Characterisation of Mixed Microbial Populations in White Mineral Dispersions

By

NICOLA DI MAIUTA

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Department of Biological Sciences
University of Warwick

Department of Research & Development Microbiology
Omya Development AG

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Contents

Chapters

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Author's Declaration

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been submitted for a higher degree to any other University or Institution. The work presented in this thesis is the achievement of original research work done by myself under the supervision of Dr. C.S. Dow, unless otherwise stated. All sources of information have been declared by means of references.

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Publications

Schwarzentruber P., Di Maiuta, N., Hubschmid, S., Vladyka, B. (2007) Protection of White Mineral Dispersions. Concepts, Strategies, Options and Limitations. Interactive CD available from Omya Development AG, Switzerland.

Di Maiuta, N., Hubschmid, S., Giuliani, N., Schwarzentruber, P. and Dow, C. S. (2009) Microbial degradation of formaldehyde in white mineral dispersions preserved with formaldehyde-releasing biocides *International Biodeterioration & Biodegradation* 63, 769-777.

Di Maiuta, N., Schwarzentruber, P. and Dow, C. S. (2010) Enhancement of the antimicrobial performance of biocidal formulations used for the preservation of White Mineral Dispersions. Under review in *Applied and Environmental Microbiology*.

Di Maiuta, N. (2010) Microbial diversity of white mineral dispersions. In preparation for publication in *Journal of Applied Microbiology*.

Presentations and Conferences Attended

Di Maiuta, N., Hubschmid, S., Giuliani, N., Schwarzentruber, P., 2007. PCR Based Identification of Methylotrophic Bacteria in White Mineral Dispersions. Poster Presentation.

Di Maiuta, N., Schwarzentruber, P., Dow, C.S., 2008. Microbial Degradation of Formaldehyde in White Mineral Dispersions Preserved with Formaldehyde-Releasing Biocides. Scientific Poster presented at the IUMS 2008 in Istanbul.

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Patents

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Membership

Since 2008, Member of the Society of Applied Microbiology

Since 2008, Member of the Society of General Microbiology

Since 2009, Member and depositor of the Culture Collection of Switzerland (CCOS)

Tables

Figures

Abbreviations

16S rRNA	16S ribosomal RNA Gene
(a/d)	Active on Dry
(a/l)	Active on Liquid
AMP	2-Amino-2-methyl-1-propanol
BfR	Federal Institute of Risk Assessment (Germany)
bp	Base Pairs
BPD	Biocidal Products Directive 98/8/EC
CA	Cluster Analysis
CCCP	Carbonyl Cyanide m-Chlorophenyl hydrazone
(c/d)	Commodity on Dry
cDNA	Complementary DNA
CFII	CellFacts II® instrument
CMIT	5-Chlor-2-methyl-4-isothiazolin-3-on
d_{50}	Average particle diameter by mass (Mass-median-diameter MMD)
Da	Dalton (unified atomic mass unit)
$DiSC_3(5)$	3,3'-dipropylthiadicarbocyanine iodide
DMO	4,4-dimethyloxazolidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDDM	(Ethylenedioxy)dimethanol
EDTA	Ethylenediaminetetraacetic acid
FA	Formaldehyde
FCM	Flow Cytometry
FDA	Food and Drug Administration
FICI	Fractional Inhibitory Concentration Index
FISH	Fluorescent *In Situ* Hybridisation
GCC	Ground Calcium Carbonate
GDA	Glutardialdehyde
gDNA	Genomic DNA
HPLC	High Pressure Liquid Chromatography
MBC	Minimal Bactericidal Concentration
MIC	Minimal Inhibitory Concentration

MIT	2-Methyl-4-isothiazolin-3-on
MP	2-methyl-1-propanol
Mw	Average relative molecular mass
NPN	1-N-phenylnapthylamine
OPP	o-phenylphenol
OTUs	Operational Taxonomic Units
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar
PCAs	Principal Component Analysis
PCC	Precipitated Calcium Carbonate
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
pKa	Acid dissociation constant
PMA	Propidium Monoazide
PMT	Photomultiplier
ppm	Parts Per Million
QACs	Quaternary Ammonium Compounds
qPCR	Quantitative Polymerase Chain Reaction
rcf	Relative Centrifugal Force
rfu	Relative Fluorescence Units
RISA	rDNA Internal Spacer Analysis
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
SYTO62	Trade name of red fluorescent stain (Invitrogen)
TCC	Total Cell Count
TMAD	Tetramethylolacetylene diurea
T-RFs	Terminal Restriction Fragments
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TSB	Tryptic Soy Broth
WMD	White Mineral Dispersions

Summary

In recent years, the microbiology of white mineral dispersions and the application of microbiocides for their preservation have taken a central role for the producer and user with the aim of maintaining high quality requirements such as brightness, rheological parameters, and odour neutrality. Additionally, new applications of mineral dispersions set to open up markets in food, cosmetics and pharmaceutical applications have aroused the interest in the microbiology of white mineral dispersions. Due to the occurrence of biocide resistant bacteria, technical limitations in the usage of biocides, as well as the more rigorous regulatory situation created by the BPD, the demand for new biocide research to ensure continuing effective WMD preservation is increasing. Despite efforts to optimise the application of microbiocides for the storage and protection of mineral dispersions, costs for preservation and disinfection are escalating. These are reasons why the current preservation strategies have been revisited and new preservation strategies have been designed.

The work described in this thesis demonstrates that the microbial diversity of white mineral dispersions is greater than previously assumed and gives detailed insight about the microbial diversity of mineral dispersions. The occurrence of microbial contamination in mineral dispersions is of a seasonal nature rather than manufacture site or product type specific. Furthermore, the incidence of biocide-resistant bacteria in mineral slurries is increasing and the microbial degradation products of biocidal compounds are disadvantageous for dispersion stability (pH and viscosity). New strategies for the preservation of mineral dispersions have been developed and biocide performance against biocide-resistance bacteria has been enhanced by combining in-use biocides with a range of non-biocidal additives.

The industrial application of these new findings contributes to a more efficient preservation of white mineral dispersions with respect to both environmental as well as financial resources and opens up a basis for alternative preservation strategies of white mineral dispersions.

CHAPTER 1

Introduction

1.1 Chemistry and Properties of White Mineral Dispersions

Omya AG in Switzerland is the world leader in the manufacture of white mineral dispersions (WMD) used in several industrial applications and supplies a vast number of mineral products from more than 160 manufacturing sites worldwide. WMD (ground and precipitated calcium carbonate, clay, and talc) are water-based mineral dispersions with a solid content ranging from 25-80 % (w/w). Calcium carbonate ($CaCO_3$) is a natural compound which is only slightly soluble in water (14 mg l^{-1} at 20 ºC). Therefore, in nature calcium carbonate in the course of the Earth's history formed sediments of limestone, chalk and marble as a consequence of continuous dissolving and deposition cycles (Figure 1a). Chemical precipitation, biochemical processes and organogenic sedimentation are the most important reactions leading to the deposition of calcium carbonate. The use of calcium carbonate as a filler and as a coating pigment has expanded in recent years across various industrial sectors. In 2003, for example, the demand for filler and coating pigments amounted to 21.2 billion tons, whereof 11.3 billion tons were used in the paper industry, 5.3 billion tons in paints and coating and 4.6 billion tons for applications in plastics (Omya, 2004).

Wet fillers and particularly aqueous dispersions of calcium carbonate, also referred to as calcium carbonate slurries, are primarily used in the paper industry, whereas dry fillers are used in coating and plastic applications. However, more than 50% of the calcium carbonate is supplied as dispersions in order to fulfil the paper machine processing requirements and because the supply chain management of such huge amounts of calcium carbonate is facilitated in the pumpable wet form. A water suspension of 75% (w/w) calcium carbonate solids and 25% (w/w) water is subject to sedimentation of the particles and unable to flow. Therefore, the high solid content of calcium carbonate slurries accentuates the need for dispersion of the mineral particles by means of highly active polyacrylic acid systems in the form of neutralised alkaline polyacrylate salts. Dispersants allow an increase in the solid content of a mineral dispersion up to 78% (w/w) while maintaining the desired viscosity of approx. 300-500 mPa·s. The mechanism of stabilisation is not fully elucidated, however, the carboxyl group of the polyacrylate has been shown to interact with the surface of the mineral particles as well as with the cationic metals (Taylor and Sigmund, 2010).

The pH of calcium carbonate slurries ranges from 8 to 10 and the particle size distribution is of importance for the type of product to be processed in the down-stream applications. In general, coarse calcium carbonate slurries have a particle size distribution of at least 90% (w/w) in the range of 1 to 5 µm and the mean particle size d_{50} value is < 2 µm. Fine calcium carbonate slurries have a particle size distribution of 50% to 99% (w/w) less than 2 µm and the mean particle size d_{50} value is < 1 µm. Figure 1b shows a scanning electron micrograph of crushed marble used to manufacture calcium carbonate dispersions.

(a)

(b)

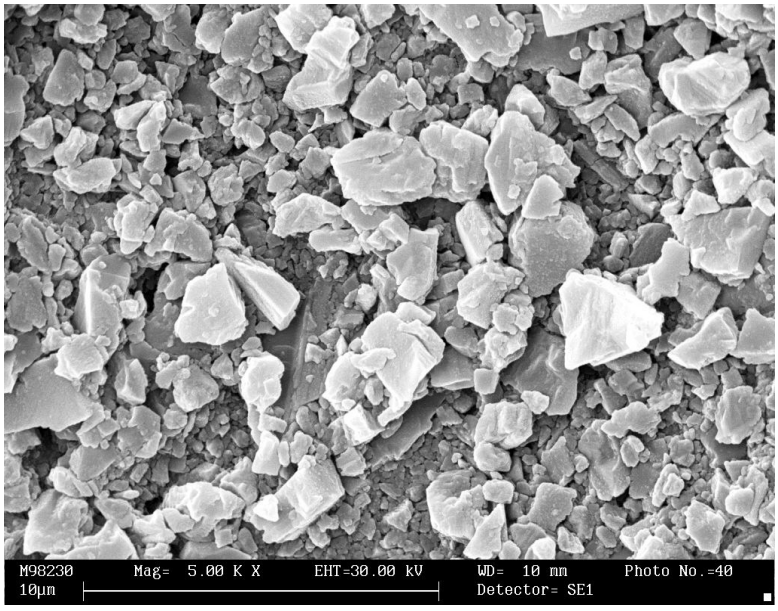


Figure 1. (a) Marble quarry in Carrara (Italy). (b) Scanning electron micrograph (5000x magnification) of Hydrocarb 90 calcium carbonate slurry ground from marble quarried in Gummern (Austria).

The production of high solid content slurries facilitates the storage and transportation and at the same time, it increases the transport density and cost-effectiveness. Today the transport density of 1.5 tonnes calcium carbonate per cubic meter is achieved for 78% slurry which is 2-4 times higher than the transport density of dry calcium carbonate (Rohleder and Huwald, 2001). The development of innovative dispersing agents and advances in production technologies will facilitate the manufacture of more highly concentrated white mineral dispersions in the future.

1.1.1 Application of WMD in Different Industries

Calcium carbonate is a natural raw material and offers a low-cost and ecologically safe product of economic importance which is used in countless industrial applications. Chalk, limestone and marble as a source of calcium carbonate have been used for centuries, e.g. for sculptures. After the Second World War, calcium carbonate attained increasing importance in various industrial fields such as in civil engineering, chemical and agricultural industries, as a filler and coating in paper, paints and coatings, in concrete as well as more recently in water treatment, pharmaceuticals, cosmetics and food applications. In practice, aqueous preparations and especially suspensions or dispersions of water-insoluble mineral solids are used.

