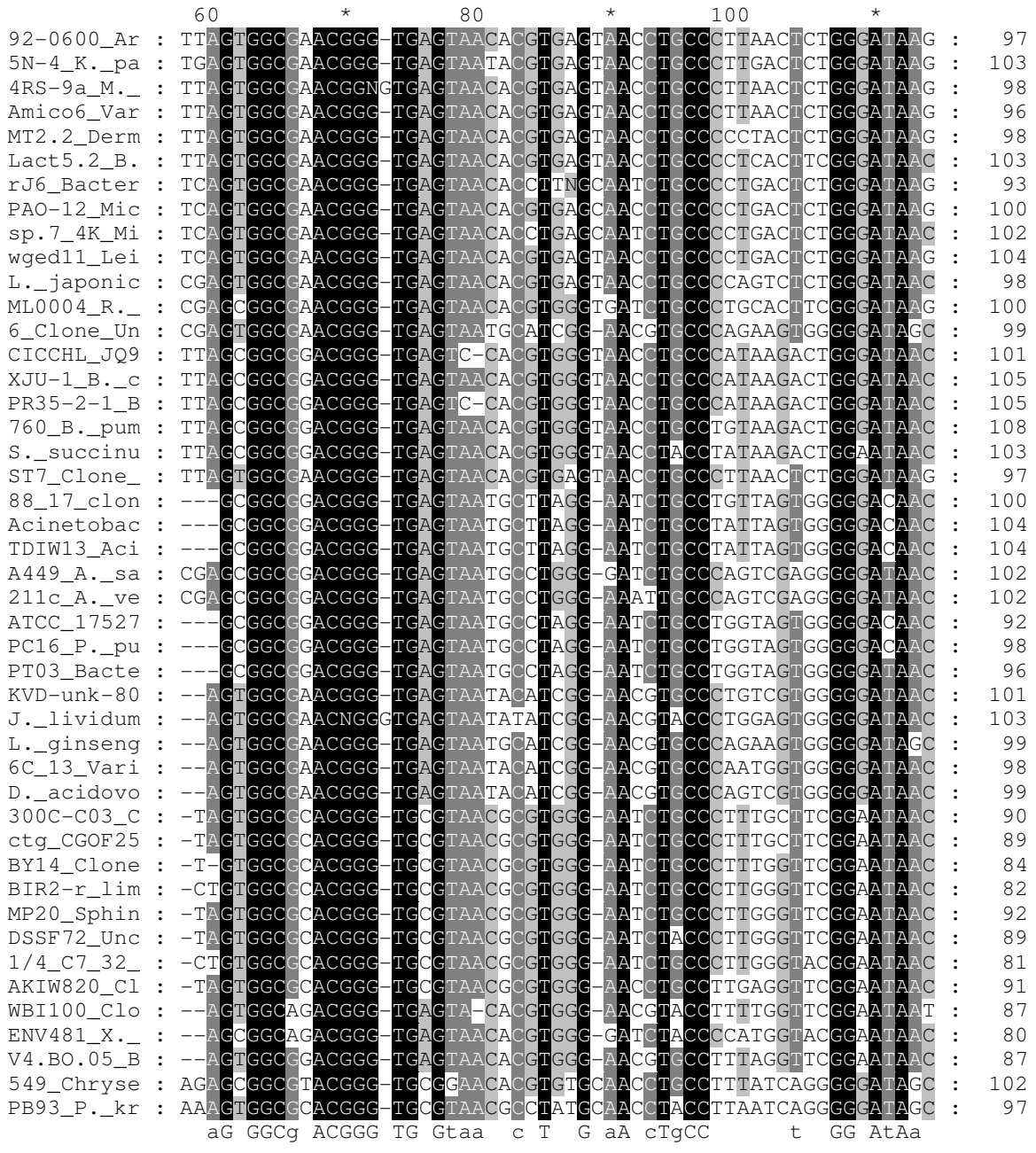
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KVD-unk-80 : --AGTGGCGAACGGG-TGAGTAATACATCGG-AACGTGCCCTGTCGTGGGGGATAAC : 101
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1/4_C7_32_ : -CTGTGGCGCACGGG-TGCGTAACGCGTGGG-AATCTGCCCTTGGGTACGGAATAAC : 81
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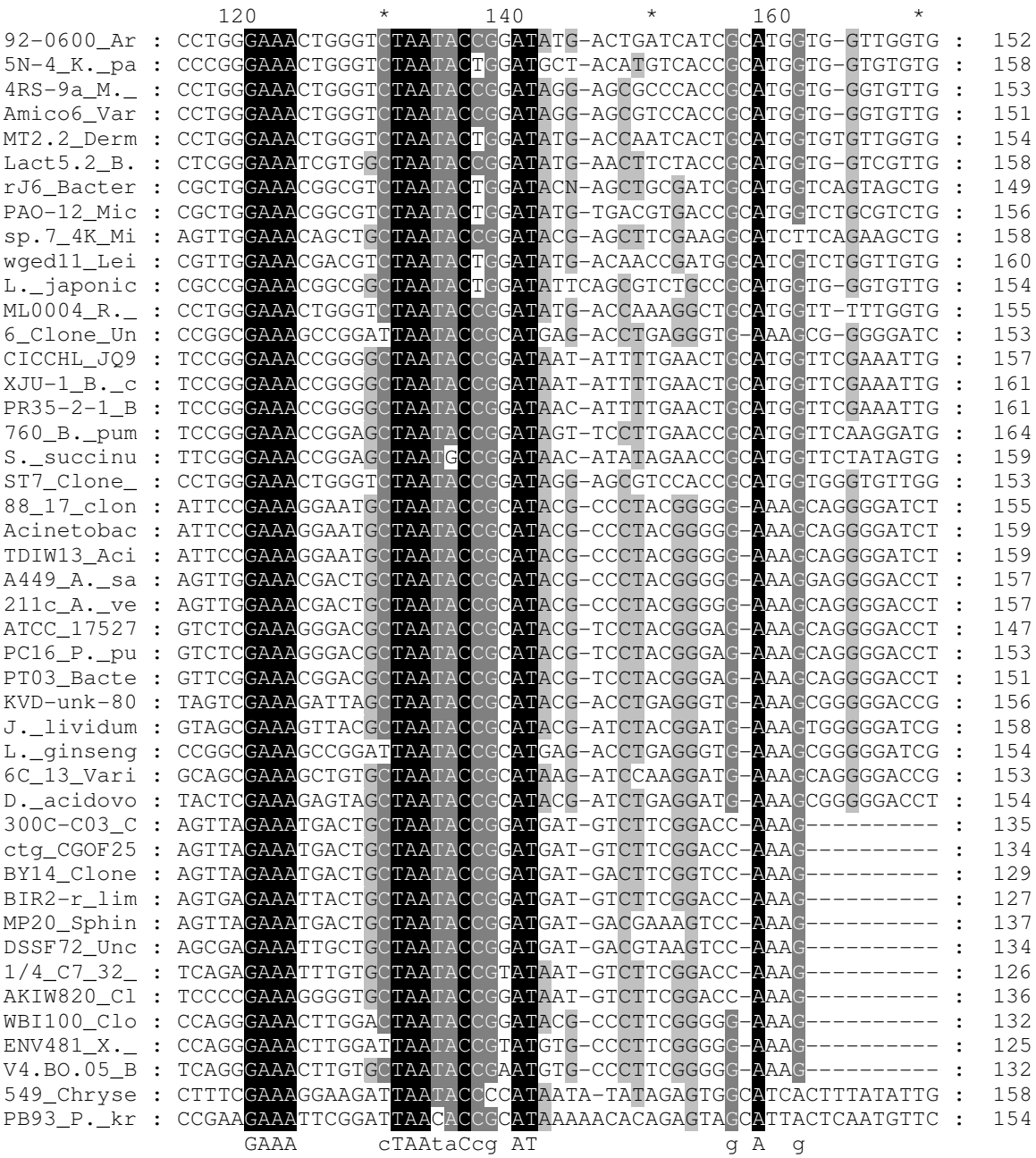
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4RS-9a_M._ : CCTGGGAAACTGGGTCTAATACCGGATAGG-AGCGCCCACCGCATGGTG-GGTGTTG : 153
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MT2.2_Derm : CCTGGGAAACTGGGTCTAATACTGGATATG-ACCAATCACTGCATGGTGTGTTGGTG : 154
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sp.7_4K_Mi : AGTTGGAAACAGCTGCTAATACCGGATACG-AGCTTCGAAGGCATCTTCAGAAGCTG : 158
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L._japonic : CGCCGGAAACGGCGGCTAATACTGGATATTCAGCGTCTGCCGCATGGTG-GGTGTTG : 154
ML0004_R._ : CCTGGGAAACTGGGTCTAATACCGGATATG-ACCAAAGGCTGCATGGTT-TTTGGTG : 155
6_Clone_Un : CCGGGGAAAGCCGGATTAATACCGCATGAG-ACCTGAGGGTG-AAAGCG-GGGGATC : 153
CICCHL_JQ9 : TCCGGGAAACCGGGGCTAATACCGGATAAT-ATTTTGAACTGCATGGTTCGAAATTG : 157
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760_B._pum : TCCGGGAAACCGGAGCTAATACCGGATAGT-TCCTTGAACCGCATGGTTCAAGGATG : 164
S._succinu : TTCGGGAAACCGGAGCTAATGCCGGATAAC-ATATAGAACCGCATGGTTCTATAGTG : 159
ST7_Clone_ : CCTGGGAAACTGGGTCTAATACCGGATAGG-AGCGTCCACCGCATGGTGGGTGTTGG : 153
88_17_clon : ATTCCGAAAGGAATGCTAATACCGCATACG-CCCTACGGGGG-AAAGCAGGGGATCT : 155
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KVD-unk-80 : TAGTCGAAAGATTAGCTAATACCGCATACG-ACCTGAGGGTG-AAAGCGGGGGACCG : 156
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L._ginseng : CCGGCGAAAGCCGGATTAATACCGCATGAG-ACCTGAGGGTG-AAAGCGGGGGATCG : 154
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D._acidovo : TACTCGAAAGAGTAGCTAATACCGCATACG-ATCTGAGGATG-AAAGCGGGGGACCT : 154
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ctg_CGOF25 : AGTTAGAAATGACTGCTAATACCGGATGAT-GTCTTCGGACC-AAAG---------- : 134
BY14_Clone : AGTTAGAAATGACTGCTAATACCGGATGAT-GACTTCGGTCC-AAAG---------- : 129
BIR2-r_lim : AGTGAGAAATTACTGCTAATACCGGATGAT-GTCTTCGGACC-AAAG---------- : 127
MP20_Sphin : AGTTAGAAATGACTGCTAATACCGGATGAT-GACGAAAGTCC-AAAG---------- : 137
DSSF72_Unc : AGCGAGAAATTGCTGCTAATACCGGATGAT-GACGTAAGTCC-AAAG---------- : 134
1/4_C7_32_ : TCAGAGAAATTTGTGCTAATACCGTATAAT-GTCTTCGGACC-AAAG---------- : 126
AKIW820_Cl : TCCCCGAAAGGGGTGCTAATACCGGATAAT-GTCTTCGGACC-AAAG---------- : 136
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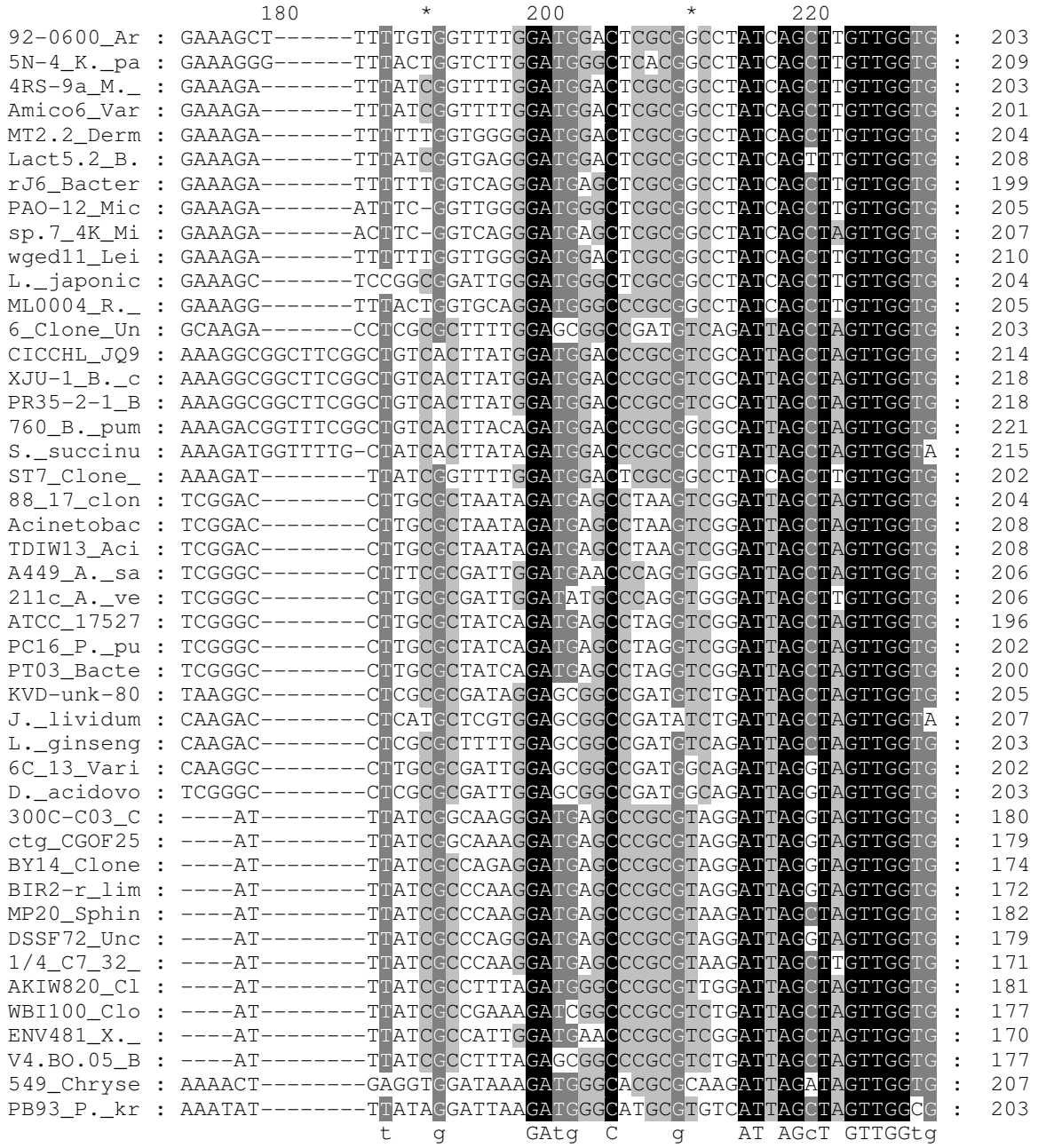
V4.BO.05_B : TCAGGGAAACTTGTGCTAATACCGAATGTG-CCCTTCGGGGG-AAAG---------- : 132
549_Chryse : CTTTCGAAAGGAAGATTAATACCCCATAATA-TATAGAGTGGCATCACTTTATATTG : 158
PB93_P._kr : CCGAAGAAATTCGGATTAACACCGCATAAAAACACAGAGTAGCATTACTCAATGTTC : 154
GAAA cTAAtaCcg AT g A g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5N-4_K._pa : GAAAGGG------TTTACTGGTCTTGGATGGGCTCACGGCCTATCAGCTTGTTGGTG : 209
4RS-9a_M._ : GAAAGA-------TTTATCGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTG : 203
Amico6_Var : GAAAGA-------TTTATCGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTG : 201
MT2.2_Derm : GAAAGA-------TTTTTTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTG : 204
Lact5.2_B. : GAAAGA-------TTTATCGGTGAGGGATGGACTCGCGGCCTATCAGTTTGTTGGTG : 208
rJ6_Bacter : GAAAGA-------TTTTTTGGTCAGGGATGAGCTCGCGGCCTATCAGCTTGTTGGTG : 199
PAO-12_Mic : GAAAGA-------ATTTC-GGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTG : 205
sp.7_4K_Mi : GAAAGA-------ACTTC-GGTCAGGGATGAGCTCGCGGCCTATCAGCTAGTTGGTG : 207
wged11_Lei : GAAAGA-------TTTTTTGGTTGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTG : 210
L._japonic : GAAAGC-------TCCGGCGGATTGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTG : 204
ML0004_R._ : GAAAGG-------TTTACTGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTG : 205
6_Clone_Un : GCAAGA-------CCTCGCGCTTTTGGAGCGGCCGATGTCAGATTAGCTAGTTGGTG : 203
CICCHL_JQ9 : AAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG : 214
XJU-1_B._c : AAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG : 218
PR35-2-1_B : AAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG : 218
760_B._pum : AAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTG : 221
S._succinu : AAAGATGGTTTTG-CTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTA : 215
ST7_Clone_ : AAAGAT--------TTATCGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTG : 202
88_17_clon : TCGGAC--------CTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTG : 204
Acinetobac : TCGGAC--------CTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTG : 208
TDIW13_Aci : TCGGAC--------CTTGCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTG : 208
A449_A._sa : TCGGGC--------CTTTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTG : 206
211c_A._ve : TCGGGC--------CTTGCGCGATTGGATATGCCCAGGTGGGATTAGCTTGTTGGTG : 206
ATCC_17527 : TCGGGC--------CTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG : 196
PC16_P._pu : TCGGGC--------CTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG : 202
PT03_Bacte : TCGGGC--------CTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG : 200
KVD-unk-80 : TAAGGC--------CTCGCGCGATAGGAGCGGCCGATGTCTGATTAGCTAGTTGGTG : 205
J._lividum : CAAGAC--------CTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTA : 207
L._ginseng : CAAGAC--------CTCGCGCTTTTGGAGCGGCCGATGTCAGATTAGCTAGTTGGTG : 203
6C_13_Vari : CAAGGC--------CTTGCGCGATTGGAGCGGCCGATGGCAGATTAGGTAGTTGGTG : 202
D._acidovo : TCGGGC--------CTCGCGCGATTGGAGCGGCCGATGGCAGATTAGGTAGTTGGTG : 203
300C-C03_C : ----AT--------TTATCGGCAAGGGATGAGCCCGCGTAGGATTAGGTAGTTGGTG : 180
ctg_CGOF25 : ----AT--------TTATCGGCAAAGGATGAGCCCGCGTAGGATTAGGTAGTTGGTG : 179
BY14_Clone : ----AT--------TTATCGCCAGAGGATGAGCCCGCGTAGGATTAGGTAGTTGGTG : 174
BIR2-r_lim : ----AT--------TTATCGCCCAAGGATGAGCCCGCGTAGGATTAGGTAGTTGGTG : 172
MP20_Sphin : ----AT--------TTATCGCCCAAGGATGAGCCCGCGTAAGATTAGCTAGTTGGTG : 182
DSSF72_Unc : ----AT--------TTATCGCCCAGGGATGAGCCCGCGTAGGATTAGGTAGTTGGTG : 179
1/4_C7_32_ : ----AT--------TTATCGCCCAAGGATGAGCCCGCGTAAGATTAGCTTGTTGGTG : 171
AKIW820_Cl : ----AT--------TTATCGCCTTTAGATGGGCCCGCGTTGGATTAGCTAGTTGGTG : 181
WBI100_Clo : ----AT--------TTATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTAGTTGGTG : 177
ENV481_X._ : ----AT--------TTATCGCCATTGGATGAACCCGCGTCGGATTAGCTAGTTGGTG : 170
V4.BO.05_B : ----AT--------TTATCGCCTTTAGAGCGGCCCGCGTCTGATTAGCTAGTTGGTG : 177
549_Chryse : AAAACT--------GAGGTGGATAAAGATGGGCACGCGCAAGATTAGATAGTTGGTG : 207
PB93_P._kr : AAATAT--------TTATAGGATTAAGATGGGCATGCGTGTCATTAGCTAGTTGGCG : 203
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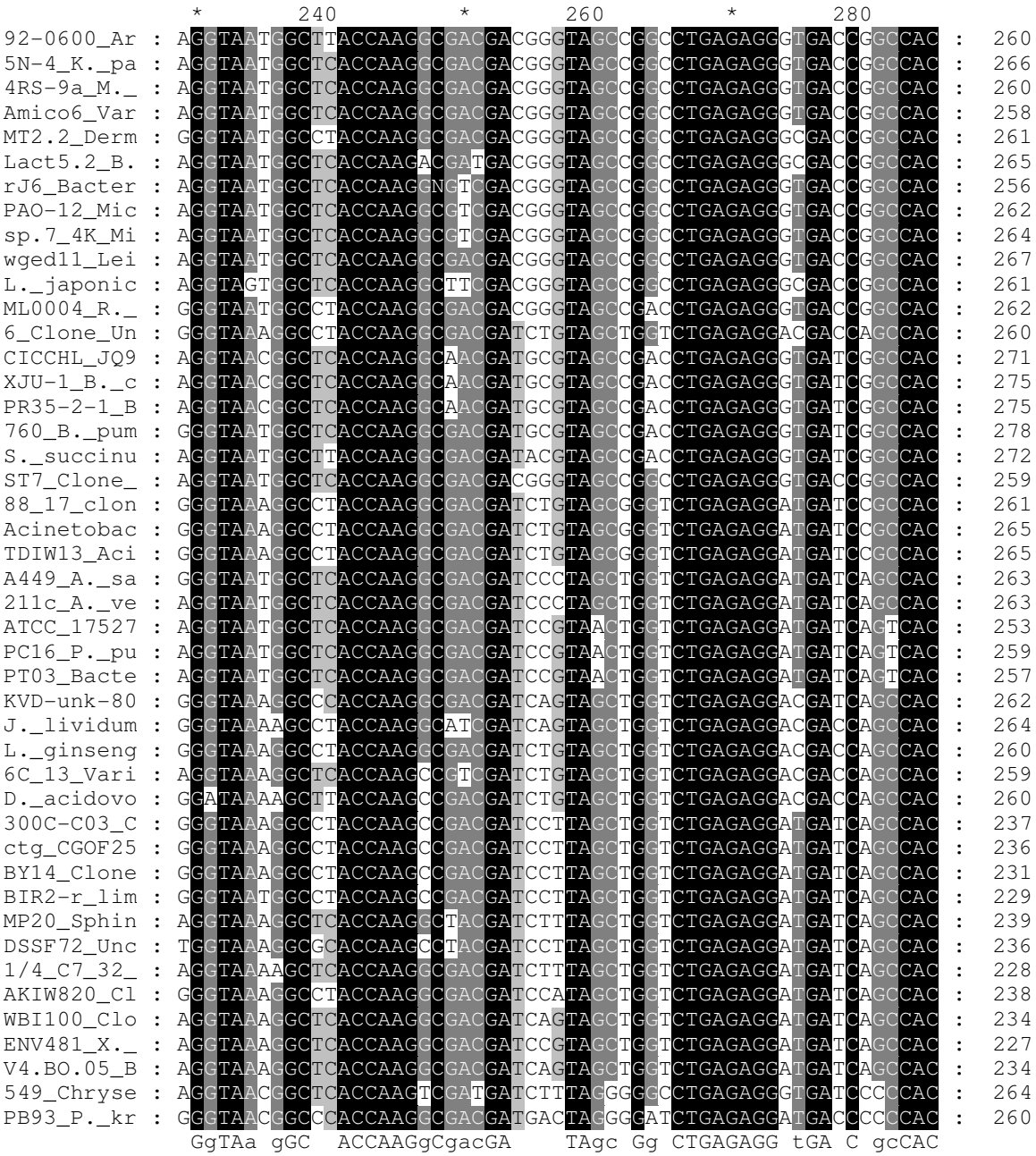
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4RS-9a_M._ : AGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC : 260
Amico6_Var : AGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC : 258
MT2.2_Derm : GGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC : 261
Lact5.2_B. : AGGTAATGGCTCACCAAGACGATGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC : 265
rJ6_Bacter : AGGTAATGGCTCACCAAGGNGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC : 256
PAO-12_Mic : AGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC : 262
sp.7_4K_Mi : AGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC : 264
wged11_Lei : AGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC : 267
L._japonic : AGGTAGTGGCTCACCAAGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC : 261
ML0004_R._ : GGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAGGGTGACCGGCCAC : 262
6_Clone_Un : GGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCAC : 260
CICCHL_JQ9 : AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC : 271
XJU-1_B._c : AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC : 275
PR35-2-1_B : AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC : 275
760_B._pum : GGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC : 278
S._succinu : AGGTAATGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCAC : 272
ST7_Clone_ : AGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC : 259
88_17_clon : GGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCAC : 261
Acinetobac : GGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCAC : 265
TDIW13_Aci : GGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCAC : 265
A449_A._sa : GGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCAC : 263
211c_A._ve : AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCAC : 263
ATCC_17527 : AGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC : 253
PC16_P._pu : AGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC : 259
PT03_Bacte : AGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC : 257
KVD-unk-80 : GGGTAAAGGCCCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGATCAGCCAC : 262
J._lividum : GGGTAAAAGCCTACCAAGGCATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCAC : 264
L._ginseng : GGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCAC : 260
6C_13_Vari : AGGTAAAGGCTCACCAAGCCGTCGATCTGTAGCTGGTCTGAGAGGACGACCAGCCAC : 259
D._acidovo : GGATAAAAGCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCAC : 260
300C-C03_C : GGGTAAAGGCCTACCAAGCCGACGATCCTTAGCTGGTCTGAGAGGATGATCAGCCAC : 237
ctg_CGOF25 : GGGTAAAGGCCTACCAAGCCGACGATCCTTAGCTGGTCTGAGAGGATGATCAGCCAC : 236
BY14_Clone : GGGTAAAGGCCTACCAAGCCGACGATCCTTAGCTGGTCTGAGAGGATGATCAGCCAC : 231
BIR2-r_lim : GGGTAATGGCCTACCAAGCCGACGATCCTTAGCTGGTCTGAGAGGATGATCAGCCAC : 229
MP20_Sphin : AGGTAAAGGCTCACCAAGGCTACGATCTTTAGCTGGTCTGAGAGGATGATCAGCCAC : 239
DSSF72_Unc : TGGTAAAGGCGCACCAAGCCTACGATCCTTAGCTGGTCTGAGAGGATGATCAGCCAC : 236
1/4_C7_32_ : AGGTAAAAGCTCACCAAGGCGACGATCTTTAGCTGGTCTGAGAGGATGATCAGCCAC : 228
AKIW820_Cl : GGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCAC : 238
WBI100_Clo : AGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCAC : 234
ENV481_X._ : AGGTAAAGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCAC : 227
V4.BO.05_B : AGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCAC : 234
549_Chryse : AGGTAACGGCTCACCAAGTCGATGATCTTTAGGGGCCTGAGAGGGTGATCCCCCAC : 264
PB93_P._kr : GGGTAACGGCCCACCAAGGCGACGATGACTAGGGGATCTGAGAGGATGACCCCCAC : 260
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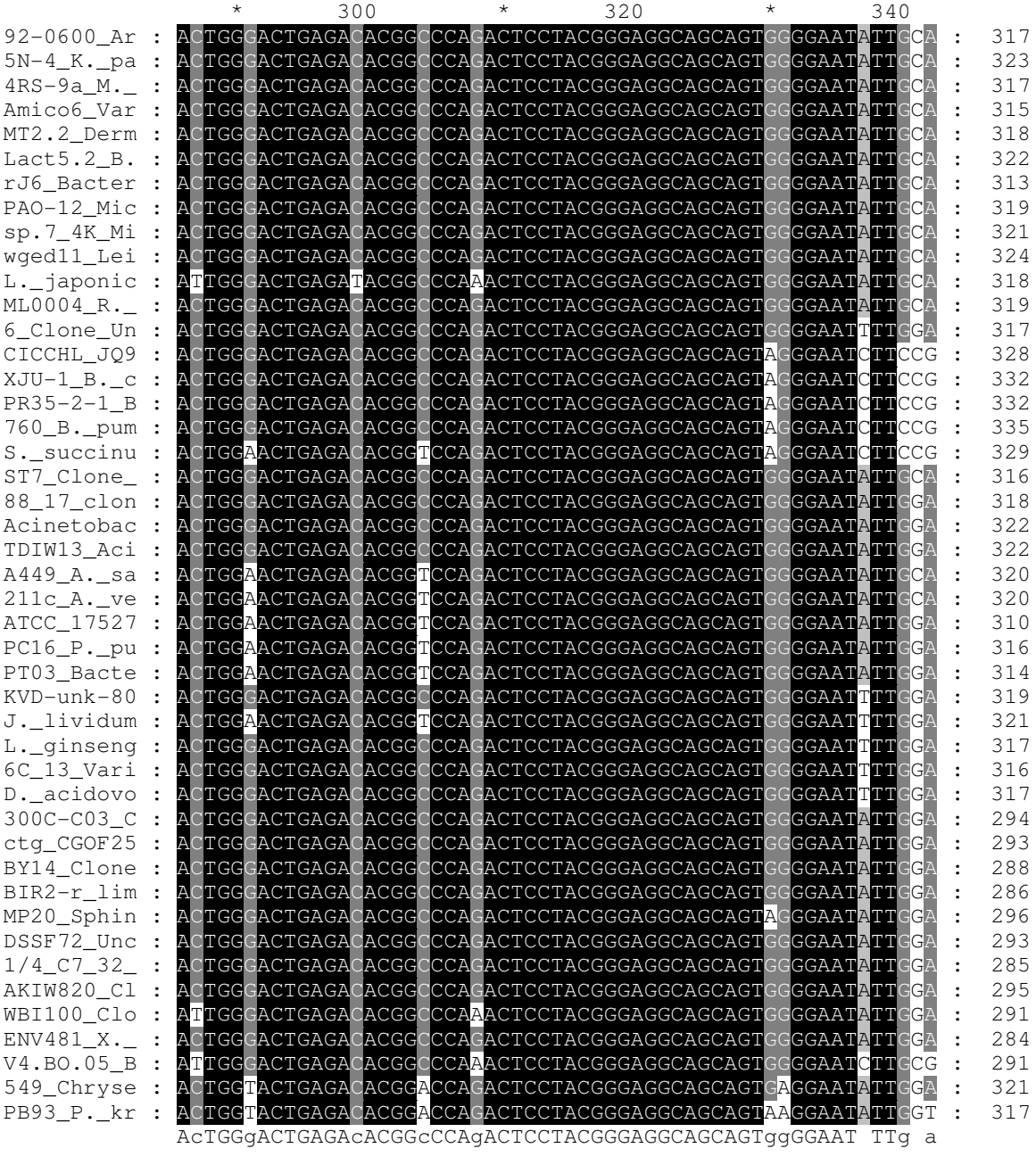