Additional forms of synthetically produced calcium carbonate are the precipitated calcium carbonate (PCC) and the surface modified calcium carbonate (MCC). These artificially produced calcium carbonate products can be optimally adapted to the required conditions of specific applications by engineering specific features and also to applications in innovative areas.

1.1.1.1 GCC

The term GCC (ground calcium carbonate) refers to ground natural calcium carbonate suitable for use as a filler both in the dry or wet formulation. The natural calcium carbonate varieties suitable to produce GCC are chalk, limestone and marble. Chalk was the first calcium carbonate mineral used as a filler in paints and putty because of its finely dispersed occurrence in nature. Depending on the area of application and the application requirements chalk is sized directly after washing or later in the dried form. Chalk offers several advantages in term of human health and

safety (key for household, food, cosmetic and pharmaceutical commodities), simple processing as well as high brightness and coatability. However, the low mineral metamorphosis and biogenesis as well as the frequently found impurities such as silicon dioxide, organic residues, sulphites and sulphides impair achieving the greatest possible index of whiteness of calcium carbonate (Huwald and Rohleder, 2001).

In contrast, limestone and its metamorphism product marble, have a much higher degree of brightness than chalk and when the paper, paint and coating industry expressed the need for brighter fillers the way to the exploitation of these new calcium carbonate materials was paved. In most cases, to attain the high degree of purity and brightness both marble and particularly limestone have to be processed to remove impurities. Nevertheless, marble offers the best properties in terms of brightness due to the natural exclusion of impurities during its metamorphosis. Limestone and marble also differ from chalk in their particle size distribution, abrasivity and opacity. Whilst a natural chalk displays a broad particle size distribution and large specific area, the crystalline limestone and marble exhibit a tight particle size distribution. Despite intensive grinding and classifying, the particle size distribution of comminuted minerals ultimately depends on the structure of the starting material (Huwald and Rohleder, 2001).

1.1.1.2 PCC

The term PCC (precipitated calcium carbonate) refers to synthetically produced calcium carbonate formed as a result of carbon dioxide precipitation of lime milk. Nevertheless, the complex petrogenesis and metamorphosis of marble in nature cannot be replicated in the laboratory. The higher volume of PCC compared to GCC is the main benefit provided to industry, however PCC has similar application fields to the marble fillers and because of the controllable purity it is used in pharmaceutical products (Huwald and Rohleder, 2001). The development of nano-sized PCC with specific features has recently been launched and is subject of future research.

1.1.1.3 MCC

The term MCC (modified calcium carbonate) refers to surface-modified synthetically produced calcium carbonate. The structure and surface of MCC is influenced by varying the reaction conditions such as pressure, temperature and time as well as by the addition of chemicals to the precipitation which influences the final crystal form and particle size distribution (Ridgway*, et al.*, 2004).

1.1.2 Processing of White Mineral Dispersions

The route from rock to the calcium carbonate filler comprises a range of technical processing steps and even though the mineral can be extracted without excessive technical investment the installation of a quarry also must fulfil quality requirements and economic criteria (Rohleder and Huwald, 2001). The first step before any extractions are carried out involves the prospecting of the deposit and thus the qualification of the minerals in terms of quality and quantity prior to opening a quarry. The method of mineral extraction strongly depends on the properties and conditions of the deposit and the environment of the quarry. The schematic processing of calcium carbonate in the dry and wet state is illustrated in detail in Figure 2. Mineral dressing of calcium carbonate initially consists of crushing, grinding and classifying of the extracted raw material. Subsequently, the raw material undergoes a sorting and washing step to eliminate foreign minerals and to clean the material from soil and coarse clay prior to dry or wet processing. The sorting of marble in most of the cases is more challenging than those of limestone because of the brightness requirements and the content of dark accessory materials generally referred as to “acidic insoluble residues” (Rohleder and Huwald, 2001). In the occurrence of ore impurities or fine embedded foreign minerals, a flotation process is carried out. Dry products are subject to a sequence of coarse grinding in ball mills and classifying by air in bladed rotor separators. Wet calcium carbonate undergoes a primary wet grinding step followed by a wet fine grinding step to the final concentration (solid content) in the presence of dispersant. Calcium carbonate slurries are typically prepared by wet grinding marble in the presence of a radically polymerized polyacrylic acid wherein the carboxylic acid groups are neutralized by alkali metals.

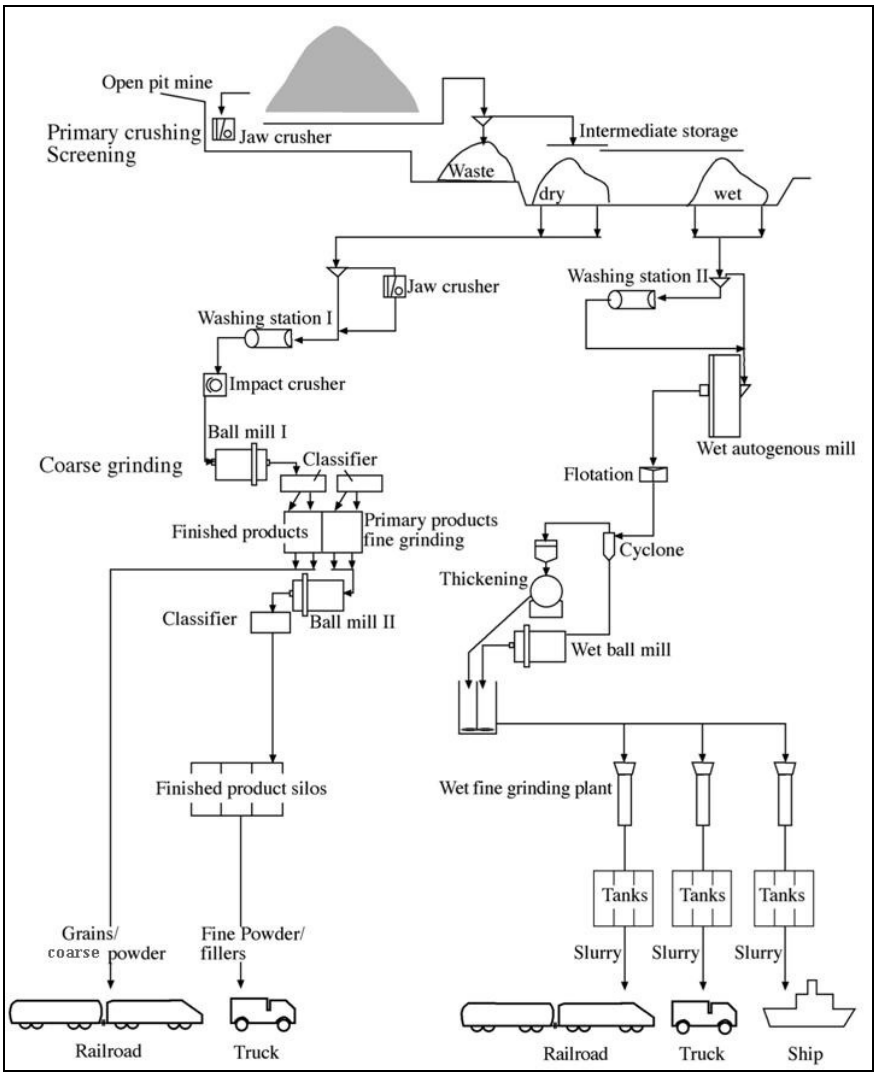


Figure 2. Schematic processing of both dry and wet calcium carbonate products (Rohleder and Huwald, 2001).

The storage, packaging and supply of the end product is an important part of the production process and also essential to guarantee the availability of the products in the required quantities at any time to the customer. Dry products are stored in silos up to 1,000 m^3 whereas slurries are stored in tanks of up to 3,500 m^3 volume. The logistic route to the customer involves the transportation of the products by trucks, railway wagons and in the case of slurry products via sea by freighters. In the case of calcium carbonate slurries the stability relating to the rheological properties, high solids content and dispersibility are key features and as a result of the water present in the wet products the microbiologically related problems take their origin.

1.2 Microbiology of WMD and the need for Preservation

Microorganisms are found ubiquitously in varied environments and are able to survive under extreme conditions. This also applies for WMD. Although the alkaline pH of WMD ranges from 8 to 10, and the water content from 25-80 % (w/w), the presence of various salts and the available oxygen are sufficient to promote an adequate microbial propagation environment (Figure 3). Moreover, WMD contain a number of biologically key compounds such as nitrogen, sulphur, and phosphorus as well as various micronutrients (personal communication D. Frey). The natural organic deposits and the polyacrylic acids of the dispersant are the main carbon and energy source. However, the neutralised dispersant is a rich source of sodium, potassium and magnesium but the bacterial degradation of acrylic polymers is limited to the fraction with an average relative molecular mass (Mw) of 1,000-4,500 Da (Kawai, 1993, Kawai*, et al.*, 1994). Acrylic polymers with a Mw greater than 3,500 Da will adsorp onto the mineral surface, however, low-Mw acrylic polymers are subject to biodegradation in WMD (Schwarzentruber, 2003a).

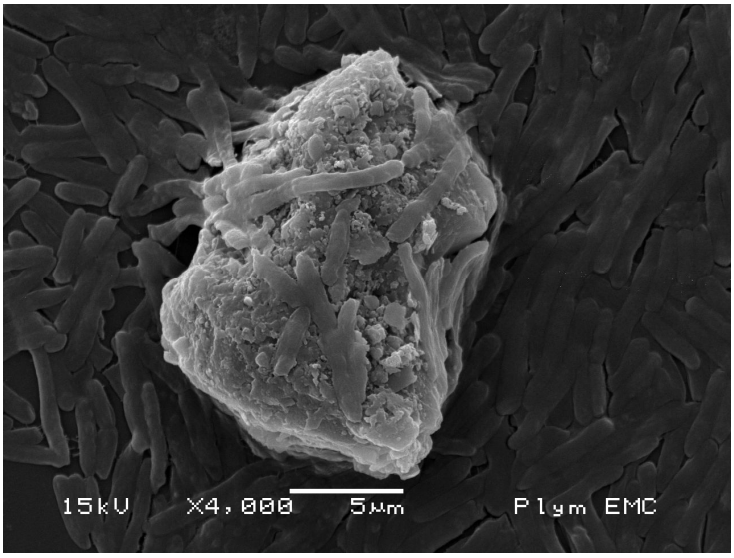


Figure 3. Scanning electron micrograph (4000x magnification) of a Hydrocarb 90 calcium carbonate slurry particle covered with bacterial cells (*Pseudomonas* sp.).

The slurry production occurs in an "open" system and the raw material, various additives, the tap water and the tendency to use process water recirculation systems are significant sources of microbial contamination. During the wet fine grinding step

of calcium carbonate slurries temperatures of up to 110 ºC can be reached for fine products. Even though this represents to a large extent a thermal disinfection treatment, in the field it has been seen that some microorganisms are able to overcome this physiological stress by persisting due to protective mechanisms, e.g. production of extracellular slime layers (Schwarzentruber, 2003b). In the post-manufacture storage tanks and during transportation the moderate temperatures (25 to 45 ºC) are favourable to bacterial growth. In some cases where flash coolers are used to cool down the product after the wet grinding step these devices have been shown to be a major source of contamination (Schwarzentruber, 2003b).