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4RS-9a_M._ : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 317
Amico6_Var : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 315
MT2.2_Derm : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 318
Lact5.2_B. : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 322
rJ6_Bacter : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 313
PAO-12_Mic : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 319
sp.7_4K_Mi : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 321
wged11_Lei : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 324
L._japonic : ATTGGGACTGAGATACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 318
ML0004_R._ : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 319
6_Clone_Un : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA : 317
CICCHL_JQ9 : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG : 328
XJU-1_B._c : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG : 332
PR35-2-1_B : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG : 332
760_B._pum : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG : 335
S._succinu : ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG : 329
ST7_Clone_ : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 316
88_17_clon : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 318
Acinetobac : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 322
TDIW13_Aci : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 322
A449_A._sa : ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 320
211c_A._ve : ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA : 320
ATCC_17527 : ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 310
PC16_P._pu : ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 316
PT03_Bacte : ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 314
KVD-unk-80 : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA : 319
J._lividum : ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA : 321
L._ginseng : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA : 317
6C_13_Vari : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA : 316
D._acidovo : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA : 317
300C-C03_C : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 294
ctg_CGOF25 : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 293
BY14_Clone : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 288
BIR2-r_lim : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 286
MP20_Sphin : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGA : 296
DSSF72_Unc : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 293
1/4_C7_32_ : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 285
AKIW820_Cl : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 295
WBI100_Clo : ATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 291
ENV481_X._ : ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA : 284
V4.BO.05_B : ATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCG : 291
549_Chryse : ACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGA : 321
PB93_P._kr : ACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTAAGGAATATTGGT : 317
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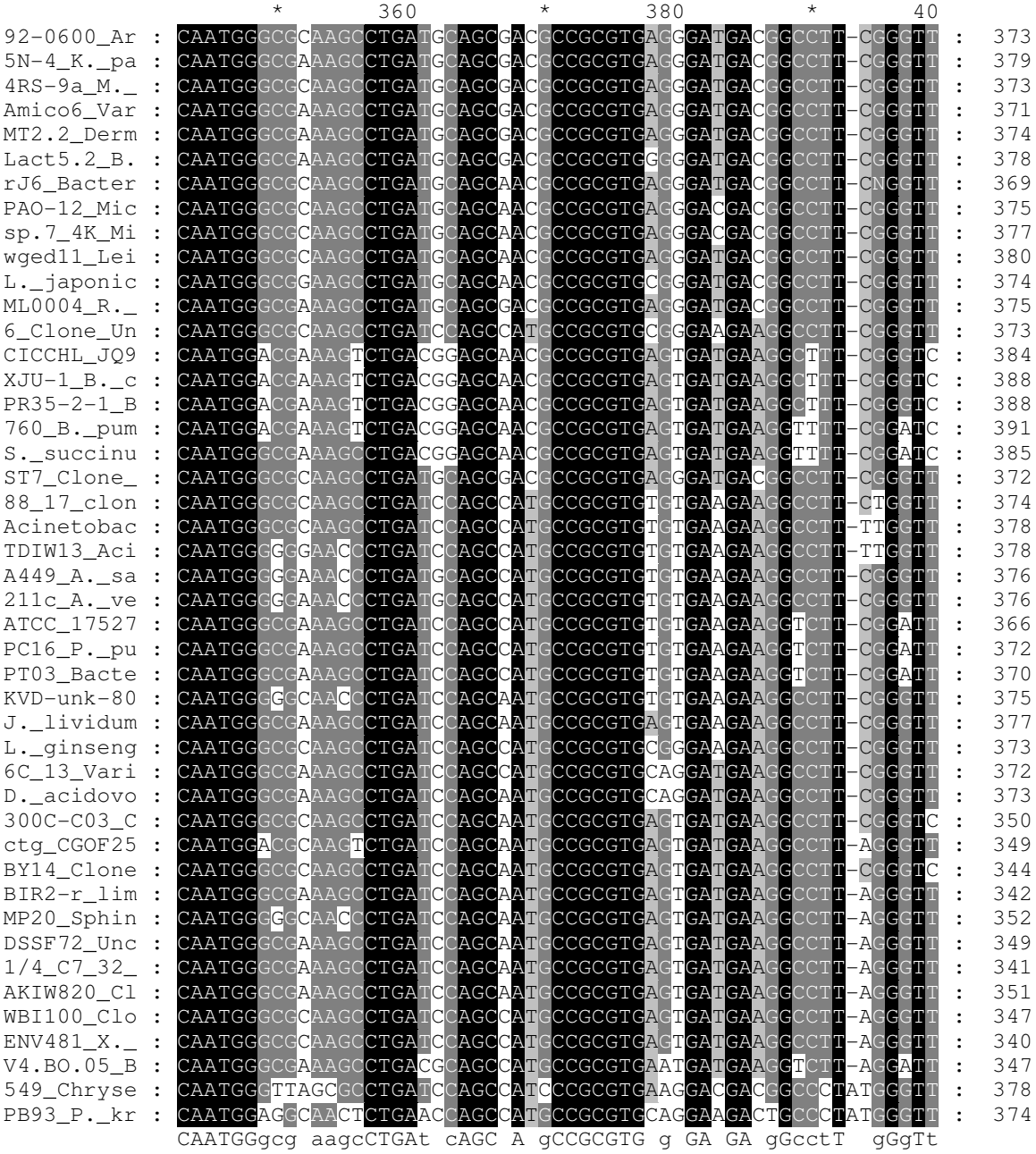
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5N-4_K._pa : CAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGACGGATGACGGCCTT-CGGGTT : 379
4RS-9a_M._ : CAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGACGGATGACGGCCTT-CGGGTT : 373
Amico6_Var : CAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGACGGATGACGGCCTT-CGGGTT : 371
MT2.2_Derm : CAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGACGGATGACGGCCTT-CGGGTT : 374
Lact5.2_B. : CAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTT-CGGGTT : 378
rJ6_Bacter : CAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGACGGATGACGGCCTT-CNGGTT : 369
PAO-12_Mic : CAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGACGGACGACGGCCTT-CGGGTT : 375
sp.7_4K_Mi : CAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGACGGACGACGGCCTT-CGGGTT : 377
wged11_Lei : CAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGACGGATGACGGCCTT-CGGGTT : 380
L._japonic : CAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGGCCTT-CGGGTT : 374
ML0004_R._ : CAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGACGGATGACGGCCTT-CGGGTT : 375
6_Clone_Un : CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCGGGAAGAAGGCCTT-CGGGTT : 373
CICCHL_JQ9 : CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGACTGATGAAGGCTTT-CGGGTC : 384
XJU-1_B._c : CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGACTGATGAAGGCTTT-CGGGTC : 388
PR35-2-1_B : CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGACTGATGAAGGCTTT-CGGGTC : 388
760_B._pum : CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGACTGATGAAGGTTTT-CGGATC : 391
S._succinu : CAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGACTGATGAAGGTTTT-CGGATC : 385
ST7_Clone_ : CAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGACGGATGACGGCCTT-CGGGTT : 372
88_17_clon : CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT-CTGGTT : 374
Acinetobac : CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT-TTGGTT : 378
TDIW13_Aci : CAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT-TTGGTT : 378
A449_A._sa : CAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTT-CGGGTT : 376
211c_A._ve : CAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTT-CGGGTT : 376
ATCC_17527 : CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT-CGGATT : 366
PC16_P._pu : CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT-CGGATT : 372
PT03_Bacte : CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT-CGGATT : 370
KVD-unk-80 : CAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTT-CGGGTT : 375
J._lividum : CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGACTGAAGAAGGCCTT-CGGGTT : 377
L._ginseng : CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCGGGAAGAAGGCCTT-CGGGTT : 373
6C_13_Vari : CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTT-CGGGTT : 372
D._acidovo : CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGCAGGATGAAGGCCTT-CGGGTT : 373
300C-C03_C : CAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-CGGGTC : 350
ctg_CGOF25 : CAATGGACGCAAGTCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 349
BY14_Clone : CAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-CGGGTC : 344
BIR2-r_lim : CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 342
MP20_Sphin : CAATGGGCGCAACCCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 352
DSSF72_Unc : CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 349
1/4_C7_32_ : CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 341
AKIW820_Cl : CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 351
WBI100_Clo : CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 347
ENV481_X._ : CAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGACTGATGAAGGCCTT-AGGGTT : 340
V4.BO.05_B : CAATGGGCGAAAGCCTGACGCAGCCATGCCGCGTGAATGATGAAGGTCTT-AGGATT : 347
549_Chryse : CAATGGGTTAGCGCCTGATCCAGCCATCCCGCGTGAAGGACGACGGCCCTATGGGTT : 378
PB93_P._kr : CAATGGAGCCAACTCTGAACCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTT : 374
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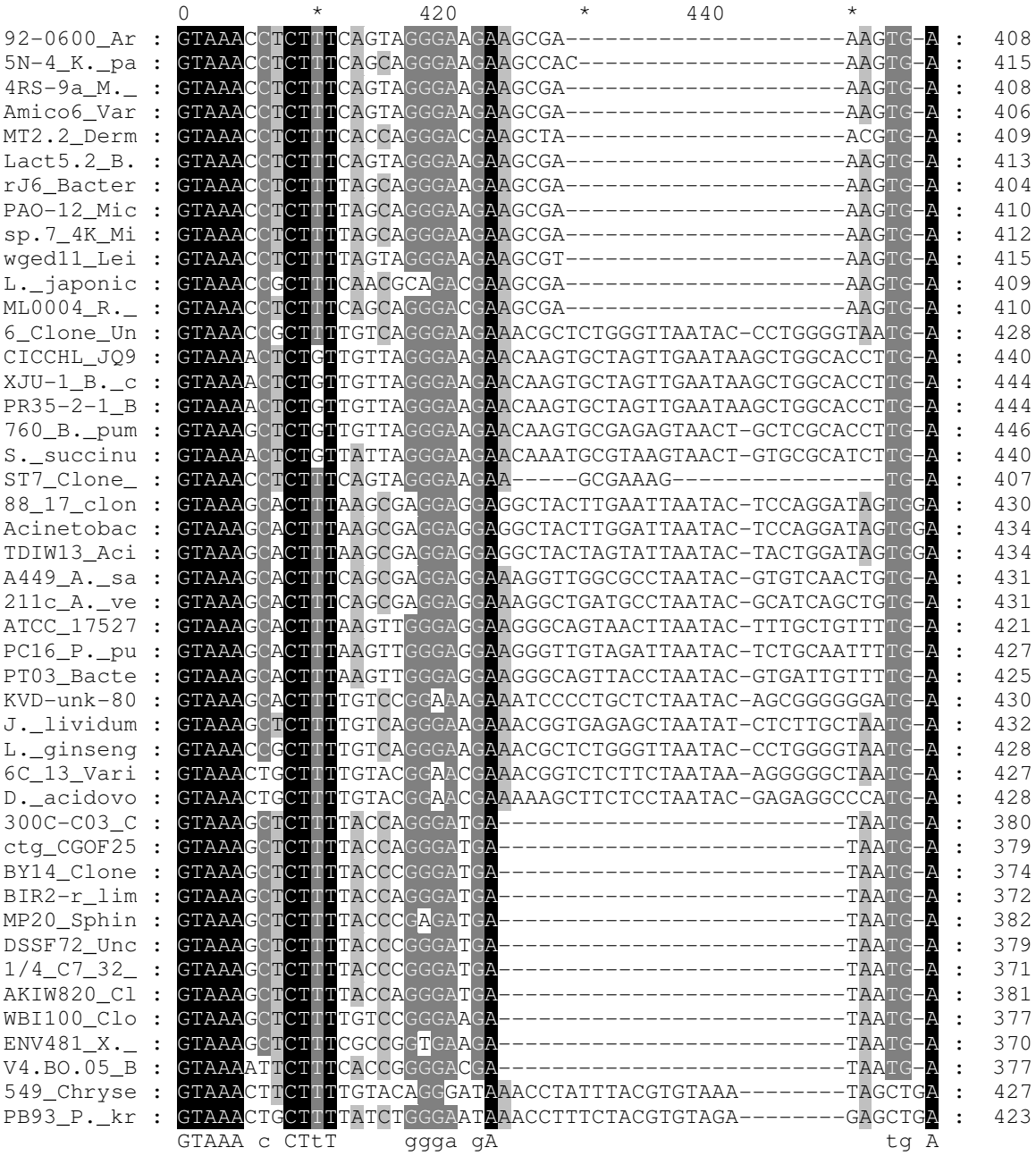
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5N-4_K._pa : GTAAACCTCTTTCAGCAGGGAAGAAGCCAC----------AAGTG-A : 415
4RS-9a_M._ : GTAAACCTCTTTCAGTAGGGAAGAAGCGA----------AAGTG-A : 408
Amico6_Var : GTAAACCTCTTTCAGTAGGGAAGAAGCGA----------AAGTG-A : 406
MT2.2_Derm : GTAAACCTCTTTCACCAGGGACGAAGCTA----------ACGTG-A : 409
Lact5.2_B. : GTAAACCTCTTTCAGTAGGGAAGAAGCGA----------AAGTG-A : 413
rJ6_Bacter : GTAAACCTCTTTTAGCAGGGAAGAAGCGA----------AAGTG-A : 404
PAO-12_Mic : GTAAACCTCTTTTAGCAGGGAAGAAGCGA----------AAGTG-A : 410
sp.7_4K_Mi : GTAAACCTCTTTTAGCAGGGAAGAAGCGA----------AAGTG-A : 412
wged11_Lei : GTAAACCTCTTTTAGTAGGGAAGAAGCGT----------AAGTG-A : 415
L._japonic : GTAAACCGCTTTCAACGCAGACGAAGCGA----------AAGTG-A : 409
ML0004_R._ : GTAAACCTCTTTCAGCAGGGACGAAGCGA----------AAGTG-A : 410
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CICCHL_JQ9 : GTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTG-A : 440
XJU-1_B._c : GTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTG-A : 444
PR35-2-1_B : GTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTG-A : 444
760_B._pum : GTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTAACT-GCTCGCACCTTG-A : 446
S._succinu : GTAAAACTCTGTTATTAGGGAAGAACAAATGCGTAAGTAACT-GTGCGCATCTTG-A : 440
ST7_Clone_ : GTAAACCTCTTTCAGTAGGGAAGAA-----GCGAAAG----------TG-A : 407
88_17_clon : GTAAAGCACTTTAAGCGAGGAGGAGGCTACTTGAATTAATAC-TCCAGGATAGTGGA : 430
Acinetobac : GTAAAGCACTTTAAGCGAGGAGGAGGCTACTTGGATTAATAC-TCCAGGATAGTGGA : 434
TDIW13_Aci : GTAAAGCACTTTAAGCGAGGAGGAGGCTACTAGTATTAATAC-TACTGGATAGTGGA : 434
A449_A._sa : GTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATAC-GTGTCAACTGTG-A : 431
211c_A._ve : GTAAAGCACTTTCAGCGAGGAGGAAAGGCTGATGCCTAATAC-GCATCAGCTGTG-A : 431
ATCC_17527 : GTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAACTTAATAC-TTTGCTGTTTTG-A : 421
PC16_P._pu : GTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATAC-TCTGCAATTTTG-A : 427
PT03_Bacte : GTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATAC-GTGATTGTTTTG-A : 425
KVD-unk-80 : GTAAAGCACTTTTGTCCGGAAAGAAATCCCCTGCTCTAATAC-AGCGGGGGGATG-A : 430
J._lividum : GTAAAGCTCTTTTGTCAGGGAAGAAACGGTGAGAGCTAATAT-CTCTTGCTAATG-A : 432
L._ginseng : GTAAACCGCTTTTGTCAGGGAAGAAACGCTCTGGGTTAATAC-CCTGGGGTAATG-A : 428
6C_13_Vari : GTAAACTGCTTTTGTACGGAACGAAACGGTCTCTTCTAATAA-AGGGGGCTAATG-A : 427
D._acidovo : GTAAACTGCTTTTGTACGGAACGAAAAAGCTTCTCCTAATAC-GAGAGGCCCATG-A : 428
300C-C03_C : GTAAAGCTCTTTTACCAGGGATGA----------TAATG-A : 380
ctg_CGOF25 : GTAAAGCTCTTTTACCAGGGATGA----------TAATG-A : 379
BY14_Clone : GTAAAGCTCTTTTACCCGGGATGA----------TAATG-A : 374
BIR2-r_lim : GTAAAGCTCTTTTACCAGGGATGA----------TAATG-A : 372
MP20_Sphin : GTAAAGCTCTTTTACCCGAGATGA----------TAATG-A : 382
DSSF72_Unc : GTAAAGCTCTTTTACCCGGGATGA----------TAATG-A : 379
1/4_C7_32_ : GTAAAGCTCTTTTACCCGGGATGA----------TAATG-A : 371
AKIW820_Cl : GTAAAGCTCTTTTACCAGGGATGA----------TAATG-A : 381
WBI100_Clo : GTAAAGCTCTTTTGTCCGGGAAGA----------TAATG-A : 377
ENV481_X._ : GTAAAGCTCTTTCGCCGGTGAAGA----------TAATG-A : 370
V4.BO.05_B : GTAAAATTCTTTTCACCGGGGACGA----------TAATG-A : 377
549_Chryse : GTAAACTTCTTTTGTACAGGGATAAACCTATTTACGTGTAAA--------TAGCTGA : 427
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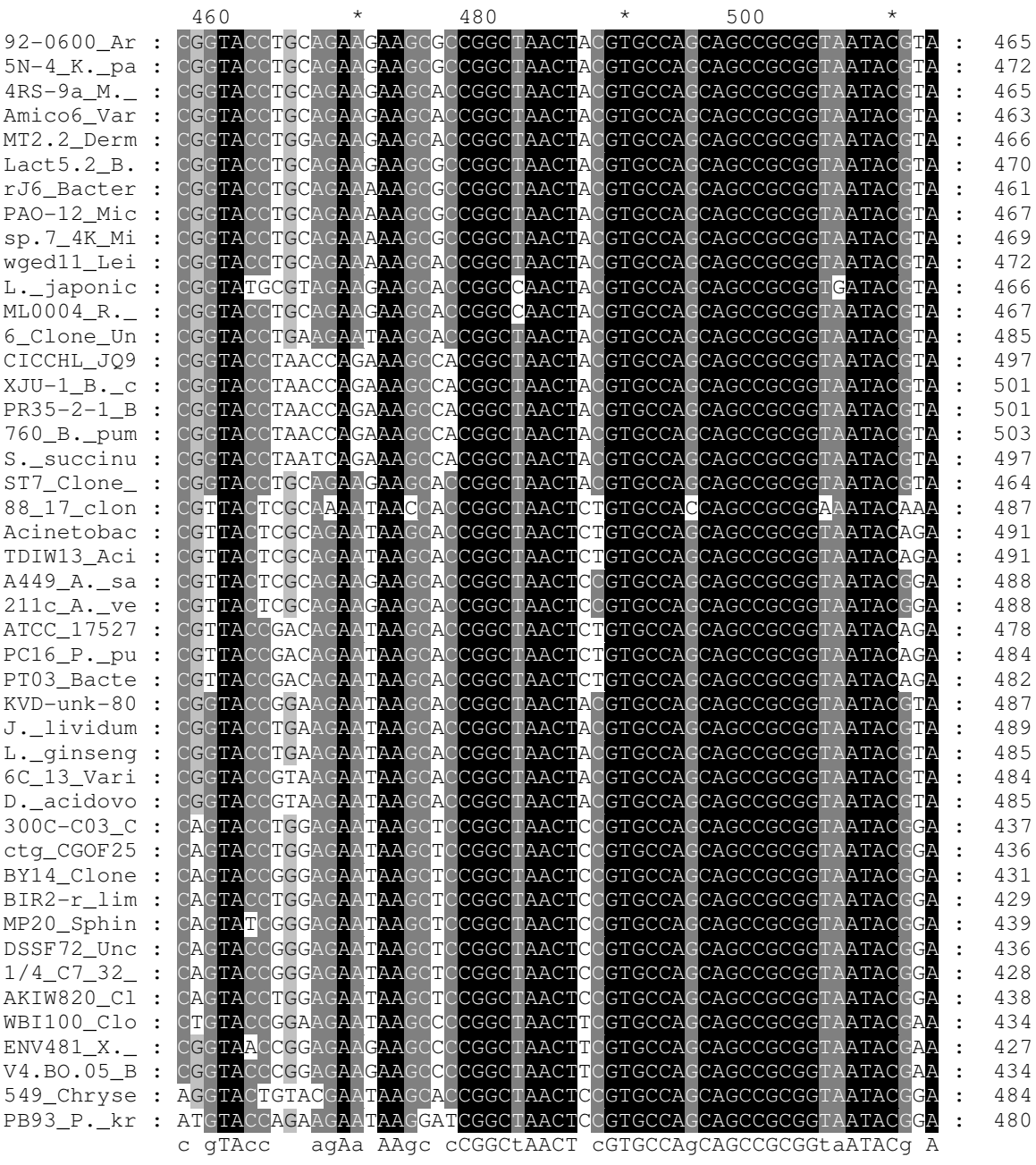
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4RS-9a_M._ : CGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 465
Amico6_Var : CGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 463
MT2.2_Derm : CGGTACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 466
Lact5.2_B. : CGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 470
rJ6_Bacter : CGGTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 461
PAO-12_Mic : CGGTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 467
sp.7_4K_Mi : CGGTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 469
wged11_Lei : CGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 472
L._japonic : CGGTATGCGTAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTGATACGTA : 466
ML0004_R._ : CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTA : 467
6_Clone_Un : CGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 485
CICCHL_JQ9 : CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 497
XJU-1_B._c : CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 501
PR35-2-1_B : CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 501
760_B._pum : CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 503
S._succinu : CGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 497
ST7_Clone_ : CGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 464
88_17_clon : CGTTACTCGCAAATAACGACCGGCTAACTCTGTGCCACCAGCCGCGGAATACAAA : 487
Acinetobac : CGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA : 491
TDIW13_Aci : CGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA : 491
A449_A._sa : CGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 488
211c_A._ve : CGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 488
ATCC_17527 : CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA : 478
PC16_P._pu : CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA : 484
PT03_Bacte : CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA : 482
KVD-unk-80 : CGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 487
J._lividum : CGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 489
L._ginseng : CGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 485
6C_13_Vari : CGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 484
D._acidovo : CGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA : 485
300C-C03_C : CAGTACCTGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 437
ctg_CGOF25 : CAGTACCTGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 436
BY14_Clone : CAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 431
BIR2-r_lim : CAGTACCTGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 429
MP20_Sphin : CAGTATCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 439
DSSF72_Unc : CAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 436
1/4_C7_32_ : CAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 428
AKIW820_Cl : CAGTACCTGGAGAATAAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 438
WBI100_Clo : CTGTACCGGAAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA : 434
ENV481_X._ : CGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA : 427
V4.BO.05_B : CGGTACCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA : 434
549_Chryse : AGGTACTGTACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 484
PB93_P._kr : ATGTACCAGAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA : 480
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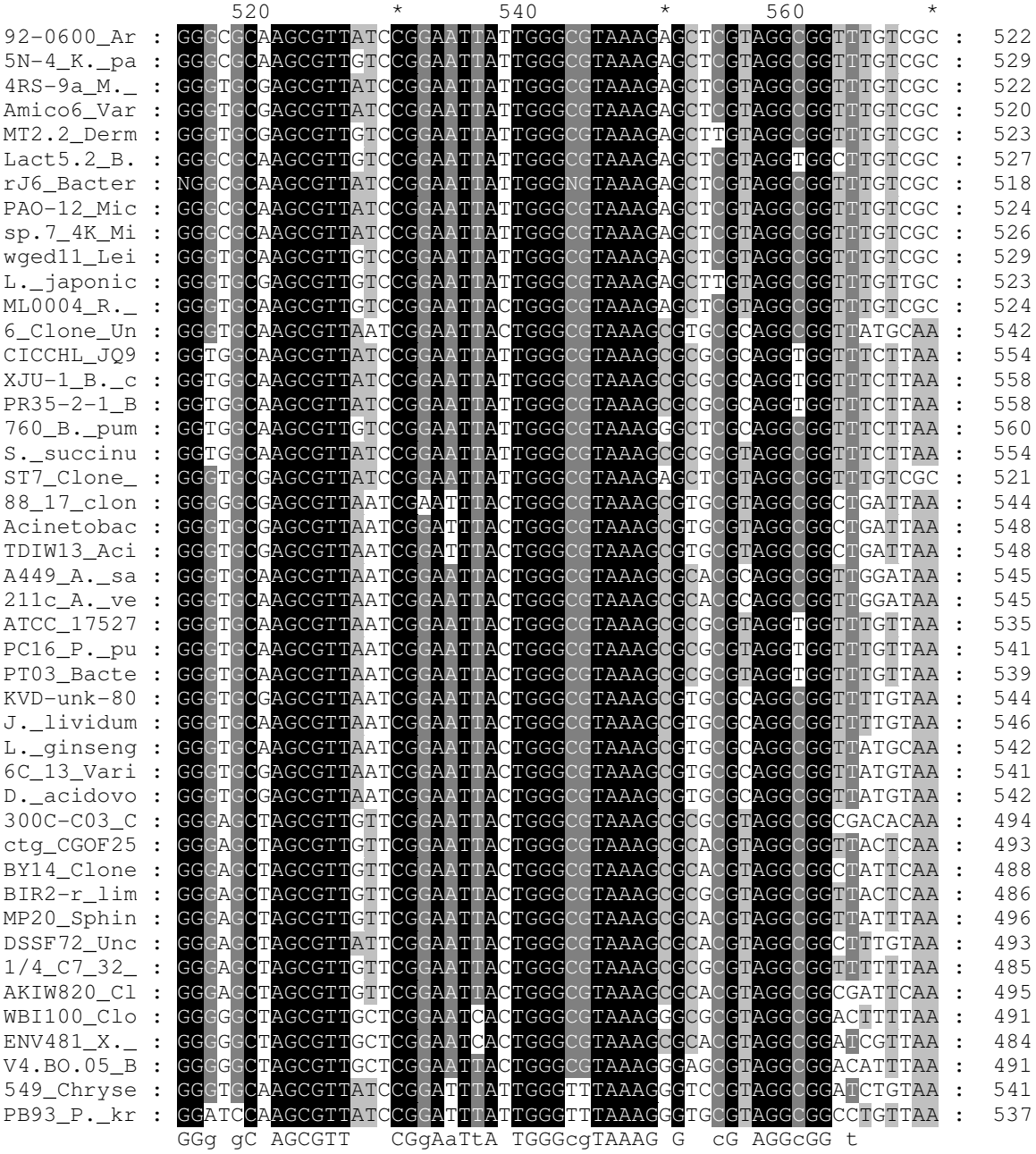
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4RS-9a_M._ : GGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC : 522
Amico6_Var : GGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC : 520
MT2.2_Derm : GGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGC : 523
Lact5.2_B. : GGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGTGGCTTGTCGC : 527
rJ6_Bacter : NGGCGCAAGCGTTATCCGGAATTATTGGGNGTAAAGAGCTCGTAGGCGGTTTGTCGC : 518
PAO-12_Mic : GGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC : 524
sp.7_4K_Mi : GGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC : 526
wged11_Lei : GGGTGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC : 529
L._japonic : GGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTTGC : 523
ML0004_R._ : GGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC : 524
6_Clone_Un : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTATGCAA : 542
CICCHL_JQ9 : GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAA : 554
XJU-1_B._c : GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAA : 558
PR35-2-1_B : GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAA : 558
760_B._pum : GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGCTCGCAGGCGGTTTCTTAA : 560
S._succinu : GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAA : 554
ST7_Clone_ : GGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC : 521
88_17_clon : GGGGGCGAGCGTTAATCGAATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTGATTAA : 544
Acinetobac : GGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTGATTAA : 548
TDIW13_Aci : GGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTGATTAA : 548
A449_A._sa : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAA : 545
211c_A._ve : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAA : 545
ATCC_17527 : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAA : 535
PC16_P._pu : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAA : 541
PT03_Bacte : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAA : 539
KVD-unk-80 : GGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAA : 544
J._lividum : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAA : 546
L._ginseng : GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTATGCAA : 542
6C_13_Vari : GGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTATGTAA : 541
D._acidovo : GGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTATGTAA : 542
300C-C03_C : GGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCGACACAA : 494
ctg_CGOF25 : GGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGTTACTCAA : 493
BY14_Clone : GGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCTATTCAA : 488
BIR2-r_lim : GGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGTTACTCAA : 486
MP20_Sphin : GGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGTTATTTAA : 496
DSSF72_Unc : GGGAGCTAGCGTTATTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCTTTGTAA : 493
1/4_C7_32_ : GGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAA : 485
AKIW820_Cl : GGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGCGATTCAA : 495
WBI100_Clo : GGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGCGCGTAGGCGGACTTTTAA : 491
ENV481_X._ : GGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGCGCACGTAGGCGGATCGTTAA : 484
V4.BO.05_B : GGGGGCTAGCGTTGCTCGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAA : 491
549_Chryse : GGGTGCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTCCGTAGGCGGATCTGTAA : 541
PB93_P._kr : GGATCCAAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCCTGTTAA : 537
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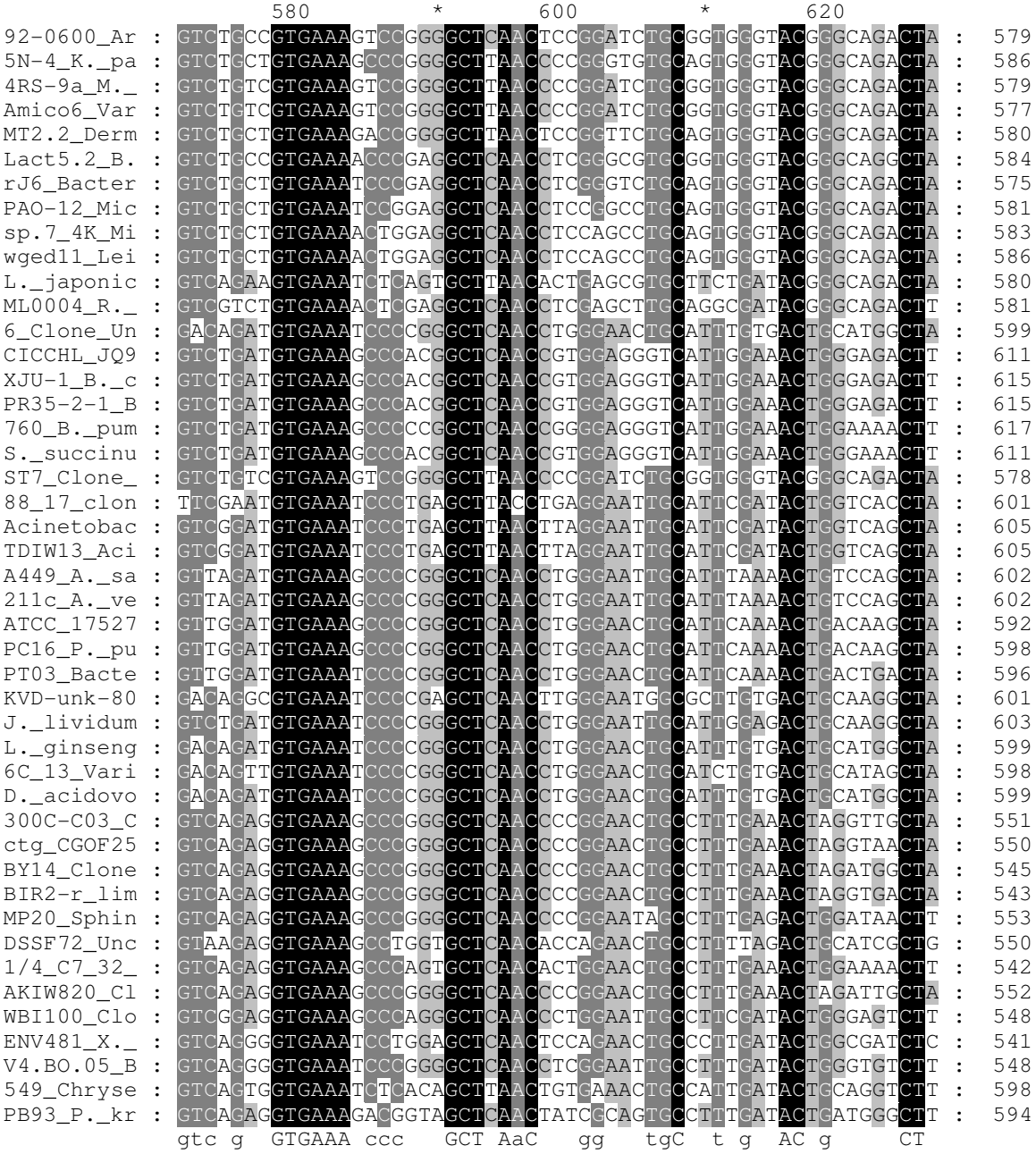
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5N-4_K._pa : GTCTGCCTGTGAAAGCCCGGGGCTTAACCCCGGGTGTGCAGTGGGTACGGCAGACTA : 586
4RS-9a_M._ : GTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGCAGACTA : 579
Amico6_Var : GTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGCAGACTA : 577
MT2.2_Derm : GTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGCAGACTA : 580
Lact5.2_B. : GTCTGCCGTGAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGCAGGCTA : 584
rJ6_Bacter : GTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGTCTGCAGTGGGTACGGCAGACTA : 575
PAO-12_Mic : GTCTGCTGTGAAATCCGGAGGCTCAACCTCCGGCCTGCAGTGGGTACGGCAGACTA : 581
sp.7_4K_Mi : GTCTGCTGTGAAAACTGGAGGCTCAACCTCCAGCCTGCAGTGGGTACGGCAGACTA : 583
wged11_Lei : GTCTGCTGTGAAAACTGGAGGCTCAACCTCCAGCCTGCAGTGGGTACGGCAGACTA : 586
L._japonic : GTCAGAAGTGAAATCTCAGTGCTTAACACTGAGCGTGCTTCTGATACGGGCAGACTA : 580
ML0004_R._ : GTCGTCTGTGAAAACTCGAGGCTCAACCTCGAGCTTGCAGGCGATACGGGCAGACTT : 581
6_Clone_Un : GACAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGCATGGCTA : 599
CICCHL_JQ9 : GTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTT : 611
XJU-1_B._c : GTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTT : 615
PR35-2-1_B : GTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTT : 615
760_B._pum : GTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGAAAACTT : 617
S._succinu : GTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTT : 611
ST7_Clone_ : GTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGGCAGACTA : 578
88_17_clon : TTCGAATGTGAAATCCCTGAGCTTACCTGAGGAATTGCATTCGATACTGGTCACCTA : 601
Acinetobac : GTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGTCAGCTA : 605
TDIW13_Aci : GTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGTCAGCTA : 605
A449_A._sa : GTTACATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTA : 602
211c_A._ve : GTTACATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTA : 602
ATCC_17527 : GTTGCATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTA : 592
PC16_P._pu : GTTGCATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACAAGCTA : 598
PT03_Bacte : GTTGCATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTA : 596
KVD-unk-80 : GACAGGCGTGAAATCCCCGAGCTCAACTTGGGAATGGCGCTTGTGACTGCAAGGCTA : 601
J._lividum : GTCTGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTGGAGACTGCAAGGCTA : 603
L._ginseng : GACACATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGCATGGCTA : 599
6C_13_Vari : GACACTTGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGTGACTGCATAGCTA : 598
D._acidovo : GACACATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGTGACTGCATGGCTA : 599
300C-C03_C : GTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAACTGCCTTTGAAACTAGGTTGCTA : 551
ctg_CGOF25 : GTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAACTGCCTTTGAAACTAGGTAACTA : 550
BY14_Clone : GTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAACTGCCTTTGAAACTAGATGGCTA : 545
BIR2-r_lim : GTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAACTGCCTTTGAAACTAGGTGACTA : 543