The deterioration of WMD by microorganism has to be taken seriously. Bacterial counts > 10^6 cfu ml^{-1} can affect the physiochemical properties of white mineral dispersions (Figure 4), which consequently lead to considerable problems in the down-stream processing by the user and negatively affect the commercial value.

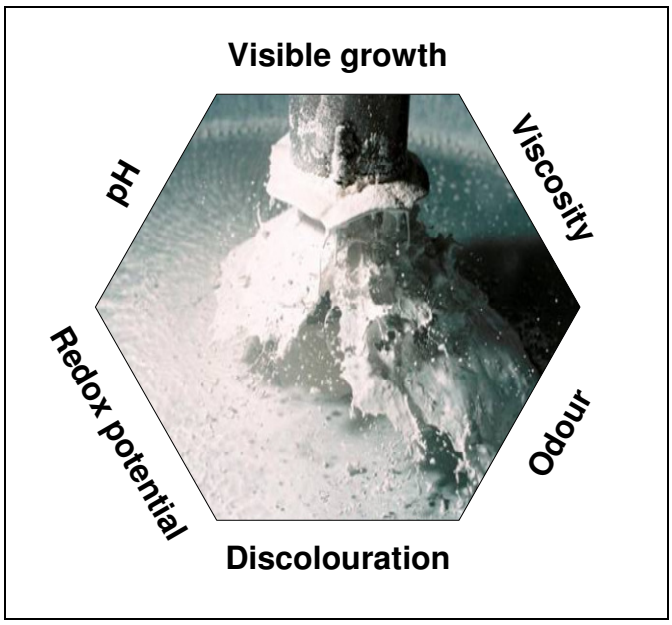


Figure 4. Physiochemical properties affected as a result of microbial contamination in white mineral dispersions.

Bacterial contamination of WMD often leads to acidification of the product and hence to changes in the dispersion stability in terms of viscosity (Schwarzentruber, 2003b). A decrease in pH is usually associated with an increase in viscosity and consequently involves alteration of the rheological parameters such as fluidity and

pumpability which are required to ensure trouble-free handling to the final user. Discolouration and development of unpleasant odours can occur under certain conditions when oxygenation (agitation/stirring) of the WMD product stops and aerobic growth is replaced by anaerobic growth. The anaerobic growth of microorganisms in WMD is generally connected with a decrease of the redox potential. In addition, highly contaminated products represent the risk of biofilm formation.

1.2.1 Monitoring of Contamination in WMD

A handful of scientific investigations in the field of microbiology and white mineral dispersions are available and only recently the research activities in the microbiology and preservation of WMD have been intensified. Although the technical application and the dosage of biocides for WMD preservation have been well studied, little is known with regards to the chemical interaction involving biocides and mixed microbial populations. The effect on the physiology of the bacteria following biocide addition to WMD is poorly understood.

It is often necessary to quantify the microbial population present in WMD and traditionally bacterial populations have been monitored by means of classical cultivation approaches such as plate count methods or the CellFacts II® (CFII) technology. By combining flow impedance and fluorescence, the CFII instrument provides additionally information about the physiological status of the individual cells of a microbial population in real-time (Schwarzentruber, 2002). In the simplest case, enumeration of bacterial cells in WMD is carried out to detect whether bacterial contamination is present or not. Indisputably, conventional culture methods such as the plate count method are only able to disclose bacteria that form visible colonies on the agar media and fail to detect bacterial species which for a variety of reasons are not cultivable. Additionally, the time-dependent response of microorganisms for visible growth on nutrient agar to enable a plate count is retrospectively reporting the microbial situation at the sampling point at some time in the past rather than the actual situation.

The term “viability” and “culturability” are often mistakenly used as being synonymous (Kell*, et al.*, 1998). In the field it has been seen that after biocide

addition microorganisms are able to overcome this physiological stress by persisting in a so called “dormant state”. This “non-growth” state is adopted by bacteria as a response to stress due to a variety of adverse environmental conditions (Keep*, et al.*, 2006a). Whereas the extreme form of persistence is the formation of spores. The dormant state is characterized by very low or zero metabolic activity of the cells accompanied by a lack of proliferation. However, under certain conditions resuscitation and a reversion to culturability is possible (Kell and Young, 2000). This survival condition is distinct from the starvation response and is sometimes referred to as the Viable-But-Non-Culturable (VBNC) state (Keep*, et al.*, 2006b). It has been proposed that in WMD, once the concentration of the biocide has decreased, the cells are able to recover and start a new growth cycle (Schwarzentruber, 2003b). Whilst classical microbiological monitoring methods such as plate count provide retrospective results, early determination of microbial contamination by CFII offers the possibility of reactive biocide treatment, thus the biocide dose frequency are reduced by preventing the development of persistent cells and challenging the cells during the more biocide sensitive exponential growth phase (Denyer and Stewart, 1998).

1.2.2 Application of biocides in WMD

WMD are more susceptible to microbial contamination in post-processing handling (Schwarzentruber, 2003b). Therefore, biocides play an important role in the preservation of WMD in order to maintain high hygiene and quality standards, such as brightness, rheologic parameters as well as odour neutrality. Biocides are chemical products usually applied at low concentration in the range of 0.1 to 5000 ppm (parts per million) to prevent or eliminate the contamination of processes and commodities with microorganisms. To ensure an acceptable microbiological quality of WMD, biocides are used over the entire life cycle of the preparation (production, storage, transport, use).

In the beginning when biocides became indispensable to protect WMD from microbial growth the minimal inhibitory concentration (MIC) published in the literature was adopted for preservation. However, because most of the MICs published in the literature arise from microbial studies conducted in pH neutral

aqueous media and by means of clinical bacterial isolates not representative for the WMD environment these data are of little value for use in WMD (Paulus, 2005). Additionally, there is still a general lack of agreement on the standardisation of all the components interfering with the testing method for biocides (Lambert, 2003). Nowadays the in-use concentration of biocidal compounds and formulations used in the preservation of WMD originate from the MIC studies carried out *in-situ* by Schwarzentruber (2003b).

1.2.2.1 Regulatory, Safety and Environmental Issues

The European Biocidal Products Directive 98/8/EC (BPD) was published on the 24th April 1998 and came into force on by 14th May 2000. The goal of the BPD is to harmonise and authorise the application of biocidal products and their active substances in the European Union market with the intention of minimising the health and safety as well as the environmental risks in the use of biocides. The BPD also defines the data required for the registration dossier which contains information about the biocidal performance, physical and chemical properties as well as environmental adverse effects. The BPD defines 23 product types (PTs) within 4 main groups to which the biocidal compounds belong, according to their application field. Biocides used in food (except food fumigation and feedstocks), pharmaceutical and cosmetic industries are exempt from the BPD and are regulated by application-specific directives. According to the BPD a biocide is referred to as an active substance or preparation containing one or more active components, in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means (European Parliament, 1998). Currently the number of available biocidal compounds has fallen and the quantity of products preserved with biocides is increasing. The global biocide consumption in 2009 was estimated to have a value of about $ 7 billion (www.researchandmarkets.com).

The biocides used for the preservation of white mineral dispersion fall under the product type 6 - in-can preservatives (PT6) - of the main product group 2 referring to preservatives. The closure of the notification period for existing substances of PT 6 was in October 2008 and 143 substances have been included

within the review program (Kähkönen and Nordström, 2008). Finally, for a total number of 56 substances the registration dossier has been submitted by the participant biocide supplier. Apart from the economic efficiency of biocides intended for the preservation of white mineral dispersions, maintenance of human health and environmental safety are of central significance. Figure 5 presents a quadrant diagram showing the various areas of biocide performance and risk assessment in consideration of the antimicrobial activity and specificity. In the optimal case, the policies of biocide usage comply with the balance between efficacy and toxicity of an in-use biocidal agent and are ideally located in the upper right quadrant of the biocidal activity and toxicity diagram.

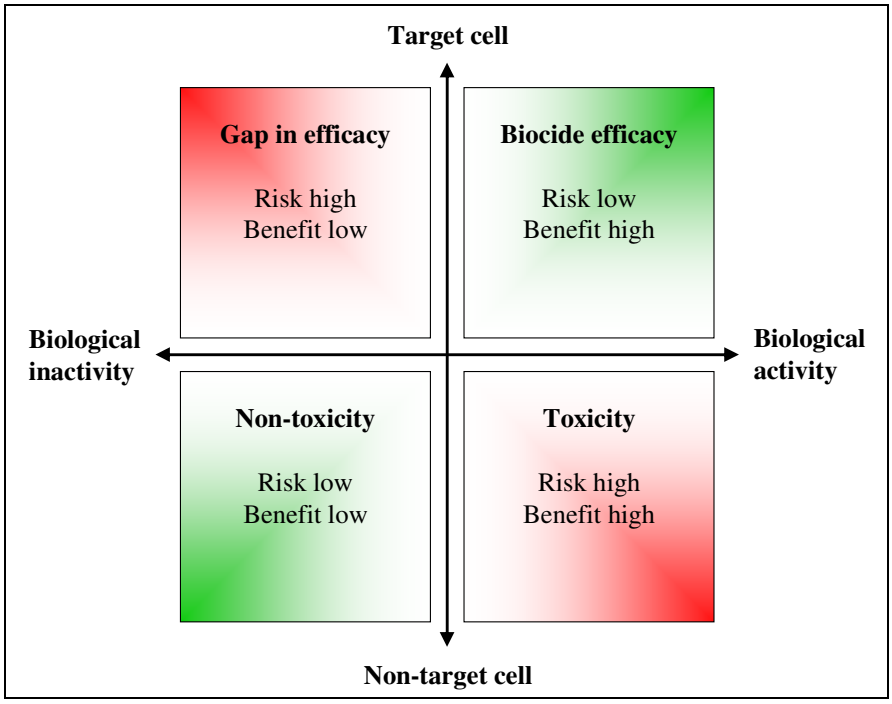


Figure 5. Biocidal activity and toxicity diagram. Adapted from (Kähkönen and Nordström, 2008). Intended areas: green. Avoided areas: red.

The emerging regulations and the implementation of the BPD have compulsorily lead to the situation wherein the development and registration of new biocidal substances have been abandoned due to the expenses of up to € 4.5 million to generate and provide the registration dossier (Kähkönen and Nordström, 2008). The principal reason is the laborious and expensive research required for the registration dossier to evaluate the risks that will arise from the proposed uses of the product. At

the same time the development of “safer” alternatives to substitute the existing biocides, to meet the emerging environmental concerns and to fulfil the criteria for eco-labelled consumer products is hindered by this regulatory framework. In the absence of biocides, microbial contamination in consumer products such as medicine and food may be dangerous to human health. On the other hand, requirements concerning the toxicity of antimicrobial substances primarily depend on the intended use. However, nowadays in addition to the antimicrobial performance the environmental sustainability as well as occupational and consumer safety is of primary importance.