MP20_Sphin : GTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAATAGCCTTTGAGACTGGATAACTT : 553
DSSF72_Unc : GTAAGAGGTGAAAGCCTGGTGCTCAACACCAGAACTGCCTTTTAGACTGCATCGCTG : 550
1/4_C7_32_ : GTCAGAGGTGAAAGCCCAGTGCTCAACACTGGAACTGCCTTTGAAACTGGAAAACTT : 542
AKIW820_Cl : GTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAACTGCCTTTGAAACTAGATTGCTA : 552
WBI100_Clo : GTCGGAGGTGAAAGCCCAGGGCTCAACCCTGGAATTGCCTTTCGATACTGGGAGTCTT : 548
ENV481_X._ : GTCAGGGGTGAAATCCTGGAGCTCAACTCCAGAACTGCCCTTGATACTGGCGATCTC : 541
V4.BO.05_B : GTCAGGGGTGAAATCCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGGGTGTCTT : 548
549_Chryse : GTCAGTGGTGAAATCTCACAGCTTAACTGTGAAACTGCCATTGATACTGCAGGTCTT : 598
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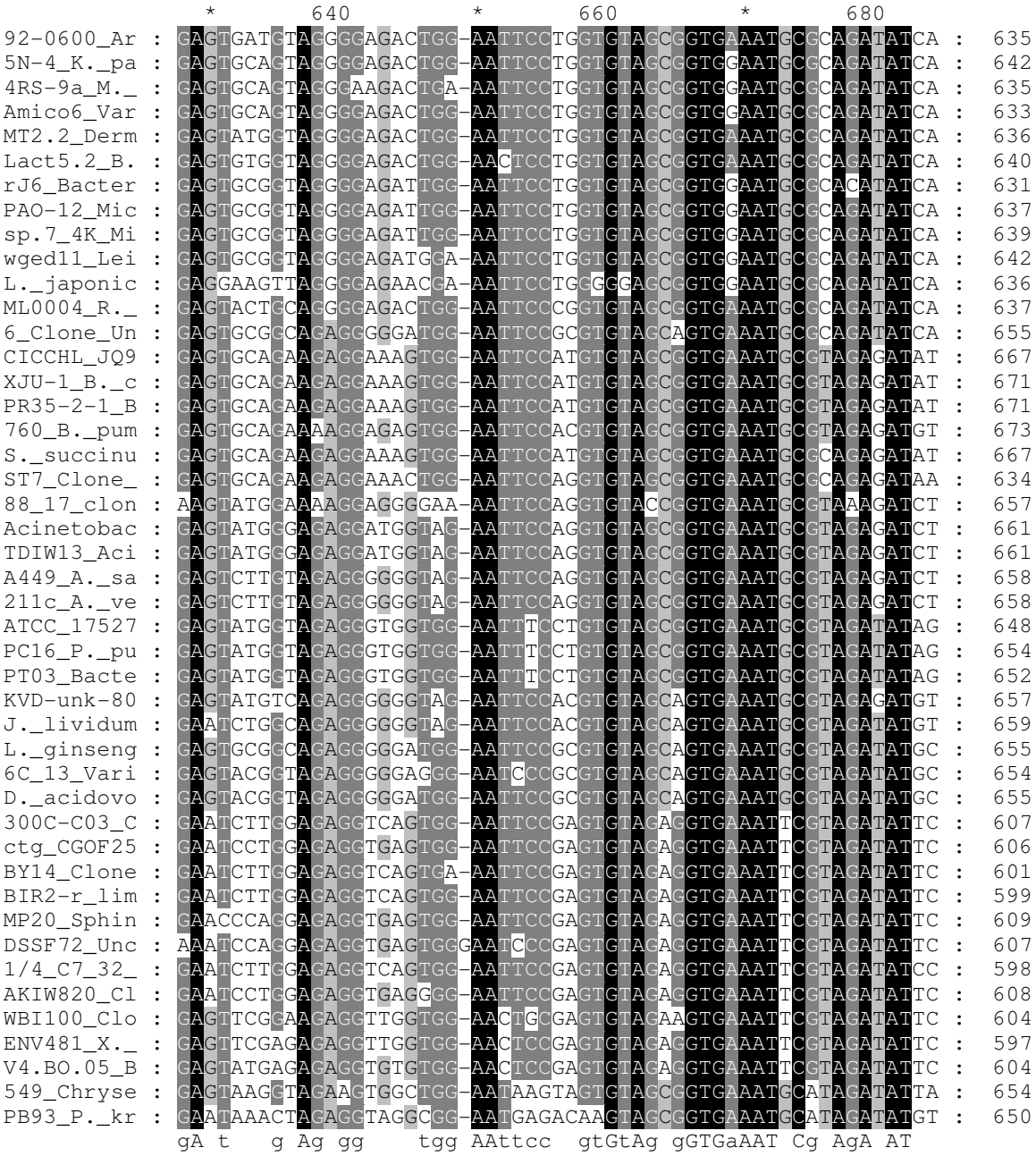
* 640 * 660 * 680
92-0600_Ar : GAGTGATCTAGGGGAGACTGG-AATTCCTGGTGTACCGGTGAAATGCGCAGATATCA : 635
5N-4_K._pa : GAGTGCACTAGGGGAGACTGG-AATTCCTGGTGTACCGGTGGAATGCGCAGATATCA : 642
4RS-9a_M._ : GAGTGCACTAGGGAAGACTGA-AATTCCTGGTGTACCGGTGGAATGCGCAGATATCA : 635
Amico6_Var : GAGTGCACTAGGGGAGACTGG-AATTCCTGGTGTACCGGTGGAATGCGCAGATATCA : 633
MT2.2_Derm : GAGTATGGTAGGGGAGACTGG-AATTCCTGGTGTACCGGTGAAATGCGCAGATATCA : 636
Lact5.2_B. : GAGTGTGGTAGGGGAGACTGG-AACTCCTGGTGTACCGGTGAAATGCGCAGATATCA : 640
rJ6_Bacter : GAGTGCGCTAGGGGAGATTGG-AATTCCTGGTGTACCGGTGGAATGCGCACATATCA : 631
PAO-12_Mic : GAGTGCGCTAGGGGAGATTGG-AATTCCTGGTGTACCGGTGGAATGCGCAGATATCA : 637
sp.7_4K_Mi : GAGTGCGCTAGGGGAGATTGG-AATTCCTGGTGTACCGGTGGAATGCGCAGATATCA : 639
wged11_Lei : GAGTGCGCTAGGGGAGATGGA-AATTCCTGGTGTACCGGTGGAATGCGCAGATATCA : 642
L._japonic : GAGGAAGTTAGGGGAGAACGA-AATTCCTGGGGGACCGGTGGAATGCGCAGATATCA : 636
ML0004_R._ : GAGTACTCCAGGGGAGACTGG-AATTCCCGGTGTACCGGTGAAATGCGCAGATATCA : 637
6_Clone_Un : GAGTGCGCCAGAGGGGGATGG-AATTCCGCGTGTACCAGTGAAATGCGCAGATATCA : 655
CICCHL_JQ9 : GAGTGCACAAGAGGAAAGTGG-AATTCCATGTGTACCGGTGAAATGCGTAGAGATAT : 667
XJU-1_B._c : GAGTGCACAAGAGGAAAGTGG-AATTCCATGTGTACCGGTGAAATGCGTAGAGATAT : 671
PR35-2-1_B : GAGTGCACAAGAGGAAAGTGG-AATTCCATGTGTACCGGTGAAATGCGTAGAGATAT : 671
760_B._pum : GAGTGCACAAAAGGAGAGTGG-AATTCCACGTGTACCGGTGAAATGCGTAGAGATGT : 673
S._succinu : GAGTGCACAAGAGGAAAGTGG-AATTCCATGTGTACCGGTGAAATGCGCAGAGATAT : 667
ST7_Clone_ : GAGTGCACAAGAGGAAACTGG-AATTCCAGGTGTACCGGTGAAATGCGCAGAGATAA : 634
88_17_clon : AAGTATGCAAAAGGAGGGGAA-AATTCCAGGTGTACCGGTGAAATGCGTAAAGATCT : 657
Acinetobac : GAGTATGCGAGAGGATGGTAG-AATTCCAGGTGTACCGGTGAAATGCGTAGAGATCT : 661
TDIW13_Aci : GAGTATGCGAGAGGATGGTAG-AATTCCAGGTGTACCGGTGAAATGCGTAGAGATCT : 661
A449_A._sa : GAGTCTTGTAGAGGGGGGTAG-AATTCCAGGTGTACCGGTGAAATGCGTAGAGATCT : 658
211c_A._ve : GAGTCTTGTAGAGGGGGGTAG-AATTCCAGGTGTACCGGTGAAATGCGTAGAGATCT : 658
ATCC_17527 : GAGTATGCTAGAGGGTGGTGG-AATTTCCTGTGTACCGGTGAAATGCGTAGATATAG : 648
PC16_P._pu : GAGTATGCTAGAGGGTGGTGG-AATTTCCTGTGTACCGGTGAAATGCGTAGATATAG : 654
PT03_Bacte : GAGTATGCTAGAGGGTGGTGG-AATTTCCTGTGTACCGGTGAAATGCGTAGATATAG : 652
KVD-unk-80 : GAGTATGTCAGAGGGGGGTAG-AATTCCACGTGTACCAGTGAAATGCGTAGAGATGT : 657
J._lividum : GAATCTGGCAGAGGGGGGTAG-AATTCCACGTGTACCAGTGAAATGCGTAGATATGT : 659
L._ginseng : GAGTGCGGCAGAGGGGGATGG-AATTCCGCGTGTACCAGTGAAATGCGTAGATATGC : 655
6C_13_Vari : GAGTACGCTAGAGGGGGAGGG-AATCCCGCGTGTACCAGTGAAATGCGTAGATATGC : 654
D._acidovo : GAGTACGCTAGAGGGGGATGG-AATTCCGCGTGTACCAGTGAAATGCGTAGATATGC : 655
300C-C03_C : GAATCTTCGAGAGGTCAGTGG-AATTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 607
ctg_CGOF25 : GAATCCTCGAGAGGTGAGTGG-AATTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 606
BY14_Clone : GAATCTTCGAGAGGTCAGTGA-AATTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 601
BIR2-r_lim : GAATCTTCGAGAGGTCAGTGG-AATTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 599
MP20_Sphin : GAACCCACGAGAGGTGAGTGG-AATTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 609
DSSF72_Unc : AAATCCACGAGAGGTGAGTGGGAATCCCGAGTGTACAGGTGAAATTCGTAGATATTC : 607
1/4_C7_32_ : GAATCTTCGAGAGGTCAGTGG-AATTCCGAGTGTACAGGTGAAATTCGTAGATATCC : 598
AKIW820_Cl : GAATCCTCGAGAGGTGAGGGG-AATTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 608
WBI100_Clo : GAGTTCGCAAGAGGTTGGTGG-AACTGCGAGTGTACAAGTGAAATTCGTAGATATTC : 604
ENV481_X._ : GAGTTCGAGAGAGGTTGGTGG-AACTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 597
V4.BO.05_B : GAGTATGAGAGAGGTGTGTGG-AACTCCGAGTGTACAGGTGAAATTCGTAGATATTC : 604
549_Chryse : GAGTAAGCTAGAAGTGGCTGG-AATAAGTAGTGTACCGGTGAAATGCATAGATATTA : 654
PB93_P._kr : GAATAAACTAGAGGTAGGCGG-AATGAGACAAGTACCGGTGAAATGCATAGATATGT : 650
gA t g Ag gg tgg AAttcc gtGtAg gGTGaAAT Cg AgA AT


* 700 * 720 * 740
92-0600_Ar : GGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGA : 692
5N-4_K._pa : GGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGCGA : 699
4RS-9a_M._ : GGAGAAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGA : 692
Amico6_Var : GGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGA : 690
MT2.2_Derm : GGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACGCTGACAAGCGA : 693
Lact5.2_B. : GGAAGAACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACACTGAGGAGCGA : 697
rJ6_Bacter : GGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGA : 688
PAO-12_Mic : GGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGA : 694
sp.7_4K_Mi : GGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGA : 696
wged11_Lei : GGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGA : 699
L._japonic : GGAGGAACACCGGGGGCGAAGGCGGTCCTCTGGGACTTTCCTGACGCTGACAAGCGA : 693
ML0004_R._ : GGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGA : 694
6_Clone_Un : GGAGGAACACCGATGGCGAAGGCAAGCCCCTGGGCATGAACTGACGCTCAGGAACGA : 712
CICCHL_JQ9 : GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGA : 724
XJU-1_B._c : GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGA : 728
PR35-2-1_B : GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGA : 728
760_B._pum : GGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGA : 730
S._succinu : GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGA : 724
ST7_Clone_ : GGAGGAACACCAATGGCGAAGGCAACTCTCTGGGCTGTAACTGACACTGAGGAGCGA : 691
88_17_clon : GGAGAAATACCGATGGCAAAGGCAGCCATCTGGCCTAATACTGACCCTGAGGGACAA : 714
Acinetobac : GGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGA : 718
TDIW13_Aci : GGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGA : 718
A449_A._sa : GGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA : 715
211c_A._ve : GGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA : 715
ATCC_17527 : GAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGA : 705
PC16_P._pu : GAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGA : 711
PT03_Bacte : GAAGGAAAACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGA : 709
KVD-unk-80 : GGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGACGTCACTGACGCTCATGCACGA : 714
J._lividum : GGAGGAACACCGATGGCGAAGGCAGCCCCCTGGGTCAAGATTGACGCTCATGCACGA : 716
L._ginseng : GGAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCTCATGCACGA : 712
6C_13_Vari : GGAGGAACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGA : 711
D._acidovo : GGAGGAACACCGATGGCGAAGGCAATCCCCTGGACCTGTACTGACGCTCATGCACGA : 712
300C-C03_C : GGAAGAACACCAGTGGCGAAGGCGACTGACTGGACAAGTATTGACGCTGAGGTGCGA : 664
ctg_CGOF25 : GGAAGAACACCAGTGGCGAAGGCGGCTCACTGGACAGGTATTGACGCTGAGGTGCGA : 663
BY14_Clone : GGAAGAACACCAGTGGCGAAGGCGACTGACTGGACAAGTATTGACGCTGAGGTGCGA : 658
BIR2-r_lim : GGAAGAACACCAGTGGCGAAGGCGACTGACTGGACAAGTATTGACGCTGAGGTGCGA : 656
MP20_Sphin : GGAAGAACACCAGTGGCGAAGGCGGCTCACTGGACTGGTATTGACGCTGAGGTGCGA : 666
DSSF72_Unc : GGAAGAACACCAGTGGCGAAGGCGGCTCACTGGACTGGTATTGACGCTGAGGTGCGA : 664
1/4_C7_32_ : GGAAGAACACCAGTGGCGAAGGCGACTGACTGGACAAGTATTGACGCTGAGGTGCGA : 655
AKIW820_Cl : GGAAGAACACCAGTGGCGAAGGCGACTCACTGGACAGGTACTGACGCTGAGGTGCGA : 665
WBI100_Clo : GCAAGAACACCGGTGGCGAAGGCGGCCAACTGGTCCGATACTGACGCTGAGGCGCGA : 661
ENV481_X._ : GGAAGAACACCAGTGGCGAAGGCGGCCAACTGGCTCGATACTGACGCTGAGGTGCGA : 654
V4.BO.05_B : GGAAGAACACCAGTGGCGAAGGCGACACACTGGCTCATTACTGACGCTGAGGCTCGA : 661
549_Chryse : CTTAGAACACCAATTGCGAAGGCAGGTCACTATGTCTTAACTGACGCTGATGGACGA : 711
PB93_P._kr : CTCAGAACACCGATTGCGAAGGCAGCTTACTATGGTTTTATTGACGCTGAGGCACGA : 707
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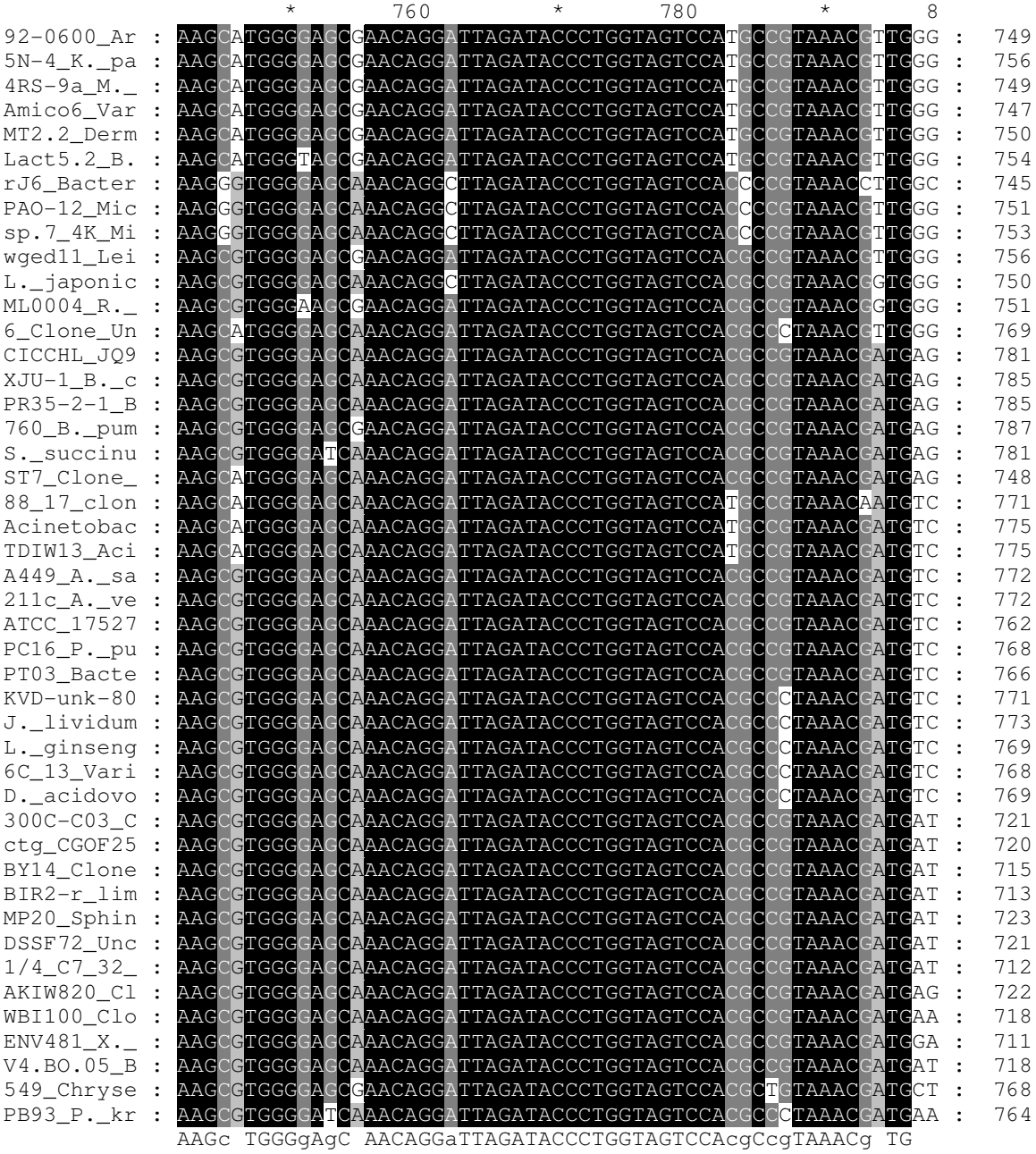
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5N-4_K._pa : AAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGG : 756
4RS-9a_M._ : AAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGG : 749
Amico6_Var : AAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGG : 747
MT2.2_Derm : AAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGG : 750
Lact5.2_B. : AAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGG : 754
rJ6_Bacter : AAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACCTTGGC : 745
PAO-12_Mic : AAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGG : 751
sp.7_4K_Mi : AAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGG : 753
wged11_Lei : AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGG : 756
L._japonic : AAGCGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGG : 750
ML0004_R._ : AAGCGTGGGAAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGG : 751
6_Clone_Un : AAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGTTGGG : 769
CICCHL_JQ9 : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG : 781
XJU-1_B._c : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG : 785
PR35-2-1_B : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG : 785
760_B._pum : AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG : 787
S._succinu : AAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG : 781
ST7_Clone_ : AAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG : 748
88_17_clon : AAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACAATGTC : 771
Acinetobac : AAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTC : 775
TDIW13_Aci : AAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTC : 775
A449_A._sa : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC : 772
211c_A._ve : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC : 772
ATCC_17527 : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC : 762
PC16_P._pu : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC : 768
PT03_Bacte : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC : 766
KVD-unk-80 : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTC : 771
J._lividum : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTC : 773
L._ginseng : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTC : 769
6C_13_Vari : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTC : 768
D._acidovo : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTC : 769
300C-C03_C : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 721
ctg_CGOF25 : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 720
BY14_Clone : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 715
BIR2-r_lim : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 713
MP20_Sphin : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 723
DSSF72_Unc : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 721
1/4_C7_32_ : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 712
AKIW820_Cl : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG : 722
WBI100_Clo : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAA : 718
ENV481_X._ : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGA : 711
V4.BO.05_B : AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAT : 718
549_Chryse : AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGCT : 768
PB93_P._kr : AAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAA : 764
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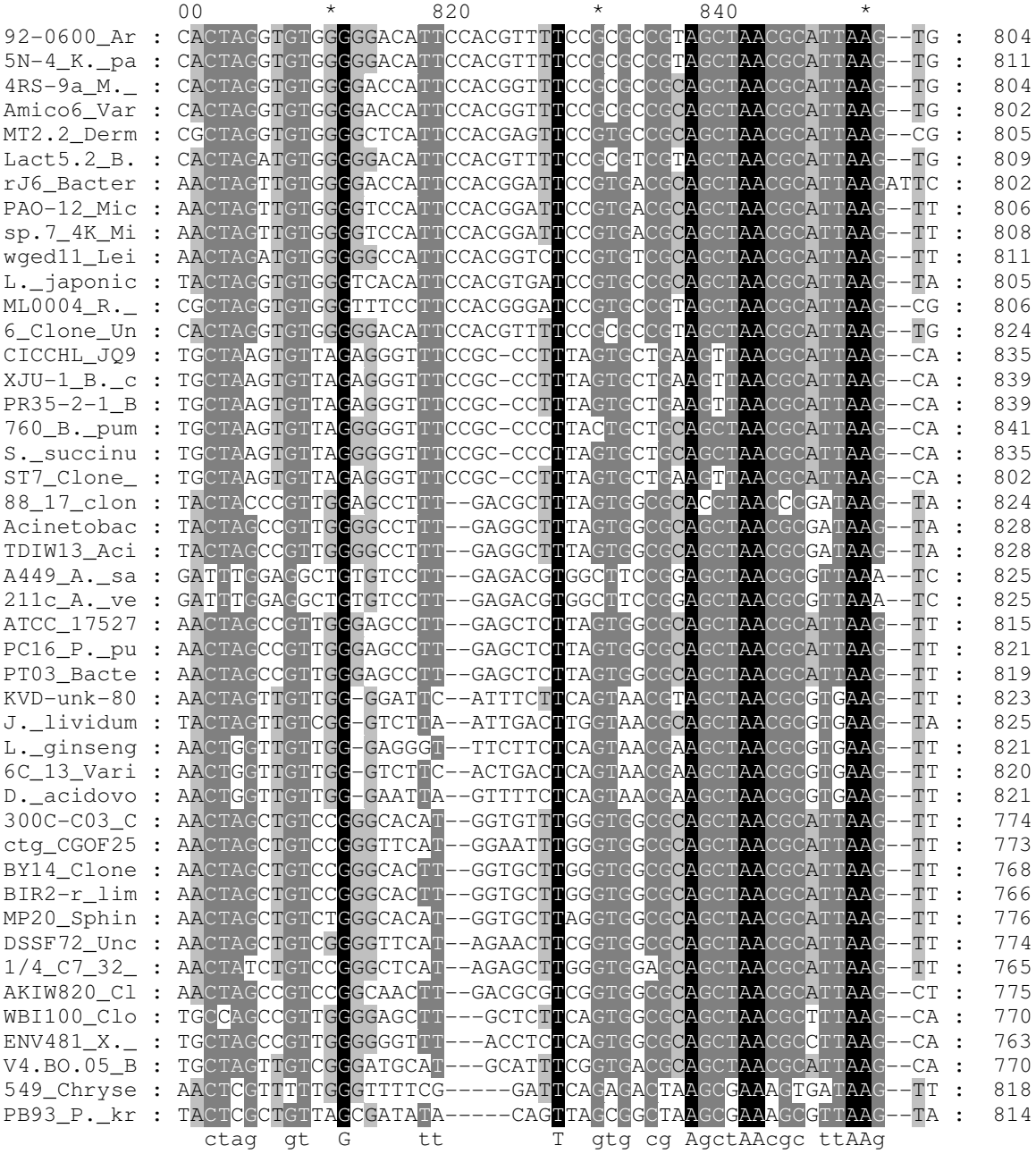
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5N-4_K._pa : CACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAG--TG : 811
4RS-9a_M._ : CACTAGGTGTGGGGACCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAG--TG : 804
Amico6_Var : CACTAGGTGTGGGGACCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAG--TG : 802
MT2.2_Derm : CGCTAGGTGTGGGGCTCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAG--CG : 805
Lact5.2_B. : CACTAGATGTGGGGGACATTCCACGTTTTCCGCGTCGTAGCTAACGCATTAAG--TG : 809
rJ6_Bacter : AACTAGTTGTGGGGACCATTCCACGGATTCCGTGACGCAGCTAACGCATTAAGATTC : 802
PAO-12_Mic : AACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACGCAGCTAACGCATTAAG--TT : 806
sp.7_4K_Mi : AACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACGCAGCTAACGCATTAAG--TT : 808
wged11_Lei : AACTAGATGTGGGGGCCATTCCACGGTCTCCGTGTCGCAGCTAACGCATTAAG--TT : 811
L._japonic : TACTAGGTGTGGGTCACATTCCACGTGATCCGTGCCGCAGCTAACGCATTAAG--TA : 805
ML0004_R._ : CGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGCTAGCTAACGCATTAAG--CG : 806
6_Clone_Un : CACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGCTAGCTAACGCATTAAG--TG : 824
CICCHL_JQ9 : TGCTAAGTGTTAGAGGGTTTCCGC-CCTTTAGTGCTCAAGTTAACGCATTAAG--CA : 835
XJU-1_B._c : TGCTAAGTGTTAGAGGGTTTCCGC-CCTTTAGTGCTCAAGTTAACGCATTAAG--CA : 839
PR35-2-1_B : TGCTAAGTGTTAGAGGGTTTCCGC-CCTTTAGTGCTCAAGTTAACGCATTAAG--CA : 839
760_B._pum : TGCTAAGTGTTAGGGGGTTTCCGC-CCCTTACTGCTCGCAGCTAACGCATTAAG--CA : 841
S._succinu : TGCTAAGTGTTAGGGGGTTTCCGC-CCCTTAGTGCTCGCAGCTAACGCATTAAG--CA : 835
ST7_Clone_ : TGCTAAGTGTTAGAGGGTTTCCGC-CCTTTAGTGCTCAAGTTAACGCATTAAG--CA : 802
88_17_clon : TACTACCCGTTGGAGCCTTT--GACGCTTTAGTGGCGCACCTAACCCGATAAG--TA : 824
Acinetobac : TACTAGCCGTTGGGGCCTTT--GAGGCTTTAGTGGCGCAGCTAACGCGATAAG--TA : 828
TDIW13_Aci : TACTAGCCGTTGGGGCCTTT--GAGGCTTTAGTGGCGCAGCTAACGCGATAAG--TA : 828
A449_A._sa : GATTTCGACGCTGTGTCCTT--GAGACGTGGCTTCCCGAGCTAACGCGTTAAA--TC : 825
211c_A._ve : GATTTCGACGCTGTGTCCTT--GAGACGTGGCTTCCCGAGCTAACGCGTTAAA--TC : 825
ATCC_17527 : AACTAGCCGTTGGGAGCCTT--GAGCTCTTAGTGGCGCAGCTAACGCATTAAG--TT : 815
PC16_P._pu : AACTAGCCGTTGGGAGCCTT--GAGCTCTTAGTGGCGCAGCTAACGCATTAAG--TT : 821
PT03_Bacte : AACTAGCCGTTGGGAGCCTT--GAGCTCTTAGTGGCGCAGCTAACGCATTAAG--TT : 819
KVD-unk-80 : AACTAGTTGTTGG-GGATTC--ATTTCTTCAGTAACGTAGCTAACGCGTGAAG--TT : 823
J._lividum : TACTAGTTGTCGG-GTCTTA--ATTGACTTGGTAACGCAGCTAACGCGTGAAG--TA : 825
L._ginseng : AACTGGTTGTTGG-GAGGGT--TTCTTCTCAGTAACCAAGCTAACGCGTGAAG--TT : 821
6C_13_Vari : AACTGGTTGTTGG-GTCTTC--ACTGACTCAGTAACCAAGCTAACGCGTGAAG--TT : 820
D._acidovo : AACTGGTTGTTGG-GAATTA--GTTTTCTCAGTAACCAAGCTAACGCGTGAAG--TT : 821
300C-C03_C : AACTAGCTGTCCGGGCACAT--GGTGTTTGGGTGGCGCAGCTAACGCATTAAG--TT : 774
ctg_CGOF25 : AACTAGCTGTCCGGGTTCAT--GGAATTTGGGTGGCGCAGCTAACGCATTAAG--TT : 773
BY14_Clone : AACTAGCTGTCCGGGCACTT--GGTGCTTGGGTGGCGCAGCTAACGCATTAAG--TT : 768
BIR2-r_lim : AACTAGCTGTCCGGGCACTT--GGTGCTTGGGTGGCGCAGCTAACGCATTAAG--TT : 766
MP20_Sphin : AACTAGCTGTCTGGGCACAT--GGTGCTTAGGTGGCGCAGCTAACGCATTAAG--TT : 776
DSSF72_Unc : AACTAGCTGTCGGGGTTCAT--AGAACTTCGGTGGCGCAGCTAACGCATTAAG--TT : 774
1/4_C7_32_ : AACTATCTGTCCGGGCTCAT--AGAGCTTGGGTGGACGCAGCTAACGCATTAAG--TT : 765
AKIW820_Cl : AACTAGCCGTCCGGCAACTT--GACGCGTCGGTGGCGCAGCTAACGCATTAAG--CT : 775
WBI100_Clo : TGCCAGCCGTTGGGGAGCTT---GCTCTTCAGTGGCGCAGCTAACGCTTTAAG--CA : 770
ENV481_X._ : TGCTAGCCGTTGGGGGGTTT---ACCTCTCAGTGGCGCAGCTAACGCCTTAAG--CA : 763
V4.BO.05_B : TGCTAGTTGTCGGGATGCAT---GCATTTCGGTGACGCAGCTAACGCATTAAG--CA : 770
549_Chryse : AACTCGTTTTTGGGTTTTCG-----GATTCAGAGACTAAGCGAAAGTGATAAG--TT : 818
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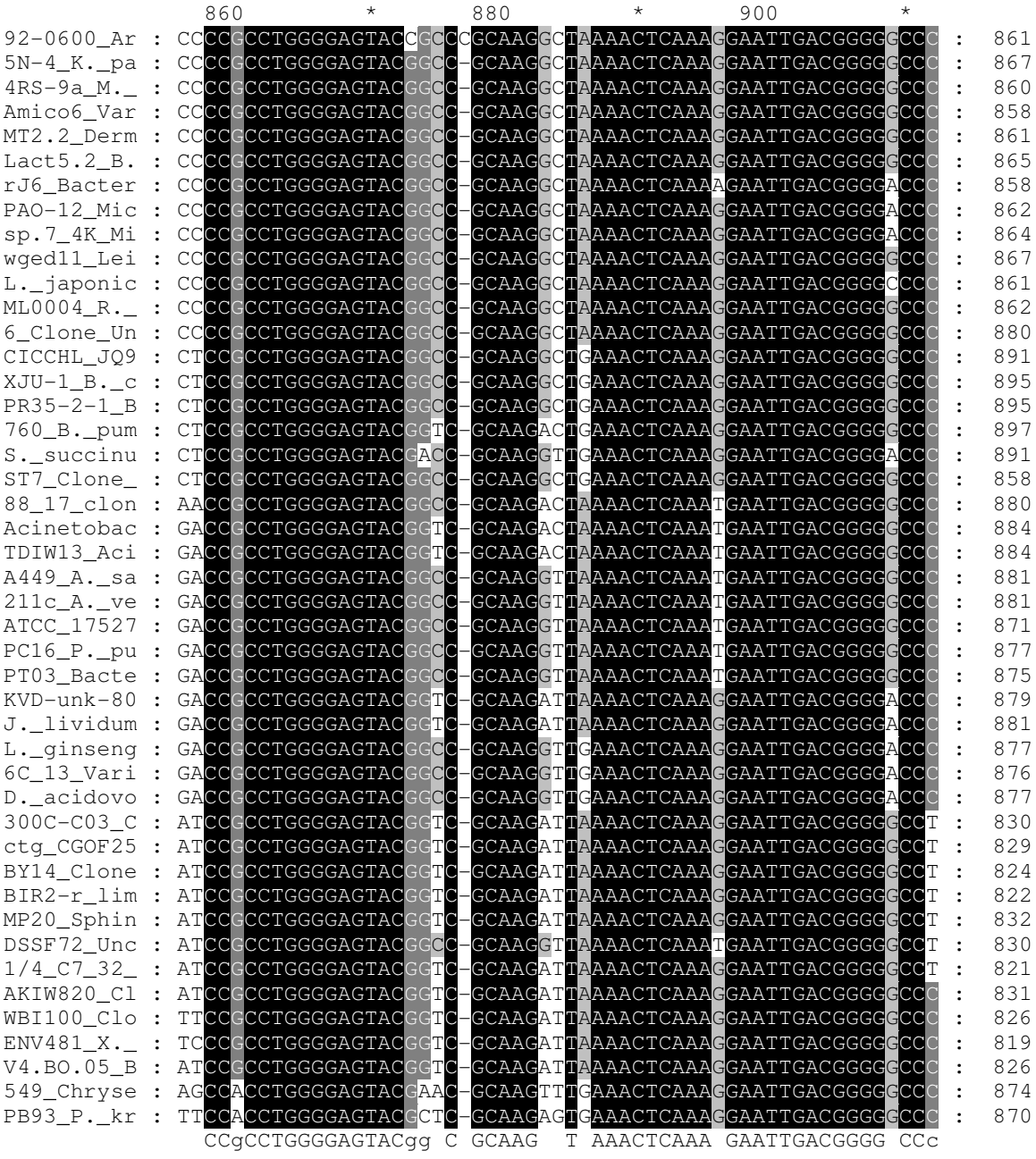
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4RS-9a_M._ : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC : 860
Amico6_Var : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC : 858
MT2.2_Derm : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC : 861
Lact5.2_B. : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC : 865
rJ6_Bacter : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAAGAATTGACGGGGACCC : 858
PAO-12_Mic : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCC : 862
sp.7_4K_Mi : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCC : 864
wged11_Lei : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC : 867
L._japonic : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGCCCC : 861
ML0004_R._ : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC : 862
6_Clone_Un : CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC : 880
CICCHL_JQ9 : CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCC : 891
XJU-1_B._c : CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCC : 895
PR35-2-1_B : CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCC : 895
760_B._pum : CTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCC : 897
S._succinu : CTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCC : 891
ST7_Clone_ : CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCC : 858
88_17_clon : AACCGCCTGGGGAGTACGGCCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCC : 880
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TDIW13_Aci : GACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCC : 884
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211c_A._ve : GACCGCCTGGGGAGTACGGCGCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC : 881
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PC16_P._pu : GACCGCCTGGGGAGTACGGCGCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC : 877
PT03_Bacte : GACCGCCTGGGGAGTACGGCGCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC : 875
KVD-unk-80 : GACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCC : 879
J._lividum : GACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCC : 881
L._ginseng : GACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCC : 877
6C_13_Vari : GACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCC : 876
D._acidovo : GACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCC : 877
300C-C03_C : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCT : 830
ctg_CGOF25 : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCT : 829
BY14_Clone : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCT : 824
BIR2-r_lim : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCT : 822
MP20_Sphin : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCT : 832
DSSF72_Unc : ATCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCT : 830
1/4_C7_32_ : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCT : 821
AKIW820_Cl : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCC : 831
WBI100_Clo : TTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCC : 826
ENV481_X._ : TCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCC : 819
V4.BO.05_B : ATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCC : 826
549_Chryse : AGCCACCTGGGGAGTACGAACGCAAGTTTGAAACTCAAAGGAATTGACGGGGGCCC : 874
PB93_P._kr : TTCCACCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGGCCC : 870
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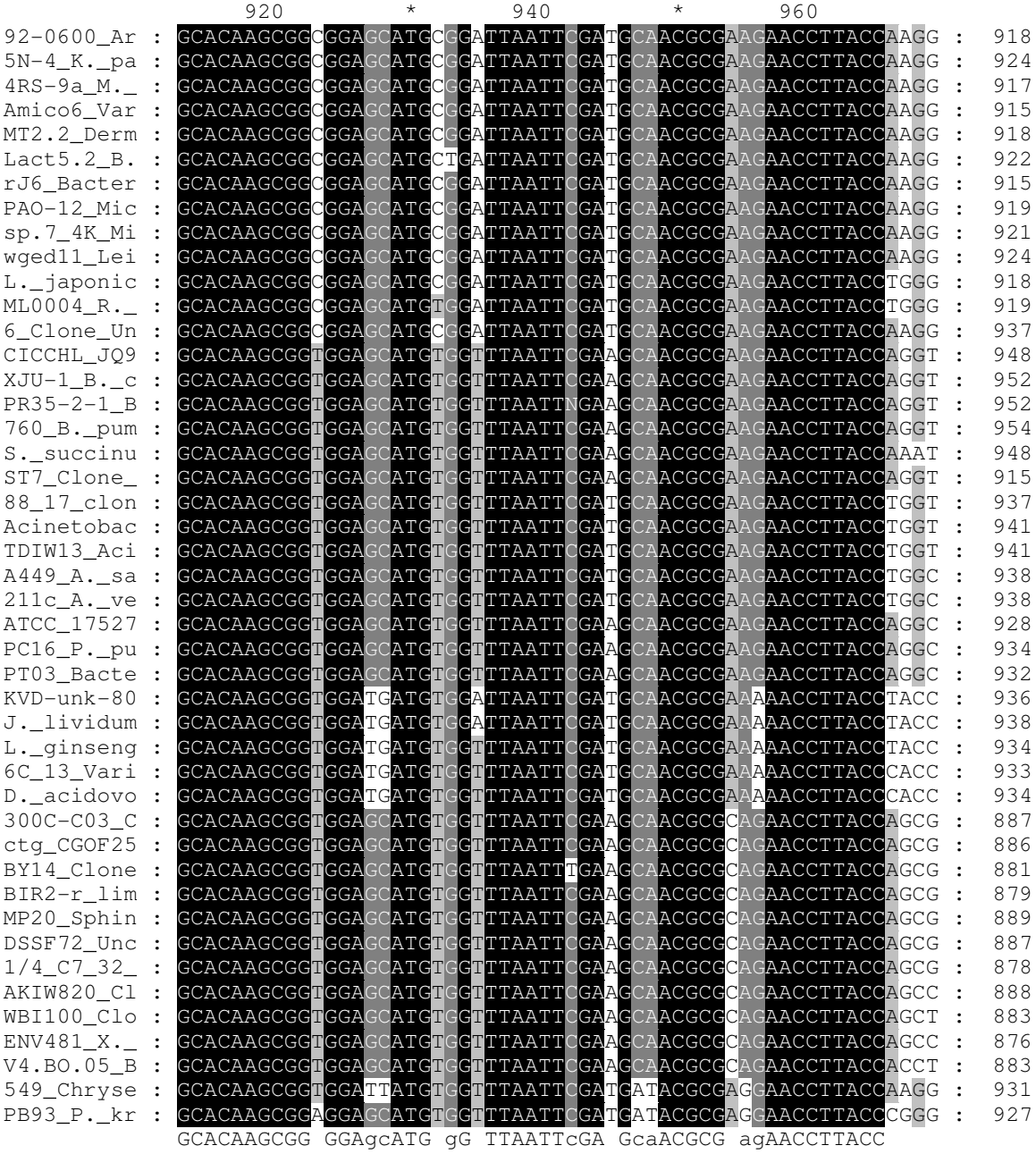
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5N-4_K._pa : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 924
4RS-9a_M._ : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 917
Amico6_Var : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 915
MT2.2_Derm : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 918
Lact5.2_B. : GCACAAGCGGCGGAGCATGCTGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 922
rJ6_Bacter : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 915
PAO-12_Mic : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 919
sp.7_4K_Mi : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 921
wged11_Lei : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 924
L._japonic : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGG : 918
ML0004_R._ : GCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGG : 919
6_Clone_Un : GCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGG : 937
CICCHL_JQ9 : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT : 948
XJU-1_B._c : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT : 952
PR35-2-1_B : GCACAAGCGGTGGAGCATGTGGTTTAATTNGAAGCAACGCGAAGAACCTTACCAGGT : 952
760_B._pum : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT : 954
S._succinu : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAAT : 948
ST7_Clone_ : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT : 915
88_17_clon : GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGT : 937
Acinetobac : GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGT : 941
TDIW13_Aci : GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGT : 941
A449_A._sa : GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGC : 938
211c_A._ve : GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGC : 938
ATCC_17527 : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGC : 928
PC16_P._pu : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGC : 934
PT03_Bacte : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGC : 932
KVD-unk-80 : GCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACC : 936