1.2.2.2 Stability and Effectiveness of Biocides

The chemical stability and therefore the effective performance of biocidal formulations in WMD can be influenced by several factors. In general, the stability of biocidal compounds in a system depends on the exposure time as well as on the presence of other reactive chemicals. Grinding aids, oxidising and reductive agents as well as other chemical additives are just a few substances which can have an impact on the stability of biocides. In this context it is important to point out that in biocidal formulations the physiochemical properties of WMD can promote cross-reactions also. In the case of WMD, the effective application of biocidal formulations implies the pH compatibility of the active compounds with the alkaline pH of the product. In addition, the temperature can affect both the optimum efficacy and the stability of biocides. Most of the available biocidal compounds thermally decompose rapidly and since following the wet grinding step high temperatures of 50 to 80 °C are achieved, the dosage site of biocidal formulations to WMD has to be chosen carefully.

1.3 Know-how Transfer to the WMD Manufacturing Industry

1.3.1 Microbial Diversity of WMD

Over recent years the WMD producers, as well as their customers, had little interest in the microbial diversity arising in white mineral dispersions. The main goal of efficient and economic biocide dosage in respect to the WMD was the repression of detectable viable cells which were compromising many of the technical applications. This position was reviewed when WMD were introduced into new business areas such as food, cosmetics or even the pharmaceutical industries, hence, the knowledge of WMD bacterial community composition is becoming important.

Although a greater microbial diversity than originally assumed has been detected in WMD, only a small fraction of the total population has been elucidated (Lahtinen*, et al.*, 2006, Schwarzentruber, 2003b). However, this work focused on the characterisation of culturable bacteria rather than on the "whole" biodiversity considering unculturable bacteria as well. Schwarzentruber (2003b) identified by 16S rRNA (16S ribosomal RNA) sequencing various water and soil bacteria, primarily *Pseudomonas* sp., from calcium carbonate slurries and additionally a number of molecular techniques (RISA and FISH) were applied to investigate the microbial diversity. Since cultivation methods can only detect a small fraction of the microbial diversity of samples such as WMD, different molecular techniques have been implemented to evaluate *in-situ* microbial diversity without prior cultivation (Amann*, et al.*, 1995). Also of importance are the facts that the incidence of biocide resistant microorganisms is increasing whereas biocide compound commodities intended for use in WMD are being limited (Schwarzentruber, 2005). On the one hand the applicability of WMD is being subjected to special regulations in order to fulfil the high hygiene requirements and the prescriptive limits for pathogenic microorganisms in the newly acquired business areas. Whilst on the other hand the increasing resistance against biocides in WMD introduces the need for elucidation of the different members of mixed microbial populations in order to discover and eliminate resistant strains by combining optimal dosage to the accurate choice of the antimicrobial compounds. Furthermore, awareness of the bacterial community is assumed to provide important insights about the members populating a particular matrix such as WMD.

The quantitative analysis of clone libraries and DNA fingerprinting generated from natural microbial communities is a step towards culture-independent phylogenetic analysis (Suzuki, *et al.*, 1997). Random sequencing of clone libraries constructed either with universal primers targeting the eubacterial domain or with specific primers targeting related upper group levels of eubacteria, in relation to the fingerprinting of the community by terminal restriction fragment length polymorphism (T-RFLP) patterns has provided accurate information about community composition (Hayashi, *et al.*, 2003, Osborn, *et al.*, 2000, Wang, *et al.*, 2004). In addition, T-RFLP is a high-throughput and reproducible method that is both a qualitative and quantitative analysis with the ability to detect even rare members of a microbial community (Dorigo, *et al.*, 2005, Liu, *et al.*, 1997). By means of these patterns, it has been possible to study the seasonal and spatial succession of microbial communities in the environment (Chiu, *et al.*, 2006, Hullar, *et al.*, 2006). Ecological succession studies of microorganisms have been adapted for quantifying bacterial population structure and dynamics in soils (De Leij *et al.* 1993; Sigler and Zeyer 2004) and multivariate analysis of the acquired data offered new insight into the succession and community structure of microorganism in the environment (Ramette, 2007, Schutte, *et al.*, 2008). However, because the analysis of clone libraries is lab intensive and expensive the recently described pyrosequencing technique has been applied to perform deep sequencing of 16S rRNA amplicon libraries of mixed bacterial populations from various environments (Acosta-Martínez, *et al.*, 2008, Dowd, *et al.*, 2008, Huse, *et al.*, 2008, Liu, *et al.*, 2007, Sundquist, *et al.*, 2007). Finally, the combination of molecular techniques used in this research thesis will provide new insights about the microbial diversity of white mineral dispersions.

1.3.2 Spatial and Temporal Microbial Succession in WMD

Based on the assumption that bacterial community succession in WMD is driven by the ability of both “opportunistic” and “maintenance” bacterial types (r- and K-strategists) to respond to variable growth conditions the elucidation of bacterial community structure and succession in WMD is gaining in importance. The microbial spatial and temporal succession in WMD has never been elucidated and

will provide insights into the specificity of bacterial communities linked to the seasons in respect to the product type or production plant. Even though WMD are treated with biocide, it has been assumed that some bacterial species resist the biocide challenges and are able to recover and dominate once the environmental conditions become favourable. Above all, the purpose of investigating the disturbance response and the population distribution in WMD production lines and storage tanks before and after biocide dosage is to resolve and understand the source of microbial community diversity.

1.3.3 Bacterial Resistance in WMD

The risk of the formation of resistant bacteria should not be underestimated and the problem of resistance to antimicrobial agents, used for the preservation of WMD, is increasing (Schwarzentruber, 2005). The limited range of biocidal compounds available, inadequate preservation systems and the nearly complete neglect of house-keeping have led to the presence of resistant microorganisms which are now difficult to control. All these factors in combination may also play a role in the development of cross-resistance to biocides. In addition, other compounds such as formaldehyde and o-phenylphenol are losing acceptance by the customer and therefore it will be mandatory for the future to apply the available biocide resources efficiently.

Resistance to biocidal agents has been studied and the major mechanisms found to be reduced uptake of the biocide (impermeability or efflux) or compound degradation by acquired mutations (Russell, 2003c). In addition, the stationary-phase phenomena during which bacteria demonstrate various morphological and physiological response changes as well as stress response mechanisms also contributes to the occurrence of intrinsic resistance (McDonnell and Gerald, 2007). Furthermore, whether excess biocide usage leads to the development of bacterial resistance against biocidal compounds and antibiotics is still subject to debate (Pagès, *et al.*, 2009, Russell, 2003c). Based on the assumption of heterogenic diversity and physiology of bacterial populations inhabiting WMD it is not surprising that bacteria in WMD are not uniformly susceptible to biocides.

Finally, to overcome these problems and to guarantee the quality of WMD products in the future it has become necessary to understand the interactions between

the diverse microbial populations and the biocides. In addition, there is the need to gain more knowledge about the occurrence of bacterial resistance in white mineral dispersions. In order to keep within the utilisation limits of biocidal agents, optimisation and control of the biocide dosage will become essential in the near future. New strategies to combat resistant microorganisms in WMD have to be drawn up and potential application may solve the emerging problems, both resistance and adaptation.

1.3.4 Novel Approaches for the Preservation of WMD

In order to overcome the lack of new antimicrobial substances applicable to WMD and to avoid bacterial resistance developing in WMD other strategies have to be considered rather than increasing biocide dosage: (i) the evaluation of technical process measures to achieve eradication of resistant microorganisms from the WMD production and storage process, (ii) the use of synergistic combinations of biocides and (iii) the enhancement of the biocide agent by non-antimicrobial compounds.

Several attempts have been made to enhance the performance of biocides however the evidence of successful enhancement must be given rather than the generation of a multicomponent biocidal formulation (Hiom, 2003). Biocide "enhancers" are non-biocidal substances which do not exhibit an inherent antimicrobial activity but support and/or enhance the activity of antimicrobials. Sensitisation by various compounds such as EDTA, ascorbic acid, nerolidol, SDS, Triton-X 100, DMSO, vanilline, Poly-L-Lysine and many others have been reported (Hancock and Wong 1984; Vaara 1992; Alakomi *et al.* 2006). This approach may solve the emerging problems, either resistance or adaptation, by ensuring that an accumulation of the biocidal agent at the action site is achieved. The breakthrough of a biocidal enhancer offers a potential tool to revolutionise the consumption of biocidal agents in the WMD producing industry. Moreover, of particular importance are the specifications of enhancer/synergistic compounds due to essential chemical compatibility with WMD and economic and ecologic sustainability.

1.4 Aims of the Thesis

The aims of this project were:

- To characterise the diversity as well as the seasonal and spatial succession of microbial communities in white mineral dispersions by molecular techniques.

- To investigate the microbial diversity of viable, compromised and dead bacteria in white mineral dispersions by new discrimination methods.

- To identify and characterise biocide-resistant bacterial species in white mineral dispersions.

- To evaluate the chemically and biologically stability of biocidal compounds used to preserve white mineral dispersions.

- To evaluate the performance of so called “biocide enhancers” against biocide-resistant bacteria.

- To develop and implement alternative preservation protocols suitable for white mineral dispersions.

The application of these findings should result in a more efficient preservation of white mineral dispersions with respect to both environmental as well as financial resources. The management of these resources is considered to be a key subject in the future preservation of white mineral dispersions.

CHAPTER 2

Materials and Methods

2.1 Materials

The following solutions and buffers were, unless otherwise stated, prepared with water produced by reverse osmosis. Heat-stable solutions were sterilised in an autoclave (Varioklav Steam Steriliser; Sterico, 8602 Wangen, Switzerland) at 121 ºC and 1 bar for 15 minutes. Heat-unstable solutions were sterilised by filtration (FP 30/0.2 µm CA-S; Schleicher & Schuell, 4103 Bottmingen, Switzerland). Thermally stable and steam-stable instruments as well as plastic consumables were sterilised in an autoclave as described above.

2.1.1 Chemicals

Except as noted otherwise, all chemicals (inorganic, organic, solvents and biochemicals) were mainly obtained in *per analysis* (p.a.) grade from the following distributors:

Applied Biosystems (6343 Rotkreuz, Switzerland)
Agilent Technologies (4052 Basel, Switzerland)
BASF (67056 Ludwigshafen, Germany)
Becton & Dickinson (4123 Allschwil, Switzerland)
bioMérieux SA (1211 Genève 2, Switzerland)
Bio-Rad Laboratories (4153 Reinach, Switzerland)
BioVentures, Inc. (Murfreesboro, TN 37130 USA)
Dow Europe GmbH (8810 Horgen, Switzerland)
Fluka (9471 Buchs, Switzerland)
GE Healthcare (8152 Glattbrugg, Switzerland)
Invitrogen Inc. (4019 Basel, Switzerland)
MO BIO Laboratories Inc. (Carlsbad, CA 92010 USA)
Mycrosynth (9436 Balgach, Switzerland)
New England Biolabs (Ipswich, MA 01938 USA)
Qiagen Inc. (8634 Hombrechtikon, Switzerland)
Sigma-Aldrich Chemie GmbH (9471 Buchs, Switzerland)

2.1.2 White Mineral Dispersions (WMD)

WMD comprise water-based mineral dispersions of ground and precipitated calcium carbonate. These were calcium carbonate slurries of ground marble, limestone or chalk from different Omya production sites in Europe. Calcium carbonate slurry is typically prepared by wet grinding the primary material in the presence of 0.2 – 1.3 % (w/w) of a radically polymerized polyacrylic acid wherein the carboxylic acid groups are neutralized by alkali metals. Polyacrylic acid with a molecular weight (Mw) ranging from 3,500 Da to 15,000 Da and a polydispersity index (PDI = Mw/Mn) ranging from 2.5 to 3.5 were utilised. In general, 90 % of the particles will have an equivalent spherical diameter (esd; as measured by sedimentation using a Sedigraph 5100 series from Micrometrics) of less than 5 µm. Coarse minerals, filler or pigment materials have a particle esd generally in the range of 1 to 5 µm, at which 60% of the particle are < 1µm. Fine mineral materials have a particle esd generally less than 2 µm, at which 90% are < 2µm. For the analysed calcium carbonate slurries the solids content ranged from 50 to 80 % (w/w). The product specifications and the origin regional codes of the investigated Omya products are given in Table 1.

Table 1. Specifications and origin region codes of calcium carbonate slurries.

Trade name	Solids (%)	pH	Particle size distribution	Origin	Code	Biocide in-use
Hydrocarb 90	78	9	90% < 2 µm	Norway	H90-ME 78%	OmyAK
Covercarb 60	72	9.5	60 % < 1 µm	Norway	CC60-ME 72%	OmyAK
Hydrocarb 60	78	9	60% < 2 µm	Norway	H60-ME 78%	OmyAK
Hydrocarb 90	78	9	90% < 2 µm	Austria	H90-GU 78%	Preventol D6 forte
Covercarb 60	72	9.5	60 % < 1 µm	Austria	CC60sw-GU 72%	Preventol D6 forte
Hydrocarb 60	65	9	60% < 2 µm	Austria	H60-GU 65%	Preventol D6 forte
Hydrocarb 90	78	9	90% < 2 µm	Italy	H90-AV 78%	Preventol D7
Hydrocarb 60	78	9	60 % < 1 µm	Italy	H60-AV 72%	Preventol D7
Syncarb 52	52	10	50-65% < 2 µm	Austria	Syncarb-GO 52%	OmyAK

2.1.3 Biocides

The biocide and biocidal formulations used in this study are listed in Table 2.

Table 2. Biocidal compounds and biocidal formulations used in this study.

Trade name	CAS No.	Active compounds	Concentration (% w/w)	Supplier
OmyA	3586-55-8	(Ethylenedioxy)dimethanol	90	Rohm&Haas
OmyAK	3586-55-8	(Ethylenedioxy)dimethanol	85	Rohm&Haas
	2682-20-4	2-Methyl-4-isothiazolin-3-on	0.25	
	26172-55-4	5-Chlor-2-methyl-4-isothiazolin-3-on	0.75	
OmyBK	52-51-7	2-bromo-2-nitro-propane-1,3-diol	14	Rohm&Haas
	2682-20-4	2-Methyl-4-isothiazolin-3-on	0.35	
	26172-55-4	5-Chlor-2-methyl-4-isothiazolin-3-on	1.05	
OmyABK	3586-55-8	Ethylenedioxy)dimethanol	67.5	Rohm&Haas
	52-51-7	2-bromo-2-nitro-propane-1,3-diol	10	
	2682-20-4	2-Methyl-4-isothiazolin-3-on	0.25	
	26172-55-4	5-Chlor-2-methyl-4-isothiazolin-3-on	0.75	
Rocima 609	111-30-8	Glutardialdehyde	16.7	Rohm&Haas
	2634-33-5	1,2-Benzisothiazol-3-on	6.72	
Rocima 610	111-30-8	Glutardialdehyde	22.5	Rohm&Haas
	2682-20-4	2-Methyl-4-isothiazolin-3-on	0.31	
	26172-55-4	5-Chlor-2-methyl-4-isothiazolin-3-on	0.94	
	52-51-7	2-bromo-2-nitro-propane-1,3-diol	3	
Bioban CS-1135	51200-87-4	4,4-dimethyloxazolidine	78	Dow Microbial Control
Preventol GM	111-30-8	Glutardialdehyde	21.5	Lanxess
	2682-20-4	2-Methyl-4-isothiazolin-3-on	1.53	
	52-51-7	2-bromo-2-nitro-propane-1,3-diol	10	
Preventol GDA/IT	111-30-8	Glutardialdehyde	21.5	Lanxess
	2682-20-4	2-Methyl-4-isothiazolin-3-on	0.38	
	26172-55-4	5-Chlor-2-methyl-4-isothiazolin-3-on	1.15	
Preventol D6 forte	3586-55-8	(Ethylenedioxy)dimethanol	85	Lanxess
	2682-20-4	2-Methyl-4-isothiazolin-3-on	0.25	
	26172-55-4	5-Chlor-2-methyl-4-isothiazolin-3-on	0.75	
Preventol OF 45	90-43-7	2-Phenylphenol	45	Lanxess
Preventol D7	2682-20-4	2-Methyl-4-isothiazolin-3-on	0.38	Lanxess
	26172-55-4	5-Chlor-2-methyl-4-isothiazolin-3-on	1.15	

2.1.4 Buffers and Solutions

Alignment Bead Solution

This solution was used to tune the fluorescence detector of the CellFacts II® unit. These BODIPY dye-labelled beads are 2.5-µm in diameter and excite at 633 nm with emission in the range of 645-680 nm (Invitrogen, Cat. No. A-7312).

One drop of AlignFlow™ flow cytometry alignment beads was suspended in 5 ml 0.9% (w/v) saline solution. The solution was made daily.

$DiSC3_{(5)}$ stain solution

This cationic dye (carbocyanine with a short C3 alkyl tail) was utilised to stain the microbial cells for the CellFacts II® analysis.

A 2 mM stock solution was made by dissolving 11 mg of $DiSC3_{(5)}$ in 10 ml DMSO. To prepare a 100 µM working solution, 25 µl stock solution were dissolved in 475 µl DMSO and stored in the absence of light at 4 ºC.

Disruption Buffer

This buffer was used to detach the bacterial cells from the calcium carbonate particles.

Tris(hydroxymethyl)aminomethane (Tris) 1.12 g

Dissolved in 800 ml of 0.9% (w/v) saline solution, the pH adjusted to 8.0 with HCl and made up to 1 litre with 0.9% (w/v) saline solution. The solution was then passed trough a 0.2 µm pore size syringe filter and aliquoted into 50 ml sterile tubes.

Nycodenz® (Histodenz™)

Nycodenz® was used as a non-ionic density gradient medium for the isolation of microorganisms from WMD by centrifugation (Milling*, et al.*, 2005).

13 g of Nycodenz® were dissolved in 10 ml of water by incubation in a water bath at

70 ºC for 1 hour. The solution was then passed through a 0.2 µm pore size syringe filter and stored at 4 ºC.

Propidium monoazide (PMA) stock solution

PMA stock solution (20 mM) was prepared in 20% (v/v) DMSO and stored at -20 ºC in the dark.

Phosphate Buffered Saline (PBS)

This isotonic buffer was used to prepare dilutions of calcium carbonate slurries for the determination of total viable counts. 1 litre of buffer was made as follows:

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.27 g

Dissolved in 800 ml of water, the pH adjusted to 7.4 with HCl and made up to 1 litre with water. The solution was autoclaved at 121 ºC for 15 minutes.

Resuspension Buffer

This buffer was used to resuspend the stained bacterial cells prior to CellFacts II® measurement.

Polyethylene glycol (PEG)	0.8 g

Dissolved in 80 ml 0.9% (w/v) saline, the pH adjusted to 7.5 with 0.2 M phosphate buffer (pH 8.0) and made up to 100 ml. The solution was then passed through a 0.2 µm pore size syringe filter.

SYTO® 62 red fluorescent nucleic acid stain solution

The cell-permeant SYTO® 62 red fluorescent nucleic acid stain was utilised to stain microbial cells for CellFacts II® analysis.

The 500 µM working solution was prepared from the 5 mM stock solution (Invitrogen, Cat. No. S11344). 50 µl of the stock solution were dissolved in 450 µl DMSO and stored in the absence of light at 4 ºC.

2.1.5 Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are summarised in Table 3.

Table 3. Bacterial strains and plasmids.

Species / Name	Genotype / Identifier
Escherichia coli One Shot® TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
pCR®4-TOPO®	LacZα-*ccdB* gene fusion, kanamycin and ampicillin resistance
pUC19	control plasmid, ampicillin resistance

2.1.6 Oligonucleotide Primers

All primers were purchased from Microsynth AG (94.6 Balgach, Switzerland). Unless stated otherwise, all primers were purified using a Reverse Phase HPLC system. Modified primers (FAM) and primers used for deep amplicon sequencing were PAGE purified. The oligonucleotide primers used in this study are listed in Table 4, Table 5 and Table 6.

Table 4. Oligonucleotide primer sequences used for 16S rRNA amplification.

Name	Sequence (5'→ 3')	Target Group	Application	Reference
EUB8m_f	AGAGTTTGATCMTGGCTCAG	universal bacterial domain	16S rRNA T-RFLP Deep amplicon sequencing	(Weisburg, *et al.*, 1991)
EUB338_f	ACTCCTACGGGAGGCAGC	universal bacterial domain	16S rRNA Deep amplicon sequencing	(Amann, *et al.*, 1990)
EUB338_r	GCTGCCTCCCGTAGGAGT	universal bacterial domain	Deep amplicon sequencing	(Amann, *et al.*, 1990)
EUB1088_r	CTCGTTGCGGGACTTAACC	universal bacterial domain	16S rRNA T-RFLP	(Lee, *et al.*, 1993)
EUB515_r	TTACCGCGGCKGCTGGC	universal bacterial domain	16S rRNA Deep amplicon sequencing	(Baker, *et al.*, 2003)
EUB1492_r	GGTTACCTTGTTACGACTT	universal bacterial domain	16S rRNA	(Weisburg, *et al.*, 1991)
Fadh_f	ACGCCGACTTCAACCTGC	Formaldehyde dehydrogenase *Pseudomonas* spp.		This study
Fadh_r	GGTGTGGAAGCTGTGSGA			
Fdm_f	GATGGCCGGTAATAAAAGC	Formaldehyde dismutase *Pseudomonas* spp.		This study
Fdm_r	ACATGCCATGCGGATCGA			
M13 forward	GTAAAACGACGGCCAG	pCR4 plasmids		Invitrogen, Inc.
M13 Reverse	CAGGAAACAGCTATGAC	pCR4 plasmids		Invitrogen, Inc.

Table 5. Oligonucleotide primer sequences used for other genetic marker amplification.