J._lividum : GCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACC : 938
L._ginseng : GCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCTACC : 934
6C_13_Vari : GCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACC : 933
D._acidovo : GCACAAGCGGTGGATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACC : 934
300C-C03_C : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCG : 887
ctg_CGOF25 : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCG : 886
BY14_Clone : GCACAAGCGGTGGAGCATGTGGTTTAATTTGAAGCAACGCGCAGAACCTTACCAGCG : 881
BIR2-r_lim : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCG : 879
MP20_Sphin : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCG : 889
DSSF72_Unc : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCG : 887
1/4_C7_32_ : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCG : 878
AKIW820_Cl : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCC : 888
WBI100_Clo : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCT : 883
ENV481_X._ : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCC : 876
V4.BO.05_B : GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCACCT : 883
549_Chryse : GCACAAGCGGTGGATTATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCAAGG : 931
PB93_P._kr : GCACAAGCGGAGGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCCGGG : 927
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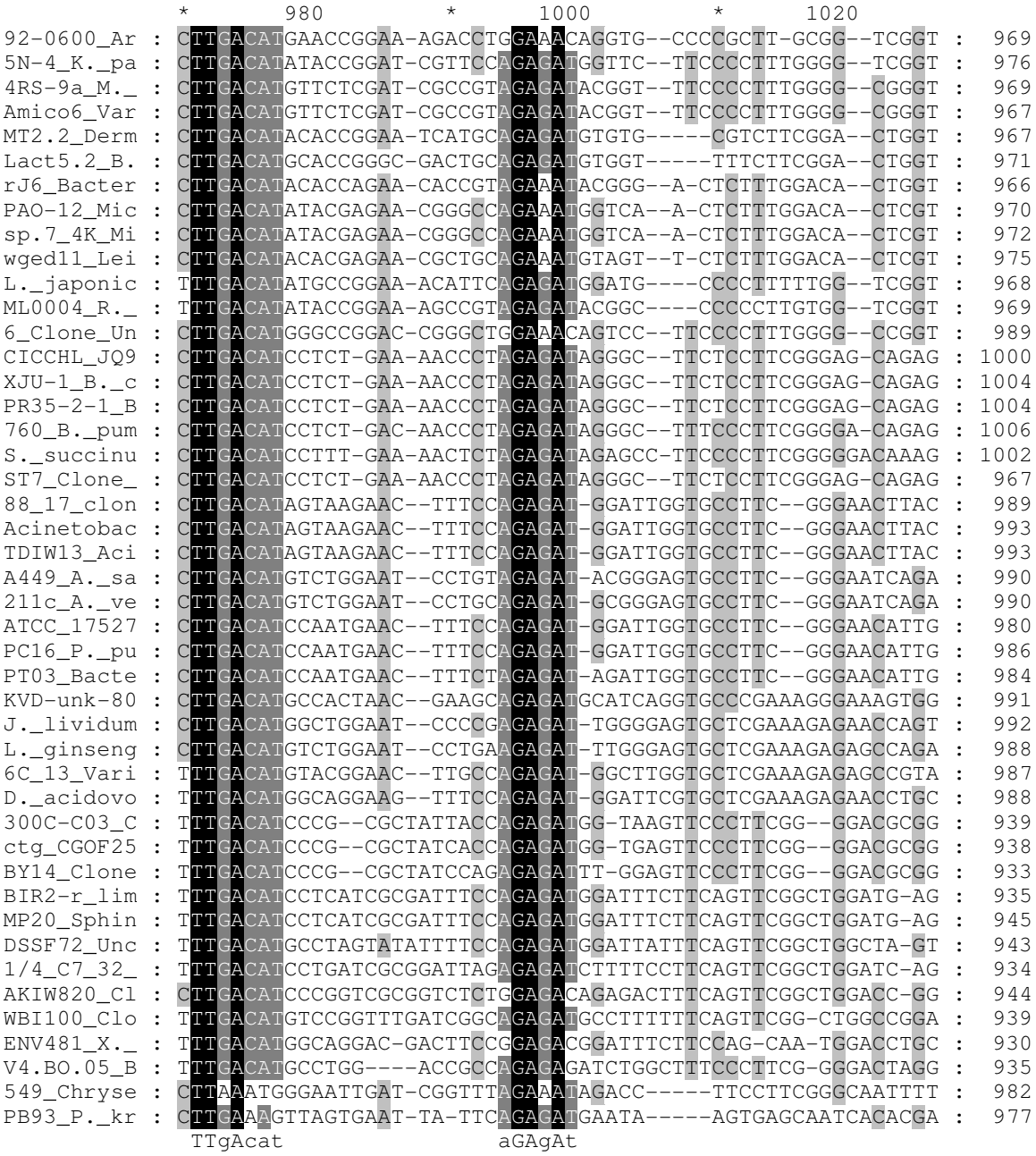
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5N-4_K._pa : CTTGACATATACCGGAT-CGTTCCAGAGATGGTTC--TTCCCCTTTGGGG--TCGGT : 976
4RS-9a_M._ : CTTGACATGTTCTCGAT-CGCCGTAGAGATACGGT--TTCCCCTTTGGGG--CGGGT : 969
Amico6_Var : CTTGACATGTTCTCGAT-CGCCGTAGAGATACGGT--TTCCCCTTTGGGG--CGGGT : 967
MT2.2_Derm : CTTGACATACACCGGAA-TCATGCAGAGATGTGTG-----CGTCTTCGGA--CTGGT : 967
Lact5.2_B. : CTTGACATGCACCGGGC-GACTGCAGAGATGTGGT-----TTTCTTCGGA--CTGGT : 971
rJ6_Bacter : CTTGACATACACCAGAA-CACCGTAGAAATACGGG--A-CTCTTTGGACA--CTGGT : 966
PAO-12_Mic : CTTGACATATACGAGAA-CGGGCCAGAAATGGTCA--A-CTCTTTGGACA--CTCGT : 970
sp.7_4K_Mi : CTTGACATATACGAGAA-CGGGCCAGAAATGGTCA--A-CTCTTTGGACA--CTCGT : 972
wged11_Lei : CTTGACATACACGAGAA-CGCTGCAGAAATGTAGT--T-CTCTTTGGACA--CTCGT : 975
L._japonic : TTTGACATATGCCGGAA-ACATTCAGAGATGGATG----CCCCTTTTTGG--TCGGT : 968
ML0004_R._ : TTTGACATATACCGGAA-AGCCGTAGAGATACGGC----CCCCCTTGTGG--TCGGT : 969
6_Clone_Un : CTTGACATGGGCCGGAC-CGGGCTGGAAACAGTCC--TTCCCCTTTGGGG--CCGGT : 989
CICCHL_JQ9 : CTTGACATCCTCT-GAA-AACCCTAGAGATAGGGC--TTCTCCTTCGGGAG-CAGAG : 1000
XJU-1_B._c : CTTGACATCCTCT-GAA-AACCCTAGAGATAGGGC--TTCTCCTTCGGGAG-CAGAG : 1004
PR35-2-1_B : CTTGACATCCTCT-GAA-AACCCTAGAGATAGGGC--TTCTCCTTCGGGAG-CAGAG : 1004
760_B._pum : CTTGACATCCTCT-GAC-AACCCTAGAGATAGGGC--TTTCCCTTCGGGGA-CAGAG : 1006
S._succinu : CTTGACATCCTTT-GAA-AACTCTAGAGATAGAGCC-TTCCCCTTCGGGGGACAAAG : 1002
ST7_Clone_ : CTTGACATCCTCT-GAA-AACCCTAGAGATAGGGC--TTCTCCTTCGGGAG-CAGAG : 967
88_17_clon : CTTGACATAGTAAGAAC--TTTCCAGAGAT-GGATTGGTGCCTTC--GGGAACTTAC : 989
Acinetobac : CTTGACATAGTAAGAAC--TTTCCAGAGAT-GGATTGGTGCCTTC--GGGAACTTAC : 993
TDIW13_Aci : CTTGACATAGTAAGAAC--TTTCCAGAGAT-GGATTGGTGCCTTC--GGGAACTTAC : 993
A449_A._sa : CTTGACATGTCTGGAAT--CCTGTAGAGAT-ACGGGAGTGCCTTC--GGGAATCAGA : 990
211c_A._ve : CTTGACATGTCTGGAAT--CCTGCAGAGAT-GCGGGAGTGCCTTC--GGGAATCAGA : 990
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PC16_P._pu : CTTGACATCCAATGAAC--TTTCCAGAGAT-GGATTGGTGCCTTC--GGGAACATTG : 986
PT03_Bacte : CTTGACATCCAATGAAC--TTTCTAGAGAT-AGATTGGTGCCTTC--GGGAACATTG : 984
KVD-unk-80 : CTTGACATGCCACTAAC--GAAGCAGAGATGCATCAGGTGCCCGAAAGGGAAAGTGG : 991
J._lividum : CTTGACATGGCTGGAAT--CCCCGAGAGAT-TGGGGAGTGCTCGAAAGAGAACCAGT : 992
L._ginseng : CTTGACATGTCTGGAAT--CCTGAAGAGAT-TTGGGAGTGCTCGAAAGAGAGCCAGA : 988
6C_13_Vari : TTTGACATGTACGGAAC--TTGCCAGAGAT-GGCTTGGTGCTCGAAAGAGAGCCGTA : 987
D._acidovo : TTTGACATGGCAGGAAG--TTTCCAGAGAT-GGATTCGTGCTCGAAAGAGAACCTGC : 988
300C-C03_C : TTTGACATCCCG--CGCTATTACCAGAGATGG-TAAGTTCCCTTCGG--GGACGCGG : 939
ctg_CGOF25 : TTTGACATCCCG--CGCTATCACCAGAGATGG-TGAGTTCCCTTCGG--GGACGCGG : 938
BY14_Clone : TTTGACATCCCG--CGCTATCCAGAGAGATTT-GGAGTTCCCTTCGG--GGACGCGG : 933
BIR2-r_lim : TTTGACATCCTCATCGCGATTTCCAGAGATGGATTTCTTCAGTTCGGCTGGATG-AG : 935
MP20_Sphin : TTTGACATCCTCATCGCGATTTCCAGAGATGGATTTCTTCAGTTCGGCTGGATG-AG : 945
DSSF72_Unc : TTTGACATGCCTAGTATATTTTCCAGAGATGGATTATTTCAGTTCGGCTGGCTA-GT : 943
1/4_C7_32_ : TTTGACATCCTGATCGCGGATTAGAGAGATCTTTTCCTTCAGTTCGGCTGGATC-AG : 934
AKIW820_Cl : CTTGACATCCCGGTCGCGGTCTCTGGAGACAGAGACTTTCAGTTCGGCTGGACC-GG : 944
WBI100_Clo : TTTGACATGTCCGGTTTGATCGGCAGAGATGCCTTTTTTCAGTTCGG-CTGGCCGGA : 939
ENV481_X._ : TTTGACATGGCAGGAC-GACTTCCGGAGACGGATTTCTTCCAG-CAA-TGGACCTGC : 930
V4.BO.05_B : TTTGACATGCCTGG----ACCGCCAGAGAGATCTGGCTTTCCCTTCG-GGGACTAGG : 935
549_Chryse : CTTAAATGGGAATTGAT-CGGTTTAGAAATAGACC-----TTCCTTCGGGCAATTTT : 982
PB93_P._kr : CTTGAAAGTTAGTGAAT-TA-TTCAGAGATGAATA-----AGTGAGCAATCACACGA : 977
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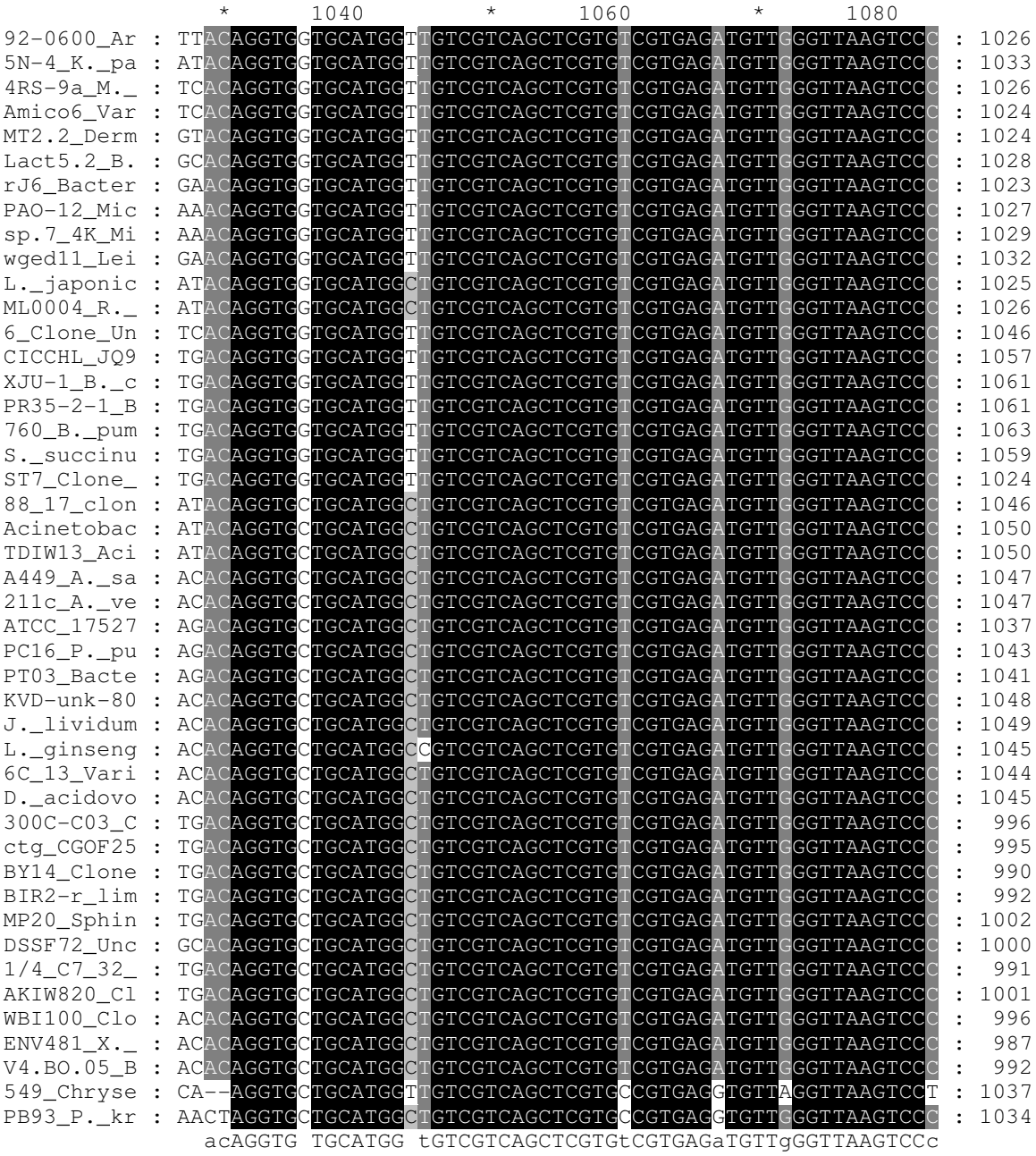
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5N-4_K._pa : ATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1033
4RS-9a_M._ : TCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1026
Amico6_Var : TCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1024
MT2.2_Derm : GTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1024
Lact5.2_B. : GCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1028
rJ6_Bacter : GAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1023
PAO-12_Mic : AAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1027
sp.7_4K_Mi : AAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1029
wged11_Lei : GAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1032
L._japonic : ATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1025
ML0004_R._ : ATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1026
6_Clone_Un : TCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1046
CICCHL_JQ9 : TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1057
XJU-1_B._c : TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1061
PR35-2-1_B : TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1061
760_B._pum : TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1063
S._succinu : TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1059
ST7_Clone_ : TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1024
88_17_clon : ATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1046
Acinetobac : ATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1050
TDIW13_Aci : ATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1050
A449_A._sa : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1047
211c_A._ve : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1047
ATCC_17527 : AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1037
PC16_P._pu : AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1043
PT03_Bacte : AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1041
KVD-unk-80 : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1048
J._lividum : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1049
L._ginseng : ACACAGGTGCTGCATGGCCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1045
6C_13_Vari : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1044
D._acidovo : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1045
300C-C03_C : TGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 996
ctg_CGOF25 : TGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 995
BY14_Clone : TGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 990
BIR2-r_lim : TGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 992
MP20_Sphin : TGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1002
DSSF72_Unc : GCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 1000
1/4_C7_32_ : TGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 991
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WBI100_Clo : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 996
ENV481_X._ : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 987
V4.BO.05_B : ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC : 992
549_Chryse : CA--AGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTTAGGTTAAGTCCT : 1037
PB93_P._kr : AACTAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCC : 1034
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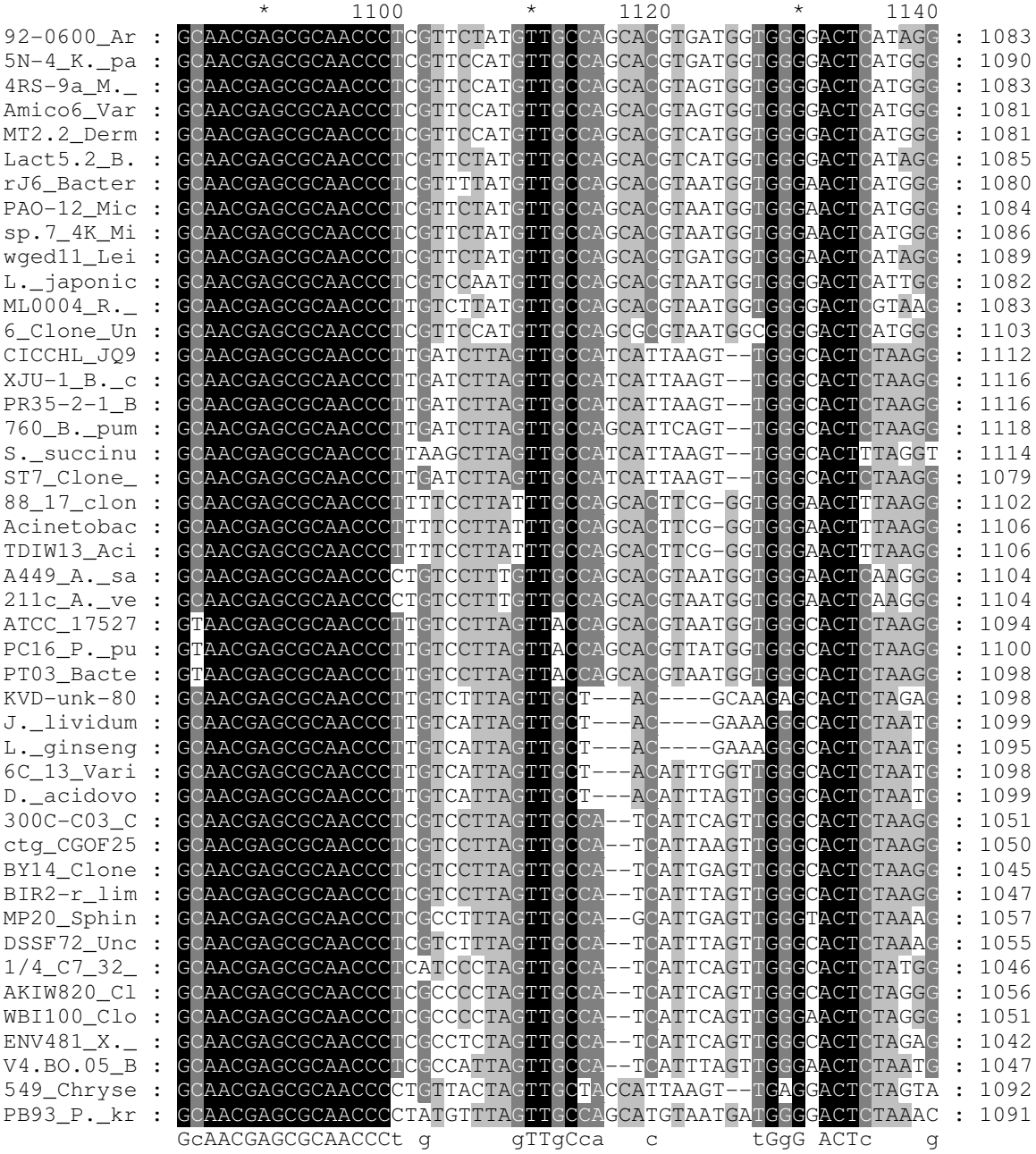
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5N-4_K._pa : GCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTGATGGTGGGGACTCATGGG : 1090
4RS-9a_M._ : GCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAGTGGTGGGGACTCATGGG : 1083
Amico6_Var : GCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAGTGGTGGGGACTCATGGG : 1081
MT2.2_Derm : GCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTCATGGTGGGGACTCATGGG : 1081
Lact5.2_B. : GCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTCATGGTGGGGACTCATAGG : 1085
rJ6_Bacter : GCAACGAGCGCAACCCTCGTTTTATGTTGCCAGCACGTAATGGTGGGAACTCATGGG : 1080
PAO-12_Mic : GCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGG : 1084
sp.7_4K_Mi : GCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGG : 1086
wged11_Lei : GCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGAACTCATAGG : 1089
L._japonic : GCAACGAGCGCAACCCTCGTCCAATGTTGCCAGCACGTAATGGTGGGGACTCATTGG : 1082
ML0004_R._ : GCAACGAGCGCAACCCTTGTCTTATGTTGCCAGCACGTAATGGTGGGGACTCGTAAG : 1083
6_Clone_Un : GCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCGCGTAATGGCGGGGACTCATGGG : 1103
CICCHL_JQ9 : GCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGT--TGGGCACTCTAAGG : 1112
XJU-1_B._c : GCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGT--TGGGCACTCTAAGG : 1116
PR35-2-1_B : GCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGT--TGGGCACTCTAAGG : 1116
760_B._pum : GCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGT--TGGGCACTCTAAGG : 1118
S._succinu : GCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGT--TGGGCACTTTAGGT : 1114
ST7_Clone_ : GCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGT--TGGGCACTCTAAGG : 1079
88_17_clon : GCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCACTTCG-GGTGGGAACTTTAAGG : 1102
Acinetobac : GCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCACTTCG-GGTGGGAACTTTAAGG : 1106
TDIW13_Aci : GCAACGAGCGCAACCCTTTTCCTTATTTGCCAGCACTTCG-GGTGGGAACTTTAAGG : 1106
A449_A._sa : GCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAGGG : 1104
211c_A._ve : GCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAGGG : 1104
ATCC_17527 : GTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGG : 1094
PC16_P._pu : GTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGG : 1100
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KVD-unk-80 : GCAACGAGCGCAACCCTTGTCTTTAGTTGCT---AC----GCAAGAGCACTCTAGAG : 1098
J._lividum : GCAACGAGCGCAACCCTTGTCATTAGTTGCT---AC----GAAAGGGCACTCTAATG : 1099
L._ginseng : GCAACGAGCGCAACCCTTGTCATTAGTTGCT---AC----GAAAGGGCACTCTAATG : 1095
6C_13_Vari : GCAACGAGCGCAACCCTTGTCATTAGTTGCT---ACATTTGGTTGGGCACTCTAATG : 1098
D._acidovo : GCAACGAGCGCAACCCTTGTCATTAGTTGCT---ACATTTAGTTGGGCACTCTAATG : 1099
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ctg_CGOF25 : GCAACGAGCGCAACCCTCGTCCTTAGTTGCCA--TCATTAAGTTGGGCACTCTAAGG : 1050
BY14_Clone : GCAACGAGCGCAACCCTCGTCCTTAGTTGCCA--TCATTGAGTTGGGCACTCTAAGG : 1045
BIR2-r_lim : GCAACGAGCGCAACCCTCGTCCTTAGTTGCCA--TCATTTAGTTGGGCACTCTAAGG : 1047
MP20_Sphin : GCAACGAGCGCAACCCTCGCCTTTAGTTGCCA--GCATTGAGTTGGGTACTCTAAAG : 1057
DSSF72_Unc : GCAACGAGCGCAACCCTCGTCTTTAGTTGCCA--TCATTTAGTTGGGCACTCTAAAG : 1055
1/4_C7_32_ : GCAACGAGCGCAACCCTCATCCCTAGTTGCCA--TCATTCAGTTGGGCACTCTATGG : 1046
AKIW820_Cl : GCAACGAGCGCAACCCTCGCCCCTAGTTGCCA--TCATTCAGTTGGGCACTCTAGGG : 1056
WBI100_Clo : GCAACGAGCGCAACCCTCGCCCCTAGTTGCCA--TCATTCAGTTGGGAACTCTAGGG : 1051
ENV481_X._ : GCAACGAGCGCAACCCTCGCCTCTAGTTGCCA--TCATTCAGTTGGGCACTCTAGAG : 1042
V4.BO.05_B : GCAACGAGCGCAACCCTCGCCATTAGTTGCCA--TCATTTAGTTGGGAACTCTAATG : 1047
549_Chryse : GCAACGAGCGCAACCCCTGTTACTAGTTGCTACCATTAAGT--TGAGGACTCTAGTA : 1092
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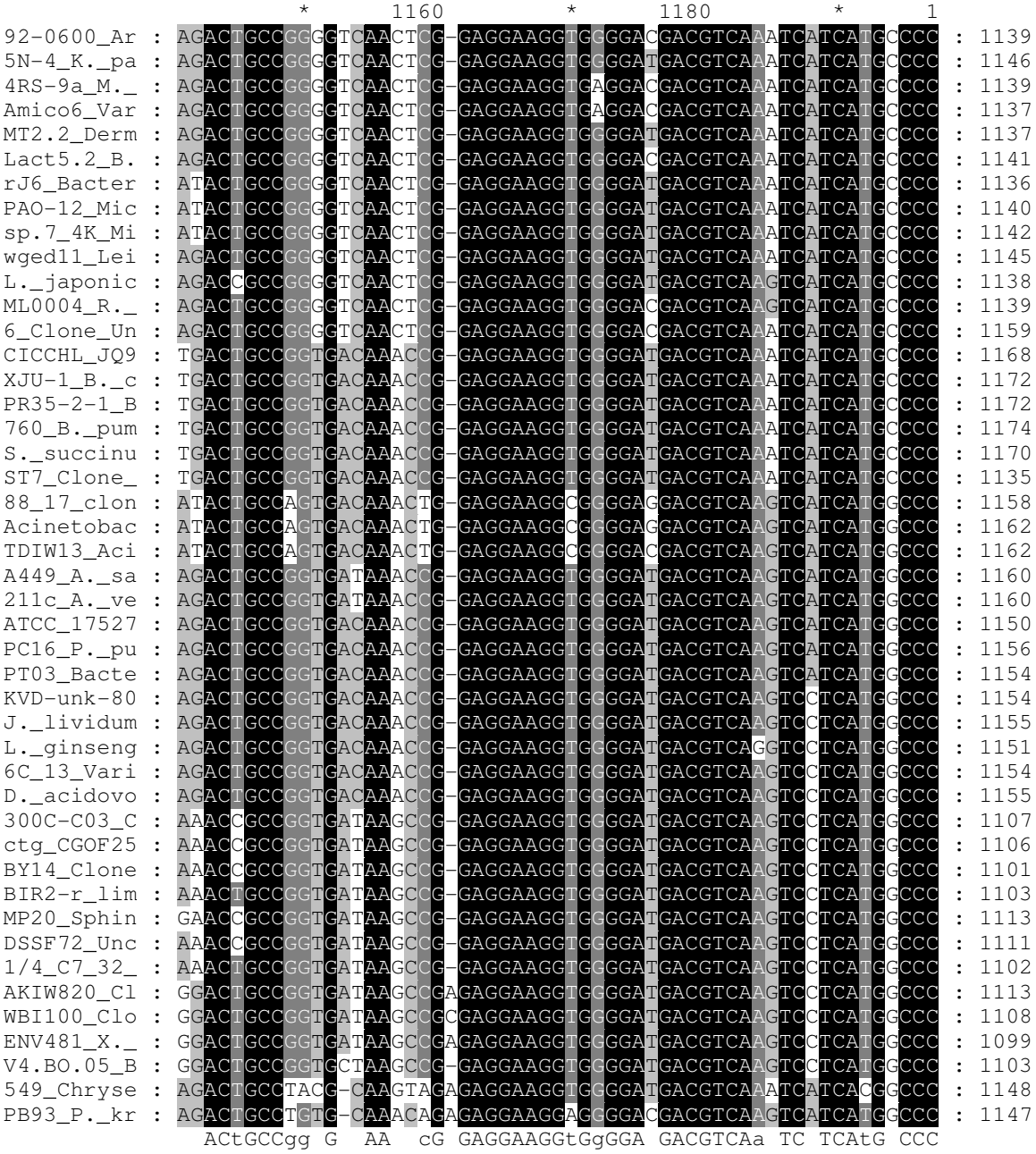
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5N-4_K._pa : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1146
4RS-9a_M._ : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGAGGACGACGTCAAATCATCATGCCCC : 1139
Amico6_Var : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGAGGACGACGTCAAATCATCATGCCCC : 1137
MT2.2_Derm : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1137
Lact5.2_B. : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGACGACGTCAAATCATCATGCCCC : 1141
rJ6_Bacter : ATACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1136
PAO-12_Mic : ATACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1140
sp.7_4K_Mi : ATACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1142
wged11_Lei : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1145
L._japonic : AGACCGCCGGGGTCAACTCG-GAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCC : 1138
ML0004_R._ : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCC : 1139
6_Clone_Un : AGACTGCCGGGGTCAACTCG-GAGGAAGGTGGGGACGACGTCAAATCATCATGCCCC : 1159
CICCHL_JQ9 : TGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1168
XJU-1_B._c : TGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1172
PR35-2-1_B : TGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1172
760_B._pum : TGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1174
S._succinu : TGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1170
ST7_Clone_ : TGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC : 1135
88_17_clon : ATACTGCCAGTGACAAACTG-GAGGAAGGCGGGGAGGACGTCAAGTCATCATGGCCC : 1158
Acinetobac : ATACTGCCAGTGACAAACTG-GAGGAAGGCGGGGAGGACGTCAAGTCATCATGGCCC : 1162
TDIW13_Aci : ATACTGCCAGTGACAAACTG-GAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCC : 1162
A449_A._sa : AGACTGCCGGTGATAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC : 1160
211c_A._ve : AGACTGCCGGTGATAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC : 1160
ATCC_17527 : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC : 1150
PC16_P._pu : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC : 1156
PT03_Bacte : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC : 1154
KVD-unk-80 : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1154
J._lividum : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1155
L._ginseng : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAGGTCCTCATGGCCC : 1151
6C_13_Vari : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1154
D._acidovo : AGACTGCCGGTGACAAACCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1155
300C-C03_C : AAACCGCCGGTGATAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1107
ctg_CGOF25 : AAACCGCCGGTGATAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1106
BY14_Clone : AAACCGCCGGTGATAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1101
BIR2-r_lim : AAACTGCCGGTGATAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1103
MP20_Sphin : GAACCGCCGGTGATAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1113
DSSF72_Unc : AAACCGCCGGTGATAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1111
1/4_C7_32_ : AAACTGCCGGTGATAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1102
AKIW820_Cl : GGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1113
WBI100_Clo : GGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1108
ENV481_X._ : GGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1099
V4.BO.05_B : GGACTGCCGGTGCTAAGCCG-GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC : 1103
549_Chryse : AGACTGCCTACG-CAAGTAGAGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCC : 1148
PB93_P._kr : AGACTGCCTGTG-CAAACAGAGAGGAAGGAGGGGACGACGTCAAGTCATCATGGCCC : 1147
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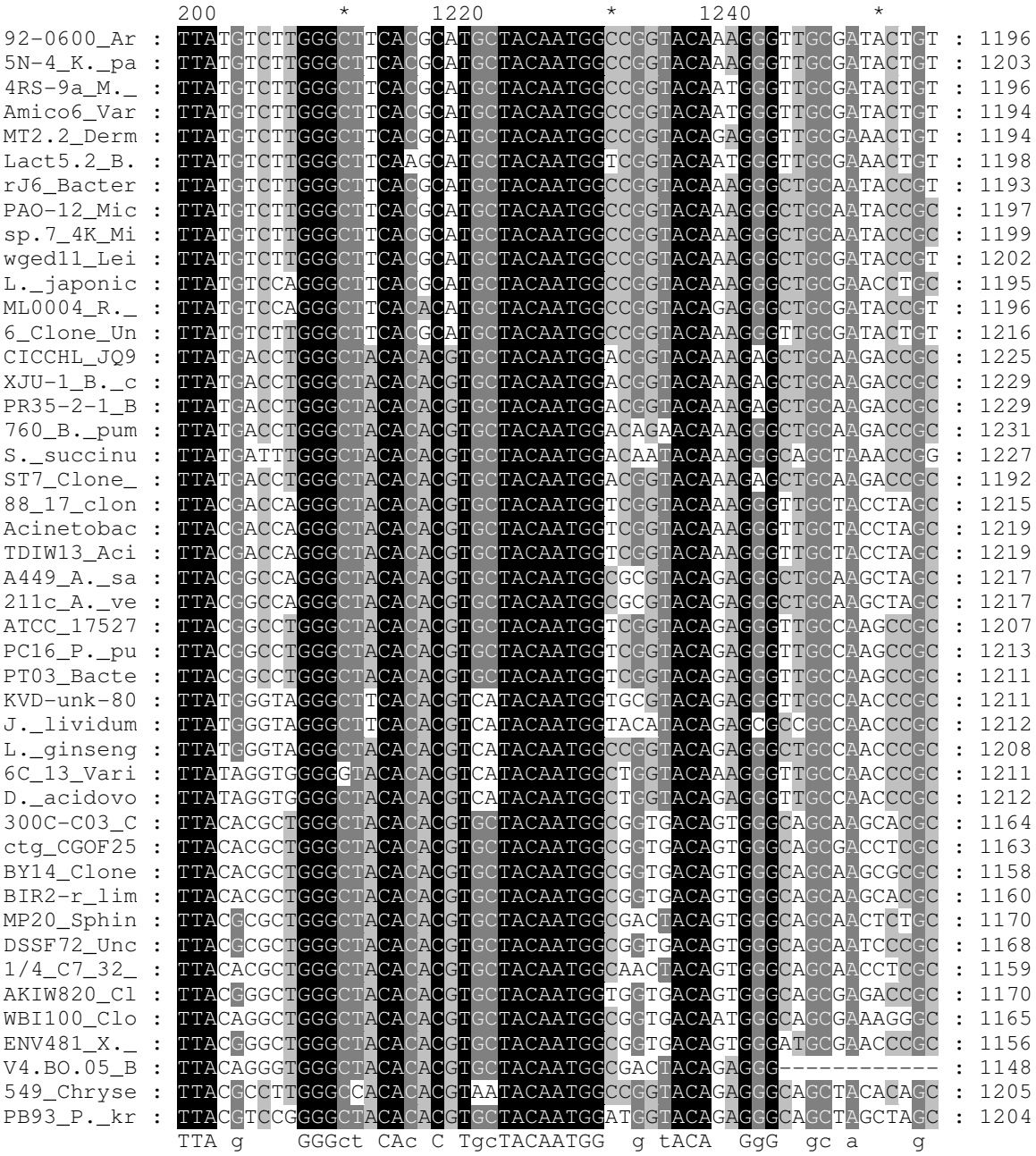
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5N-4_K._pa : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGT : 1203
4RS-9a_M._ : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGT : 1196
Amico6_Var : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGATACTGT : 1194
MT2.2_Derm : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACTGT : 1194
Lact5.2_B. : TTATGTCTTGGGCTTCAAGCATGCTACAATGGTCGGTACAATGGGTTGCGAAACTGT : 1198
rJ6_Bacter : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGT : 1193
PAO-12_Mic : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGC : 1197
sp.7_4K_Mi : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGC : 1199
wged11_Lei : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCGATACCGT : 1202
L._japonic : TTATGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCGAACCTGC : 1195
ML0004_R._ : TTATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACAGAGGGCTGCGATACCGT : 1196
6_Clone_Un : TTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGT : 1216
CICCHL_JQ9 : TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGC : 1225
XJU-1_B._c : TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGC : 1229
PR35-2-1_B : TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGC : 1229
760_B._pum : TTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCAAGACCGC : 1231
S._succinu : TTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGG : 1227
ST7_Clone_ : TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGC : 1192
88_17_clon : TTACGACCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGC : 1215
Acinetobac : TTACGACCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGC : 1219
TDIW13_Aci : TTACGACCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGC : 1219
A449_A._sa : TTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGC : 1217
211c_A._ve : TTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGC : 1217
ATCC_17527 : TTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGC : 1207
PC16_P._pu : TTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGC : 1213
PT03_Bacte : TTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGC : 1211
KVD-unk-80 : TTATGGGTAGGGCTTCACACGTCATACAATGGTGCGTACAGAGGGTTGCCAACCCGC : 1211
J._lividum : TTATGGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGCGCCGCCAACCCGC : 1212
L._ginseng : TTATGGGTAGGGCTACACACGTCATACAATGGCCGGTACAGAGGGCTGCCAACCCGC : 1208
6C_13_Vari : TTATAGGTGGGGGTACACACGTCATACAATGGCTGGTACAAAGGGTTGCCAACCCGC : 1211
D._acidovo : TTATAGGTGGGGCTACACACGTCATACAATGGCTGGTACAGAGGGTTGCCAACCCGC : 1212
300C-C03_C : TTACACGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCAAGCACGC : 1164
ctg_CGOF25 : TTACACGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCGACCTCGC : 1163
BY14_Clone : TTACACGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCAAGCGCGC : 1158
BIR2-r_lim : TTACACGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCAAGCACGC : 1160
MP20_Sphin : TTACGCGCTGGGCTACACACGTGCTACAATGGCGACTACAGTGGGCAGCAACTCTGC : 1170
DSSF72_Unc : TTACGCGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGCAGCAATCCCGC : 1168
1/4_C7_32_ : TTACACGCTGGGCTACACACGTGCTACAATGGCAACTACAGTGGGCAGCAACCTCGC : 1159
AKIW820_Cl : TTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGACCGC : 1170
WBI100_Clo : TTACAGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGCAGCGAAAGGGC : 1165
ENV481_X._ : TTACGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGATGCGAACCCGC : 1156
V4.BO.05_B : TTACAGGGTGGGCTACACACGTGCTACAATGGCGACTACAGAGGG---------- : 1148
549_Chryse : TTACGCCTTGGGCCACACACGTAATACAATGGCCGGTACAGAGGGCAGCTACACAGC : 1205
PB93_P._kr : TTACGTCCGGGGCTACACACGTGCTACAATGGATGGTACAGAGGGCAGCTAGCTAGC : 1204
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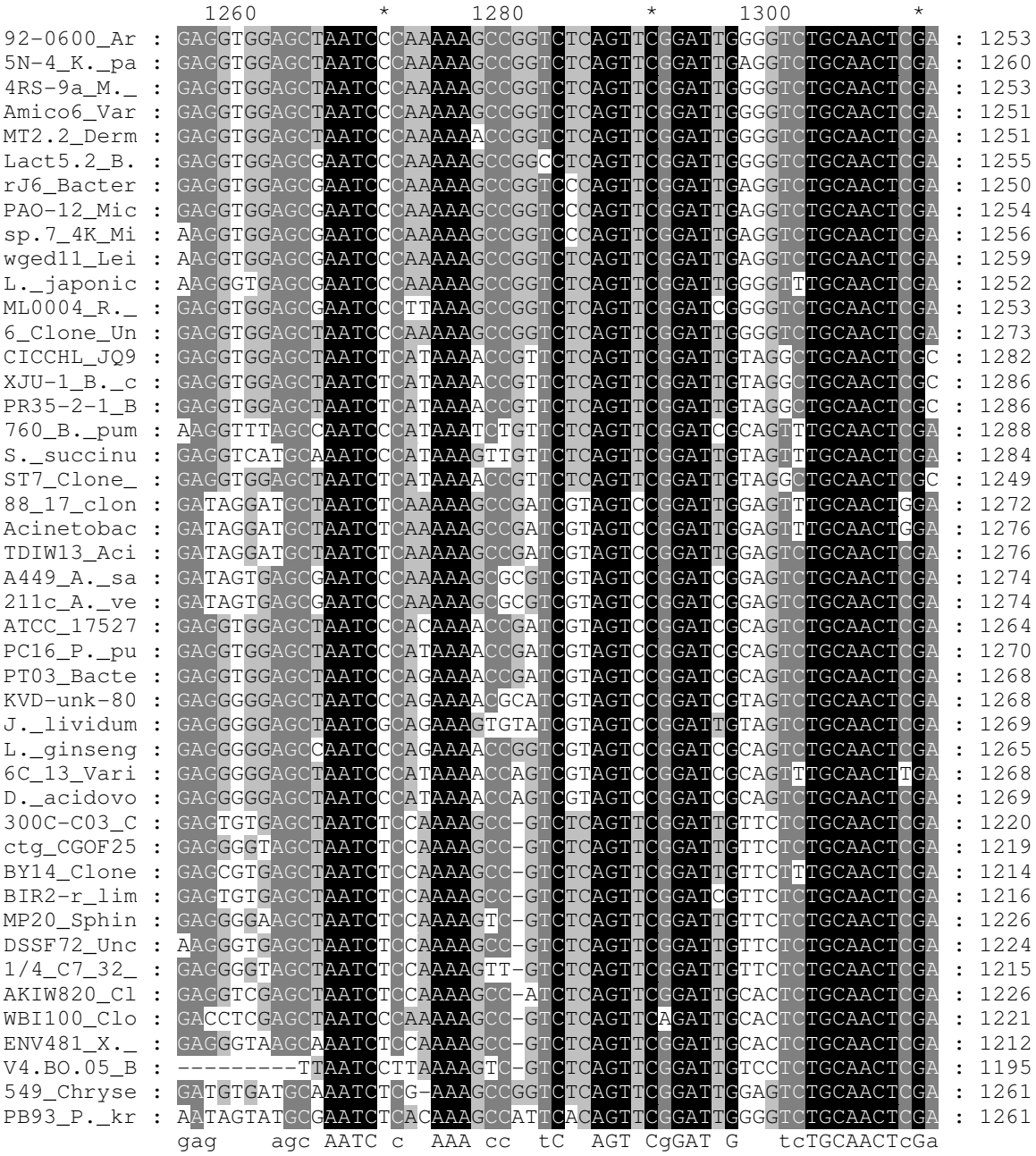
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5N-4_K._pa : GAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGA : 1260
4RS-9a_M._ : GAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGA : 1253
Amico6_Var : GAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGA : 1251
MT2.2_Derm : GAGGTGGAGCTAATCCCAAAAAACCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGA : 1251
Lact5.2_B. : GAGGTGGAGCGAATCCCAAAAAGCCGGCCTCAGTTCGGATTGGGGTCTGCAACTCGA : 1255
rJ6_Bacter : GAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGA : 1250
PAO-12_Mic : GAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGA : 1254
sp.7_4K_Mi : AAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGA : 1256
wged11_Lei : AAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGA : 1259
L._japonic : AAGGGTGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTTTGCAACTCGA : 1252
ML0004_R._ : GAGGTGGAGCGAATCCTTAAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGA : 1253
6_Clone_Un : GAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGA : 1273
CICCHL_JQ9 : GAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGC : 1282