Name	Sequence (5'→ 3')	Target	Reference
Fadh_f	ACGCCGACTTCAACCTGC	Formaldehyde dehydrogenase *Pseudomonas* spp.	This study
Fadh_r	GGTGTGGAAGCTGTGSGA		
Fdm_f	GATGGCCGGTAATAAAAGC	Formaldehyde dismutase *Pseudomonas* spp.	This study
Fdm_r	ACATGCCATGCGGATCGA		
fae1-f	GTCGGCGACGGCAAYGARGTCG	Formaldehyde activating enzyme	(Kalyuzhnaya, *et al.*, 2004)
fae1-r	GTAGTTGWANTYCTGGATCTT		
fae2-f	GCACACATCGACCTSATCATSGG	Formaldehyde activating enzyme	(Kalyuzhnaya, *et al.*, 2004)
fae2-r	CCAGTGRATGAAVACGCCRAC		

Table 6. Oligonucleotide primer sequences used for in qPCR (quantitative PCR)

Name	Sequence (5'→ 3')	Target	Reference
qFadh_f	ACGGAGGTGCCGAGCAT	Formaldehyde dehydrogenase *Pseudomonas* spp.	This study
qFadh_r	GGTCCGTACAGGCCTGGAAT		This study
qFdm_f	TGCTGGAGTGAAGCCAGGTA	Formaldehyde dismutase *Pseudomonas* spp.	This study
qFdm_r	CACACATGCCGCTCCTAACA		This study
qPseud_16S_f	GGTGCAAGCGTTAATCGGAAT	16S rRNA *Pseudomonas* spp.	This study
qPseud_16S_r	TTGGATGCAGTTCCCAGGTT		This study

2.2 General and Analytical Methods

2.2.1 Biocide Dosage

Traditionally, the biocide dosage in WMD is based on the solids content (% w/w) of the product, i.e. a certain amount of biocide or biocidal formulation in ppm denotes mg biocide per kg solids. The biocidal formulations are differentiated between active on dry (a/d) and commodity on dry (c/d). All biocide concentrations based on the solids content refer to a white mineral dispersion with a solid content of 75%. Since in this work WMD with different solids content were used, unless stated otherwise, all biocide concentrations are given relative to the weight of the aqueous phase of a white mineral dispersion with a solid content of 75% (w/w) and analogously as active on liquid (a/l) or commodity on liquid (c/l) relevant to the biocide, i.e. the concentration of biocides in the aqueous phase. In a WMD with 75% (w/w) solids the concentration of biocide in the aqueous phase is three times higher than the concentration relative to the weight of the solids.

2.2.2 Environmental Scanning Electron Microscope

Environmental Scanning Electron Microscope (ESEM) was used to take micrographs from sessile bacteria on calcium carbonate particles. Micrographs were produced from contaminated calcium carbonate slurry samples at the Plymouth Electron Microscopy Centre, University of Plymouth, UK. The following procedure was followed: Samples were fixed with 2.5% (v/v) glutardialdehyde, rinsed with distilled water and centrifuged to concentrate the particles for two minutes at 14,000 rcf on a Sigma 1-12 centrifuge. The pelleted material was air dried, mounted on SEM specimen stubs with double sided tape, gold sputter coated (Emitech K550) and examined in a JEOL 5600LV SEM at 15 kV.

2.2.3 Analytics

The aqueous phase of slurries were obtained by pressure filtration (Fann Instruments filter press series 300, special hardened filter paper 3.500 , retention 2–5 mm, 6 bar) and passed through a Sartorius (8953 Dietikon, Switzerland) 0.2 µm pore size syringe filter (Minisart RC) prior to measurement.

2.2.3.1 High Pressure Liquid Chromatography (HPLC)

HPLC analyses were performed on a Waters 600 System with in-line degasser equipped with a 717 plus autosampler and a 2996 photodiode array detector (Waters AG, 5405 Baden-Dättwil, Switzerland). A Nucleosil 120-5 C18 column 250x4.6 mm of the company Macherey-Nagel (4702 Oensingen, Switzerland) was used.

HPLC parameters for formaldehyde:

Formaldehyde and glutardialdehyde were determined after derivatisation of the slurry aqueous phase with 2,4-dinitrophenyl hydrazine (DNPH) (Bartos and Pesez, 1979, de Andrade*, et al.*, 1999).

Eluent:	Water: Acetonitrile; 50:50 v/v
Flow:	1 ml min^{-1}
Injection quantity:	10 µl
Wavelength:	353 nm
Temperature:	30 °C

HPLC parameters for glutardialdehyde:

Eluent:	Acetonitrile:Potassium dihydrogen phosphate 0.02 M; 60:40 v/v
Flow:	1 ml min^{-1}
Injection quantity:	10 µl
Wavelength:	360 nm
Temperature:	30 °C

HPLC parameters for isothiazolinones:

Eluent:	Water:Methanol; 70:30 v/v
Flow:	1 ml min^{-1}
Injection quantity:	10 µl
Wavelength:	275 nm
Temperature:	30 °C

HPLC parameters for phenolics:

For the measurement of phenolic compounds 20 g slurry are premixed with 30 g water and 5 g 1-propanol. After centrifugation at 13,000 rcf for 10 minutes, the clear solution is injected into the HPLC System.

Eluent:	Water:Methanol; 30:70 v/v
Flow:	1 ml min^{-1}
Injection quantity:	10 µl
Wavelength:	o-phenylphenol (OPP) 245 nm
	pyrocatechol 277 nm
Temperature:	35 °C

2.2.3.2 Ultra Performance Liquid Chromatography (UPLC-MS/MS)

Ultra Performance Liquid Chromatography Mass Spectrometer (UPLC-MS/MS) analyses were performed on an ACQUITY UPLC® System equipped with a Waters Xevo™ TQ MS Detector, a photodiode array detector, a column manager, binary solvent manager and an autosampler (Waters AG, 5405 Baden-Dättwil, Switzerland). The concentration of AMP (2-Amino-2-methyl-1-propanol, CAS No. 124-68-5) was determined as follows:

Sample diluent:	0.1 % (v/v) formic acid

LC-MS/MS Parameters

Column:	AQUITY UPLC® HSS T3 1.8 µm, 2.1 mm x 100 mm
Eluent:	Acetonitrile / Water / Formic Acid = 10:90:0.1 v/v
Flow:	0.4 ml min^{-1}
Injection quantity:	1 µl
Column temperature:	40°C

MS-Tune

Capillary voltages:	3.00 kV
Cone voltages:	16 V

Temperature:	350 ºC (desolvation)
Gas flow:	650 l h^{-1} (desolvation)
Cone flow:	30 l h^{-1}
MRM Mode:	90.05 --> 55.03, Time 0.00 to 2.00 min, ESI+

2.2.3.3 Ion Chromatography (IC)

Ion concentration in the calcium carbonate slurry aqueous phase was determined at room temperature by means of a Metrohm (4800 Zofingen, Switzerland) 882 Compact IC plus system with autosampler.

IC parameters for cations:

Column:	Metrosep C4 150/4.0
Eluent:	1.7 M HNO_3, 0.7 M DPA (Dipicolinic acid)
Flow:	0.9 ml min^{-1}
Injection quantity:	10 µl

IC parameters for anions (incl. formic acid):

Column:	Metrosep A Supp 5 150/4.0
Eluent:	1 M $NaHCO_3$, 3.2 M Na_2CO_3
Flow:	0.7 ml min^{-1}
Injection quantity:	20 µl

2.2.3.4 Optical Emission Spectrometry (OES)

Total ion concentration (whole calcium carbonate slurry) was determined by means of an OPTIMA 3200XL Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) system from Perkin Elmer (8603 Schwerzenbach, Switzerland). Firstly, the water of the calcium carbonate slurry samples was removed by drying the sample at 120 ºC. 8 ml of 69% v/v HNO_3 was then added to 2 g of dried sample and boiled for 5 minutes to carry out digestion. Finally, the boiled sample was cooled to 20 ºC and adjusted to 100 ml with ultrapure water (Milli-Q® integral water purification System). The lithium ions were detected in the emission line at wavelengths of 610.35 nm and 670.76 nm.

2.3 Microbiological Methods

2.3.1 Preparation of Culture Media

All culture media were sterilised by autoclaving at 121 ºC for 15 minutes.

Luria Bertani Broth (LB)

Escherichia coli and various WMD bacteria isolates were cultured in this medium. The medium contained the following compounds per litre water (pH 7.0, 25 ºC):

Tryptone (pancreatic digest of casein)	10 g
Yeast extract	5 g
NaCl	5 g

Luria Bertani Agar (LBA)

Luria Bertani Broth (LB) supplemented with 15 g l^{-1} Agar.

Mueller-Hinton Agar (MHA)

This medium, supplemented with biocide, was used for the isolation of biocide-resistant WMD bacteria. The medium contained the following compounds per litre water (pH 7.3 ± 0.1, 25 ºC):

Beef Extract Powder	2.0 g
Acid Digest of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g

Nutrient Broth (NB)

This medium was used for the cultivation of various type strains and WMD bacteria isolates. The medium contained the following compounds per litre water (pH 6.8 ± 0.2, 25 ºC):

Beef Extract	3 g
Peptone	5 g

Plate Count Agar (PCA)

This medium was used as non-selective medium for the determination of total viable counts. The medium contained the following compounds per litre water (pH 7.0 ± 0.2, 25 ºC):

Pancreatic Digest of Casein	5.0 g
Yeast Extract	2.5 g
Dextrose	1.0 g

Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA)

This medium was used for the cultivation of various type strains and WMD bacteria isolates. The medium contained the following compounds per litre water (pH 7.3 ± 0.2, 25 ºC):

Casein peptone (pancreatic)	17 g
Dipotassium hydrogen phosphate	2.5 g
Glucose	2.5 g
Sodium chloride	5 g
Soya peptone (papain digest.)	3 g

TSA: TSB supplemented with 15 g l^{-1} Agar prior to sterilisation.

2.3.2 Cultivation Conditions

Unless stated otherwise, all cultures were incubated aerobically at 30 ºC. Liquid cultures were incubated with shaking at 180 rpm on an orbital shaker. The exception was *Escherichia coli* cultures which were incubated at 37 ºC.

2.3.3 Maintenance of Cultures

Stock cultures of type strains and isolated bacteria were stored as follows:

a) short term: actual cultivation broth at 4 ºC.
b) mid to long term at - 80 ºC: broth culture supplemented with 15% (v/v) glycerol or slurry culture supplemented with 25% (v/v) glycerol.

2.3.4 Total Viable Count (TVC)

The direct plate counting method was used to determine the total viable count of bacteria cultures and calcium carbonate slurries. Samples were diluted in PBS and 100 µl of appropriate dilutions spread on nutrient agar plates using a sterile spreader. Plates were incubated at 30 ºC for 24 hours to 7 days.

2.3.5 Absorbance of Bacterial Suspensions

Absorbance at 600 nm wavelength was used to estimate the microbial concentration and to calculate the growth rate of bacteria suspensions. The absorbance was measured using a BioPhotometer® from Vaudaux-Eppendorf (4124 Schönenbuch, Switzerland).

2.4 Assessment of the Minimal Inhibitory Concentration (MIC)

2.4.1 Preservation – Challenge Testing

Challenge testing was used to determine the preservation effectiveness of antimicrobial compounds in WMD (Russell, 2003b). WMD bacteria cultures utilised for the determination of the antimicrobial activity of biocides are batch culture which have been standardised by incubation time.

50 g aliquots of sterilised white mineral dispersion were each supplemented with different amounts of biocidal compound or biocide formulation. The samples were then incubated for 3 days at 30 °C. Subsequently the samples were inoculated with 1 ml contaminated WMD culture (inherently mono or mixed bacterial species), which had previously been cultivated in WMD for 48 h at 30 ºC (>10^5 cfu ml^{-1}). After 24 h to 3 days of incubation at 30 °C the total viable count was determined using the plate count method. Samples that did not show any count (<100 cfu ml^{-1}) were re-inoculated with 1ml of the relevant WMD culture. There were no more than three inoculations performed. Cell counts greater than 10^4 cfu ml^{-1} were evaluated qualitatively by estimating the order of magnitude (10^5, 10^6 or higher). All analyses have been repeated two times with high reproducibility. Further details are given in section 4.5.2.1.

2.4.2 Curative – Disinfection Testing

The curative approach was used to determine the disinfection effectiveness of antimicrobial compounds in WMD.