XJU-1_B._c : GAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGC : 1286
PR35-2-1_B : GAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGC : 1286
760_B._pum : AAGGTTTAGCCAATCCCATAAATCTGTTCTCAGTTCGGATCGCAGTTTGCAACTCGA : 1288
S._succinu : GAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTTTGCAACTCGA : 1284
ST7_Clone_ : GAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGC : 1249
88_17_clon : GATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTTTGCAACTGGA : 1272
Acinetobac : GATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTTTGCAACTGGA : 1276
TDIW13_Aci : GATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGA : 1276
A449_A._sa : GATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGA : 1274
211c_A._ve : GATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGA : 1274
ATCC_17527 : GAGGTGGAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGA : 1264
PC16_P._pu : GAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGA : 1270
PT03_Bacte : GAGGTGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGA : 1268
KVD-unk-80 : GAGGGGGAGCTAATCCCAGAAAACGCATCGTAGTCCGGATCGTAGTCTGCAACTCGA : 1268
J._lividum : GAGGGGGAGCTAATCGCAGAAAGTGTATCGTAGTCCGGATTGTAGTCTGCAACTCGA : 1269
L._ginseng : GAGGGGGAGCCAATCCCAGAAAACCGGTCGTAGTCCGGATCGCAGTCTGCAACTCGA : 1265
6C_13_Vari : GAGGGGGAGCTAATCCCATAAAACCAGTCGTAGTCCGGATCGCAGTTTGCAACTTGA : 1268
D._acidovo : GAGGGGGAGCTAATCCCATAAAACCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGA : 1269
300C-C03_C : GAGTGTGAGCTAATCTCCAAAAGCC-GTCTCAGTTCGGATTGTTCTCTGCAACTCGA : 1220
ctg_CGOF25 : GAGGGGTAGCTAATCTCCAAAAGCC-GTCTCAGTTCGGATTGTTCTCTGCAACTCGA : 1219
BY14_Clone : GAGCGTGAGCTAATCTCCAAAAGCC-GTCTCAGTTCGGATTGTTCTTTGCAACTCGA : 1214
BIR2-r_lim : GAGTGTGAGCTAATCTCCAAAAGCC-GTCTCAGTTCGGATCGTTCTCTGCAACTCGA : 1216
MP20_Sphin : GAGGGGAAGCTAATCTCCAAAAGTC-GTCTCAGTTCGGATTGTTCTCTGCAACTCGA : 1226
DSSF72_Unc : AAGGGTGAGCTAATCTCCAAAAGCC-GTCTCAGTTCGGATTGTTCTCTGCAACTCGA : 1224
1/4_C7_32_ : GAGGGGTAGCTAATCTCCAAAAGTT-GTCTCAGTTCGGATTGTTCTCTGCAACTCGA : 1215
AKIW820_Cl : GAGGTCGAGCTAATCTCCAAAAGCC-ATCTCAGTTCGGATTGCACTCTGCAACTCGA : 1226
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V4.BO.05_B : ---------TTAATCCTTAAAAGTC-GTCTCAGTTCGGATTGTCCTCTGCAACTCGA : 1195
549_Chryse : GATGTGATGCAAATCTCG-AAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCGA : 1261
PB93_P._kr : AATAGTATGCGAATCTCACAAAGCCATTCACAGTTCGGATTGGGGTCTGCAACTCGA : 1261
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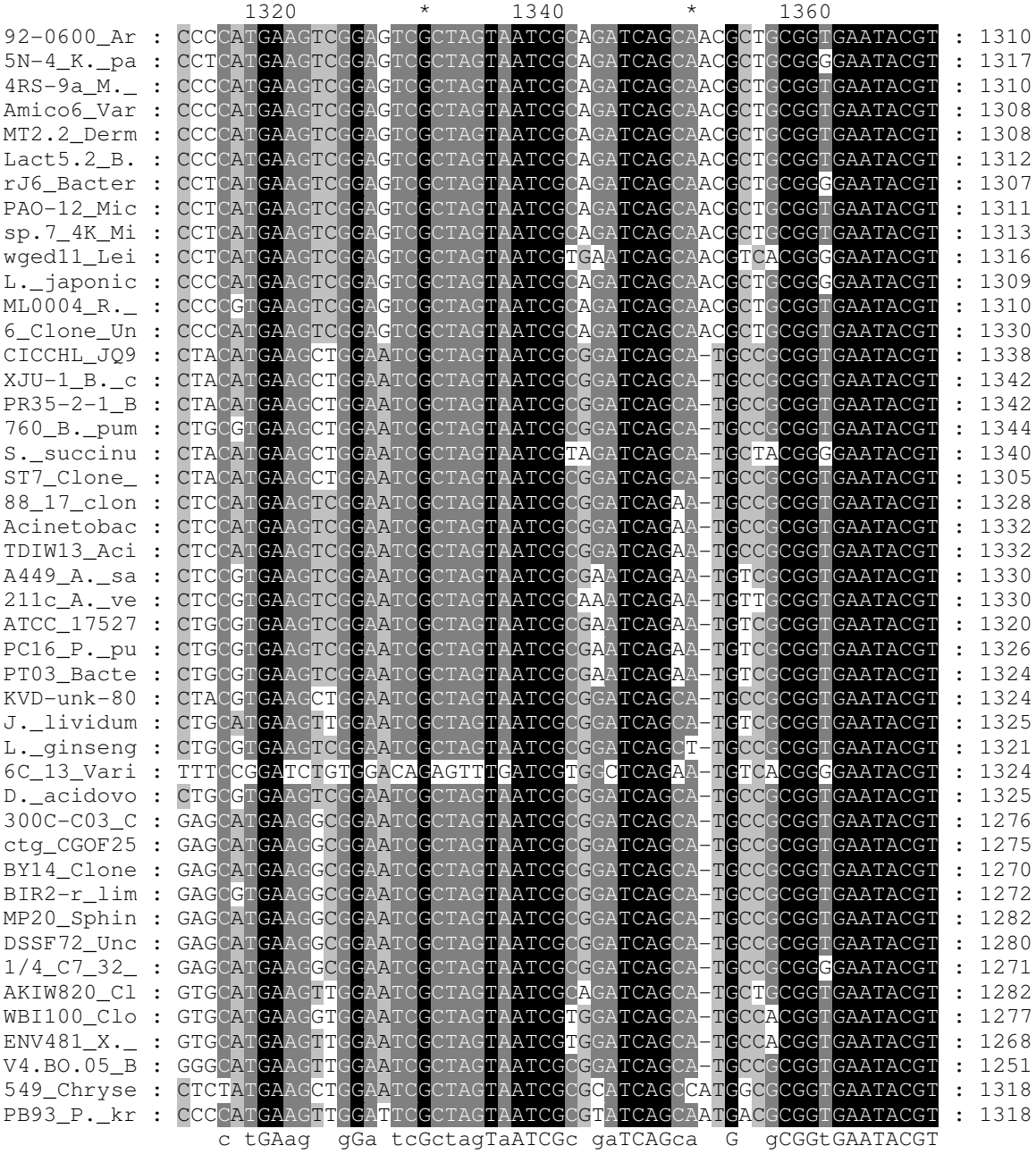
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5N-4_K._pa : CCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGGGAATACGT : 1317
4RS-9a_M._ : CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1310
Amico6_Var : CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1308
MT2.2_Derm : CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1308
Lact5.2_B. : CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1312
rJ6_Bacter : CCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGGGAATACGT : 1307
PAO-12_Mic : CCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1311
sp.7_4K_Mi : CCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1313
wged11_Lei : CCTCATGAAGTCGGAGTCGCTAGTAATCGTGAATCAGCAACGTCACGGGGAATACGT : 1316
L._japonic : CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGGGAATACGT : 1309
ML0004_R._ : CCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1310
6_Clone_Un : CCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT : 1330
CICCHL_JQ9 : CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1338
XJU-1_B._c : CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1342
PR35-2-1_B : CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1342
760_B._pum : CTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1344
S._succinu : CTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCA-TGCTACGGGGAATACGT : 1340
ST7_Clone_ : CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1305
88_17_clon : CTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAA-TGCCGCGGTGAATACGT : 1328
Acinetobac : CTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAA-TGCCGCGGTGAATACGT : 1332
TDIW13_Aci : CTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAA-TGCCGCGGTGAATACGT : 1332
A449_A._sa : CTCCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAA-TGTCGCGGTGAATACGT : 1330
211c_A._ve : CTCCGTGAAGTCGGAATCGCTAGTAATCGCAAATCAGAA-TGTTGCGGTGAATACGT : 1330
ATCC_17527 : CTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAA-TGTCGCGGTGAATACGT : 1320
PC16_P._pu : CTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAA-TGTCGCGGTGAATACGT : 1326
PT03_Bacte : CTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAA-TGTCGCGGTGAATACGT : 1324
KVD-unk-80 : CTACGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1324
J._lividum : CTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCA-TGTCGCGGTGAATACGT : 1325
L._ginseng : CTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCT-TGCCGCGGTGAATACGT : 1321
6C_13_Vari : TTTCCGGATCTGTGGACAGAGTTTGATCGTGGCTCAGAA-TGTCACGGGGAATACGT : 1324
D._acidovo : CTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1325
300C-C03_C : GAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1276
ctg_CGOF25 : GAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1275
BY14_Clone : GAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1270
BIR2-r_lim : GAGCGTGAAGGCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1272
MP20_Sphin : GAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1282
DSSF72_Unc : GAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1280
1/4_C7_32_ : GAGCATGAAGGCGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGGGAATACGT : 1271
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ENV481_X._ : GTGCATGAAGTTGGAATCGCTAGTAATCGTGGATCAGCA-TGCCACGGTGAATACGT : 1268
V4.BO.05_B : GGGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCA-TGCCGCGGTGAATACGT : 1251
549_Chryse : CTCTATGAAGCTGGAATCGCTAGTAATCGCGCATCAGCCATGGCGCGGTGAATACGT : 1318
PB93_P._kr : CCCCATGAAGTTGGATTCGCTAGTAATCGCGTATCAGCAATGACGCGGTGAATACGT : 1318
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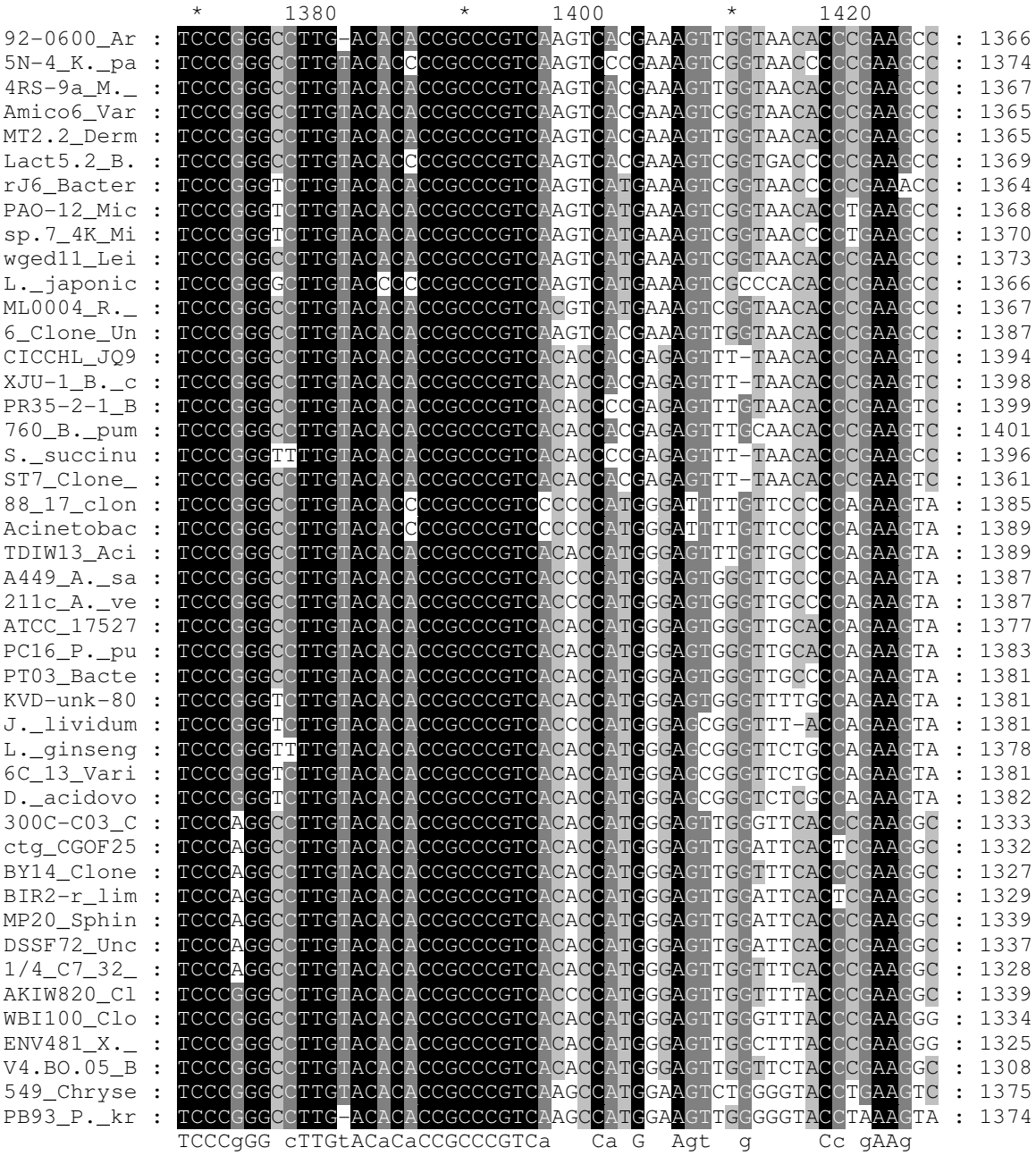
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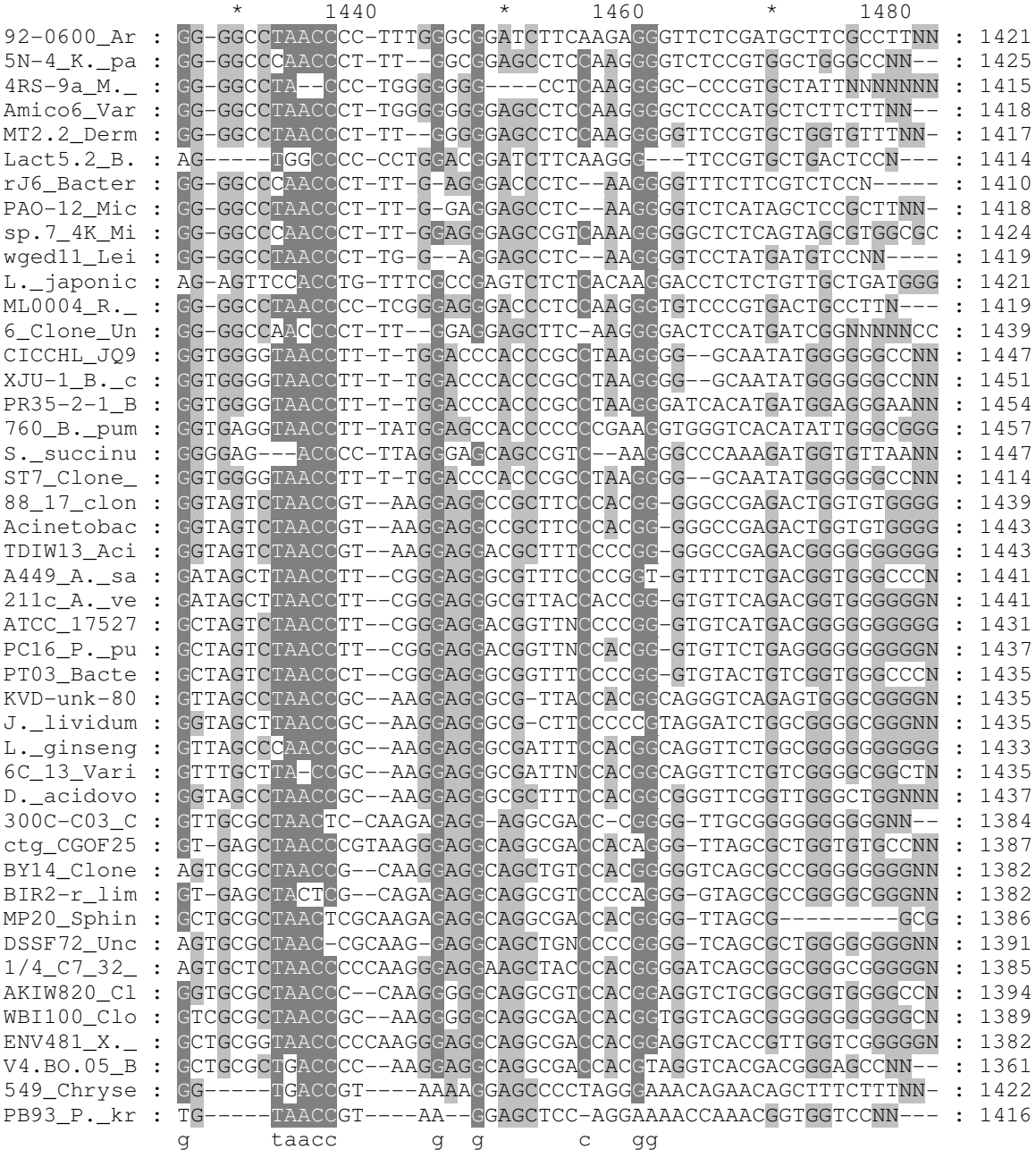
4RS-9a_M._ : TCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGAAGCC : 1367
Amico6_Var : TCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTAACACCCGAAGCC : 1365
MT2.2_Derm : TCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGAAGCC : 1365
Lact5.2_B. : TCCCGGGCCTTGTACACCCCGCCCGTCAAGTCACGAAAGTCGGTGACCCCCGAAGCC : 1369
rJ6_Bacter : TCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACCCCCGAAACC : 1364
PAO-12_Mic : TCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCTGAAGCC : 1368
sp.7_4K_Mi : TCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACCCCTGAAGCC : 1370
wged11_Lei : TCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGAAGCC : 1373
L._japonic : TCCCGGGGCTTGTACCCCCGCCCGTCAAGTCATGAAAGTCGCCCACACCCGAAGCC : 1366
ML0004_R._ : TCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCC : 1367
6_Clone_Un : TCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGAAGCC : 1387
CICCHL_JQ9 : TCCCGGGCCTTGTACACACCGCCCGTCACACCACGACAGTTT-TAACACCCGAAGTC : 1394
XJU-1_B._c : TCCCGGGCCTTGTACACACCGCCCGTCACACCACGACAGTTT-TAACACCCGAAGTC : 1398
PR35-2-1_B : TCCCGGGCCTTGTACACACCGCCCGTCACACCCCGACAGTTTGTAACACCCGAAGTC : 1399
760_B._pum : TCCCGGGCCTTGTACACACCGCCCGTCACACCACGACAGTTTGCAACACCCGAAGTC : 1401
S._succinu : TCCCGGGTTTTGTACACACCGCCCGTCACACCCCGACAGTTT-TAACACCCGAAGCC : 1396
ST7_Clone_ : TCCCGGGCCTTGTACACACCGCCCGTCACACCACGACAGTTT-TAACACCCGAAGTC : 1361
88_17_clon : TCCCGGGCCTTGTACACCCCGCCCGTCCCCCCATGGGATTTTGTTCCCCCCAGAAGTA : 1385
Acinetobac : TCCCGGGCCTTGTACACCCCGCCCGTCCCCCCATGGGATTTTGTTCCCCCCAGAAGTA : 1389
TDIW13_Aci : TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCCCCCAGAAGTA : 1389
A449_A._sa : TCCCGGGCCTTGTACACACCGCCCGTCACCCCATGGGAGTGGGTTGCCCCCAGAAGTA : 1387
211c_A._ve : TCCCGGGCCTTGTACACACCGCCCGTCACCCCATGGGAGTGGGTTGCCCCCAGAAGTA : 1387
ATCC_17527 : TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTA : 1377
PC16_P._pu : TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTA : 1383
PT03_Bacte : TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCCCCAGAAGTA : 1381
KVD-unk-80 : TCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTTGCCAGAAGTA : 1381
J._lividum : TCCCGGGTCTTGTACACACCGCCCGTCACCCCATGGGAGCGGGTTT-ACCAGAAGTA : 1381
L._ginseng : TCCCGGGTTTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTGCCAGAAGTA : 1378
6C_13_Vari : TCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTGCCAGAAGTA : 1381
D._acidovo : TCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTCTCGCCAGAAGTA : 1382
300C-C03_C : TCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGGTTCACCCGAAGGC : 1333
ctg_CGOF25 : TCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGATTCACTCGAAGGC : 1332
BY14_Clone : TCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTCACCCGAAGGC : 1327
BIR2-r_lim : TCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGATTCACTCGAAGGC : 1329
MP20_Sphin : TCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGATTCACCCGAAGGC : 1339
DSSF72_Unc : TCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGATTCACCCGAAGGC : 1337
1/4_C7_32_ : TCCCAGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTCACCCGAAGGC : 1328
AKIW820_Cl : TCCCGGGCCTTGTACACACCGCCCGTCACCCCATGGGAGTTGGTTTTACCCGAAGGC : 1339
WBI100_Clo : TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGGTTTACCCGAAGGG : 1334
ENV481_X._ : TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCCGAAGGG : 1325
V4.BO.05_B : TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCTACCCGAAGGC : 1308
549_Chryse : TCCCGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGTCTGGGGTACCTGAAGTC : 1375
PB93_P._kr : TCCCGGGCCTTG-ACACACCGCCCGTCAAGCCATGGAAGTTGGGGGTACCTAAAGTA : 1374
TCCCgGG cTTGtACaCaCCGCCCGTCa Ca G Agt g Cc gAAg



Alignment of the amino acid sequences of 45 isolates obtained from bioballs during the course of the bioreactor run. The alignment was carried out by the multiple alignment of Clustal X (1.81). Genedoc software was used for homology shading. The conserved regions are indicated with Roman numerals. The abbreviations of the isolates are given in the text. Gaps introduced into the alignment are indicated with dashes. Four shading levels were set.