Different amounts of biocidal compound or biocide formulation were added to aliquots of 50 g contaminated WMD culture (inherently mono or mixed bacterial species, $>10^5$ cfu ml^{-1}) respectively. The samples were stored at 30 °C and then the total viable count was determined using the plate count method after 24 h, 2 d and 3 d. Cell counts greater than 10^4 cfu ml^{-1} were evaluated qualitatively by estimating the order of magnitude (10^5, 10^6 or higher). All analyses have been repeated two times with high reproducibility. Further details are given in section 5.2.2.2.

2.5 CellFacts II® Analysis of WMD

Total particle counts i.e. total cell counts per ml (TCC ml^{-1}) were obtained using a CellFacts II® analyzer (CellFacts Instruments, Coventry CV4 7HS, UK). CellFacts II® combines electrical flow impedance (particle counter) and fluorescence intensity to determine cell counts and membrane potential (physiological state) (Figure 6).

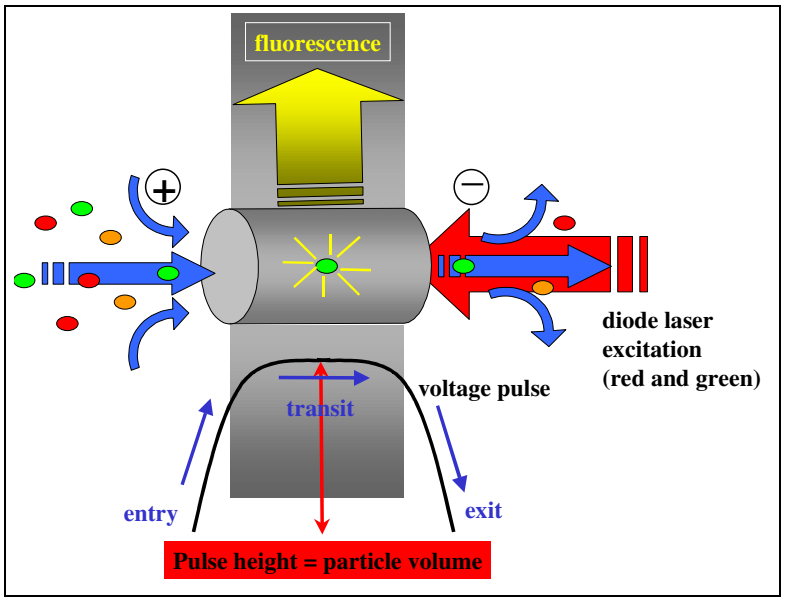


Figure 6. Schematic representation of the CellFacts II® measurement principle.

The impedance change is measured as a particle, i.e. a cell passes trough the orifice (30 µm) and the generated volume displacement of electrolyte is equivalent to the volume of the cell. A double dye system is used and the fluorescence data are acquired by exciting the samples at 635 nm (± 7 nm) via a FiberTec™ 0635 red laser diode module (25 mW) and detection via a photomultiplier at 660 nm. On the one hand, the cell-permeant SYTO62 red fluorescent nucleic acid stain diffuses through the bacterial membrane and exhibits fluorescence upon binding to nucleic acids. On the other hand, the membrane potential-sensitive probe $DiSC_3(5)$ accumulates on hyperpolarised membranes and intercalates with the membrane only if an electrical charge is present (Figure 7).

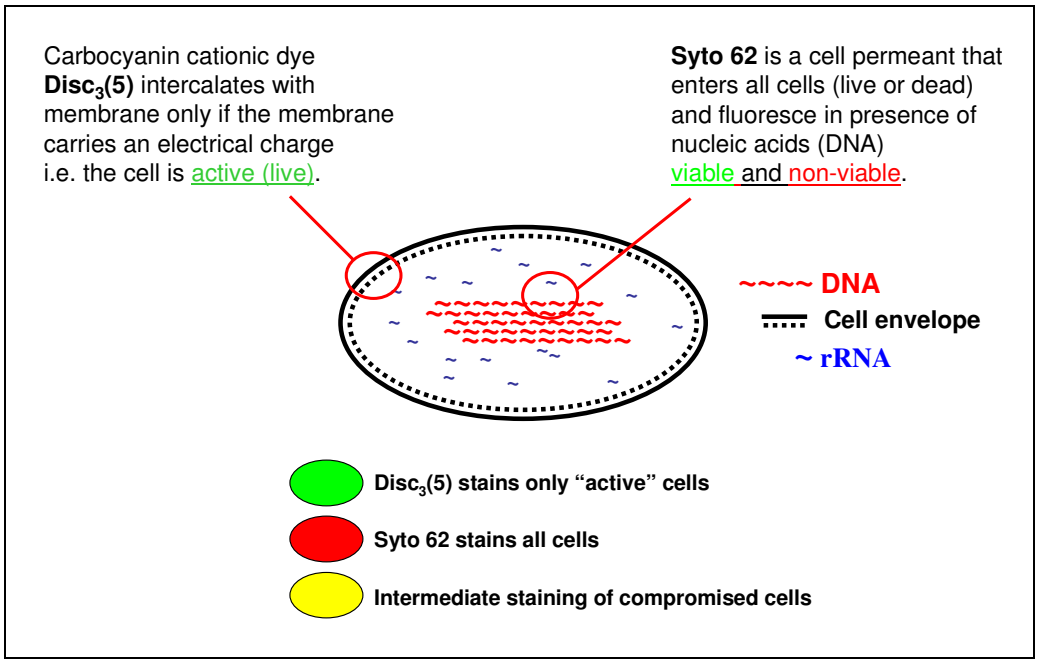


Figure 7. Double dye staining of bacterial cells to assess the membrane potential by CellFacts II®.

WMD samples were analysed as specified by the manufacturer and described elsewhere (Gabbianelli, *et al.*, 2003, Gentelet, *et al.*, 2001, Schwarzentruber, 2003b, Schwarzentruber and Gane, 2005). The computer software CellFacts II® viewer (CFII viewer) was used to process the raw data and calculate total cell number, average cell size and average fluorescence values of the analysed bacterial populations.

2.5.1 Calcium Carbonate Slurry

1 ml of WMD sample was added to 4 ml disruption buffer and mixed for 3 minutes using a vortex agitator. 1 ml of the diluted slurry was layered on the top of 300 µl Nycodenz solution and centrifuged for 6 minutes at 10,000 rcf using the soft key for gentle acceleration and deceleration. After centrifugation, the upper aqueous layer (approx. 1 ml) containing the bacterial cells was transferred to a clean tube containing 5 µl EDTA (10 mM), followed by short mixing and incubation of 5 minutes at RT. Then, 5 µl of 100 µM $DiSC_3(5)$ and 2 µl of 500 µM SYTO62 were added into the tube, mixed well and incubated for 30 minutes at RT protected from light. Nycodenz residues and excess dye were removed by centrifugation of the sample for 3 minutes at 10,000 rcf. The cell pellet was then resuspended in 1 ml resuspension buffer and analysed using the CellFacts II® instrument.

2.5.2 Bacteria Cultures

An aliquot of bacterial suspension was diluted with disruption buffer appropriately and cells were harvested by centrifugation for 3 minutes at 10,000 rcf. The cell pellet was resuspended in 1 ml resuspension buffer and 5 µl 10 mM EDTA were added, followed by short mixing and incubation of 5 minutes at RT. Then, 5 µl of 100 µM $DiSC_3(5)$ and 2 µl of 500 µM SYTO62 were added, mixed well and incubated for 30 minutes at RT protected from light. Excess dye was removed by centrifugation of the sample for 3 minutes at 10'000 rcf. The cell pellet was then resuspended in 1 ml resuspension buffer and analysed using the CellFacts II® instrument.

2.6 WMD analysis by Flow Cytometry

All flow cytometric analyses were carried out using a FACSAria™ flow cytometer from Becton & Dickinson. This flow cytometer is equipped with 3 solid state lasers, 9 fluorescent photomultiplier (PMT) detectors, 2 light scatter detectors, an aerosol management system and a high-speed sorter with a fixed-alignment cuvette flow cell. Samples were illuminated with a solid state 488 nm laser and the fluorescence was detected via 530/30 (green) and 610/20 (red) band pass filters in channel FL1 and FL3, respectively.

2.6.1 LIVE/DEAD® BacLight™ Bacterial Viability Kit

This fluorescence-based assay was used to determine bacterial cell viability by flow cytometry. This Kit (Invitrogen, Cat. No. L34856) uses the nucleic acid stains SYTO9 and Propidium Iodide (PI). The membrane-permeant SYTO9 stains bacteria with both intact and damaged membranes. PI is a membrane impermeant and generally excluded from viable cells. When both dyes are present, SYTO9 and PI compete for nucleic acid binding sites and produce green fluorescent staining of bacteria with intact cell membranes (live) and red fluorescent staining of bacteria with damaged membranes (dead). Intermediate fluorescent cells that fall outside the green and red fluorescence are referred to as cells with unknown viability or cells with a compromised membrane.

Bacterial cells from calcium carbonate slurries were separated from carbonate particles by density gradient centrifugation as described in section 2.5.1. Quadruple cell pellet were pooled and resuspended in 1 ml of disruption buffer. 5 µl of 10 mM Na-EDTA were added, followed by short mixing and incubation for 5 min at RT. Then, 1.5 µl SYTO9 and PI were added, mixed and incubated for 15 minutes at RT protected from light. Signals were acquired at a maximum speed of 1000 events per second and sheath flow rate of 0.8 – 1.2 µl min^{-1} with the amplifiers set to logarithmic amplification (log_{10} 10^4) and the trigger on the side scatter signal. Electronic compensation of the fluorescence was not used. The gains of forward scatter (FSC), side scatter (SSC) and fluorescence channels were used to adjust bacterial population position in the data plot and to minimise electronic noise. For the alignment and calibration of the laser and for daily quality control with standard settings, 1µm fluorescent calibration beads from Partec (48161 Münster, Germany; Cat. No. 05-4007) were used. Data were processed with the FACSDiva™ software and regions of interest were defined to exclude electrical noise and to gate the fluorescence parameters.

2.6.2 Fluorescence Activated Cell Sorting (FACS)

Live, dead and compromised WMD bacterial cells were sorted and then subjected to further analysis. Bacterial cells from calcium carbonate slurries were prepared for flow cytometric analysis as described above in section 2.6.1. Gates were set based on

the fluorescence value of FL1 (green) vs. FL3 (red) respectively, and related to live, dead and compromised cells. Bacterial populations in each gate were sorted simultaneously and a minimum of 50,000 total counts were acquired. The sorted cells were aseptically collected and supplemented with 1 ml sterile PBS. Sorted cells were harvested by centrifugation for 3 minutes at 10,000 rcf and stored at -80 ºC for further analysis.

2.7 Molecular Biology Techniques

2.7.1 Extraction of Nucleic Acids

DNA Extraction from White Mineral Dispersions

Genomic DNA (gDNA) was isolated from calcium carbonate slurries using the PowerSoil™ DNA Isolation Kit from MO BIO Laboratories. This Kit is suitable for isolating gDNA from various environmental samples (Feinstein*, et al.*, 2009, Francy*, et al.*, 2009). 750 µl of calcium carbonate slurry were taken as starting material, and isolation was performed as described in the standard protocol provided by the manufacturer. The extracted gDNA was stored at -21 ºC.

DNA Extraction from Liquid Cultures

Genomic bacterial DNA was extracted from 1 ml liquid culture using a DNeasy Blood & Tissue Kit from Qiagen according to the manufacturer's instructions. The extracted DNA was stored at -21 ºC.

DNA Extraction from Bacterial Colonies

DNA extraction from bacterial colonies was performed by means of heat. A single bacteria colony was resuspended in 50 µl PBS using a sterile disposable plastic loop. After heating the sample for 10 minutes at 95 ºC to lyse the bacterial cells, cell debris were removed by centrifugation at 10,000 rcf for 3 minutes. The supernatant was stored at -21 ºC.

2.7.2 Polymerase Chain Reaction (PCR)

All polymerase chain reactions were carried out on an iCycler™ thermal cycler from Bio-Rad.

2.7.2.1 Amplification of the 16S rRNA Gene

Phylogenetic analysis was performed by amplification of the partial 16S rRNA gene region using the bacterial universal primer pair EUB338_f and EUB1088_r. 12.5 µl of Qiagen HotStarTaq® Master Mix, 1 µl of each primer (10 µM), 1 µl of the extracted DNA and 9.5 µl of nuclease-free water were used for amplification. Cycling parameters included an initial denaturation for 15 minutes at 95 ºC, 30 cycles of 45 seconds at 94 ºC, 45 seconds at 56 ºC and 1 minute at 72 ºC, and a final extension for 10 minutes at 72 ºC. Sequence analysis of the 16S rRNA gene fragments was performed as described in section 2.7.6.

2.7.2.2 Amplification of the *fae* Gene

Recognition of the formaldehyde activating enzyme gene *fae* was performed by a two-step PCR as described elsewhere (Kalyuzhnaya*, et al.*, 2004, Nercessian*, et al.*, 2005b, Vorholt*, et al.*, 2000). In the first step, pre-amplification was carried out using the fae1-f/fae1-r primer pair. 12.5 µl of Qiagen HotStarTaq Mastermix, 0.1 µM of the fae1 primer pair and 100 ng of template DNA were adjusted with H_2O to a final volume of 25 µl. The annealing temperature of the pre-amplification was 45 ºC for 24 cycles. In the second step, the fae2-f/fae2-r primer pair was used. 12.5 µl of Qiagen HotStarTaq Mastermix, 0.1 µM of the fae2 primer pair and 10 µl of the PCR from step 1 were adjusted with H_2O to a final volume of 25 µl. The annealing temperature in the second step was 56 ºC for 30 cycles. Sequence analysis of the *fae* gene fragments was performed as described in 2.7.6.

2.7.3 Quantification of Bacterial Gene Expression

2.7.3.1 Isolation of RNA from Bacteria Cultures

RNA was isolated from bacteria cultures using a Qiagen RNeasy™ Protect Bacteria Mini Kit as described in the manufacturer's handbook. 1 ml of bacteria cultures was

added to 2 ml Qiagen RNAprotect™ Bacteria Reagent in order to stabilise the RNA. Enzymatic lysis of the bacteria was followed by purification of total RNA from the bacterial lysate using the Qiagen RNeasy® Mini Kit. To guarantee elimination of residual DNA from the sample, on-column DNase digestion was carried out using the Qiagen RNase-Free DNase Set. After isolation, RNA was stored at -80 ºC.

2.7.3.2 RT-PCR (Reverse Transcription PCR)

Reverse transcription of the RNA was performed by using random nonamer primer d(N)9 with the Qiagen Sensiscript RT Kit. Complementary DNA (cDNA) is first synthesized by reverse transcription and afterward an aliquot of the finished reverse-transcription reaction is used for qPCR. The cDNA reverse-transcription reaction was performed following the manufacturer's protocol utilising a primer concentration of 0.5 µM and 2 µl (< 50 ng) of template RNA.

2.7.3.3 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative Polymerase Chain Reaction (qPCR) was used to quantify gene copy number. All qPCR investigations were performed on a StepOne™ Real-Time PCR System from Applied Biosystems using the SYBR®-Green based detection chemistry and a two step strategy. Real-time PCR analysis was performed with the Power SYBR® Green PCR Master Mix from Applied Biosystems in 20 µl reactions comprising 0.2 nM of each primer and 2 µl of template from the RT-PCR reaction. The 16S rRNA gene was used to normalise the data and the relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ comparative C_t method (Rogers*, et al.*, 2007, Tasara and Stephan, 2007). To calculate the $\Delta\Delta C_t$ value, the C_t value of the calibrator (16S rRNA gene) was subtracted from the C_t value of the gene of interest (for each treatment) and followed by the subtraction of the C_t value of the untreated sample from the treated sample.

2.7.4 Propidium Monoazide PCR Analysis

Bacterial cells were isolated from calcium carbonate slurries as described for the CellFacts II® analysis in section 2.5.1 in duplicate. The duplicates of Nycodenz isolated cells were pooled, centrifuged at 10,000 rcf for 3 minutes and the pellet

resuspended in 500 µl PBS. PMA treatment and cross-linking was performed as described by Nocker *et al.* (2007a). 1.25 µl of propidium monoazide (PMA) was added to the 500 µl cell suspension to achieve a final concentration of 50 µM, followed by mixing and incubation for 5 minutes in the dark. After the incubation, tubes were placed horizontally on ice (to avoid excessive heating) about 20 cm from the light source and light exposed for 2 minutes using a 500 W halogen light source to cross-link the PMA to the DNA. Afterwards, cells were pelleted at 10,000 rcf for 3 minutes and DNA isolation was performed by means of the DNeasy Blood & Tissue Kit from Qiagen as described in the manufacturer's handbook.

2.7.5 DNA Electrophoresis

The Agilent 2100 Bioanalyzer is a microfluidics-based lab-on-a-chip platform for sizing, quantification and quality control of DNA and was used to analyse PCR products. The Bioanalyzer was operated according to the manufacturer's instructions.

2.7.6 Clone Libraries Construction

Both, 16S rRNA and genetic marker (e.g. *fae, fadh, fdm* etc.) PCR fragments were ligated into a plasmid vector prior to sequencing.

2.7.6.1 Cloning of PCR Products

PCR products were cloned by means of the TOPO® TA Cloning Kit for Sequencing from Invitrogen. PCR products were firstly purified with the Qiagen QIAquick PCR purification kit and used in the cloning reaction following a slightly modified manufacturer's protocol. 4 µl purified PCR product were used in the cloning reaction and the plasmid was then transformed into chemically competent *Escherichia coli* One Shot® TOP10 cells. As the plasmid vector pCR®4-TOPO® contains the lethal *ccd*B gene fused to the C-terminus of the LacZα fragment, the inserted DNA fragment disrupts the expression of the fusion protein and permits growth of only positive recombinants. Therefore, cells that contain plasmid without insert are not able to growth on LB plates containing 100 µg ml^{-1} ampicillin. Transformation efficiency was confirmed by setting up an additional reaction with the pUC19 control plasmid. Single colonies were then inoculated into LB medium supplemented with

100 µg ml^{-1} ampicillin and incubated at 37 ºC with shaking at 180 rpm overnight.

2.7.6.2 Plasmid Preparation

Overnight cultures of the clones from 2.7.6.1 were used for plasmid preparation. Plasmid was extracted using the Qiagen Plasmid Mini Kit according to the manufacture's protocol.

2.7.6.3 Sequencing of the Cloned DNA Fragments

The insert was sequenced by using the vector specific M13 forward and/or M13 reverse primer with BigDye® v3.1 chemistry from Applied Biosystems according to the manufacturer's protocol. Sequences were acquired on an automated genetic analyzer ABI 3130 System from Applied Biosystems and analysed by means of the Sequencing Analysis Software v5.3.1 with KB™ Basecaller v3.1 as specified by the manufacturer. Acquired sequences were compared in the GenBank database of nucleotide sequences (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (blastn). Additionally, 16S rRNA identification of bacterial species was confirmed by classification of the sequences in the Classifier RDPII Analysis Tool (Ribosomal Database Project II, http:// rdp.cme.msu.edu/). A phylogenetic tree of 16S rRNA sequences was constructed using the neighbour-joining method (Maximum Composite Likelihood) in MEGA 4 (Molecular Evolutionary Genetics Analysis) software (Kumar*, et al.*, 2008). Statistical significance was determined by performing bootstrap analyses by the neighbour-joining method (1000 data resamplings).

2.7.7 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP was used to generate a fingerprinting of a bacterial community in WMD.

2.7.7.1 Generation of T-RFs Profiles

T-RFLP analysis was performed via amplification of approx. 1000 bps of the 16S rRNA gene by PCR using bacterial universal primers. The forward primer FAM-EUB8m_f fluorescently labelled on the 5' end with 6-Carboxyfluorescein (6-FAM) was used in combination with the reverse primer EUB1088_r.

25 µl of Qiagen HotStarTaq Master Mix, 2 µl of each primer (10 µM) and 5-10 µl (ca. 20-30 ng) of the extracted DNA were adjusted with H_2O to a final volume of 50 µl. Cycling parameters included an initial denaturation for 15 minutes at 95 ºC, 30 cycles of 45 seconds at 94 ºC, 45 seconds at 54 ºC and 2.5 minutes at 72 ºC, and a final extension for 30 minutes at 72 ºC. Two independent 50 µl PCR reactions were performed for each sample and the products were combined prior to purification with the Qiagen QIAquick PCR purification kit.

For each sample 150-200 ng of the purified fluorescently labelled PCR product was digested for 3 h with 2 units of *Msp*I restriction endonuclease in a 50 µl reaction volume. After inactivation of the enzyme at 80 ºC for 20 minutes, the digested samples were purified using a Qiagen QIAquick Nucleotide Removal Kit. Thereafter, 2 µl (approx. 2 ng) of the purified Terminal Fragments (T-RFs) and 0.5 µl MapMarker®1000 X-Rhodamine labelled size standard (BioVentures) were adjusted with highly deionised formamide (Hi-Di™ formamide, Applied Biosystems) to a final volume of 20 µl and denatured at 95 ºC for 5 minutes. Fragment analysis was performed on an automated genetic analyzer ABI 3130 System from Applied Biosystems as specified by the manufacturer.

2.7.7.2 Analysis of T-RFs Profiles

T-RFs with sizes from between 50 and 1000 bps and peak heights of ≥ 25 relative fluorescence units (rfu) were analysed using the GeneMapper® v4.0 Analysis Software v2.1 and fragments that differed by less than 2 bp (base pair) were combined as they were considered to be identical. Standardisation of the DNA quantity between different samples was performed using the iterative standardisation procedure (Dunbar*, et al.*, 2001). The total DNA quantity in each profile was estimated by calculating the sum of all peak heights ≥ 25 rfu. The quotient of the profile with the lowest DNA quantity and the profile with the largest DNA quantity were used as correction factors. The DNA quantity between all profiles was standardised by multiplying each peak height in a profile with the correction factor, therefore peaks from profiles with larger DNA and close to the 25 rfu threshold often fell below the threshold and were consequently eliminated. After the first standardisation cycle, the sum of peak heights ≥ 25 rfu was recalculated and the

standardisation procedure was repeated by iteration of the sum of the peak heights, thus the DNA quantity of all profile was equal. Additionally, the relative abundance of T-RFs in a profile was calculated by dividing each peak height by the sum of all peak heights in a profile. A few T-RFs with a peak height less than 1% were not considered for further analysis, because they represented a negligible quantity of the total T-RFs from each profile.

The standardised abundance data were transformed to a Bray-Curtis distance matrix and used to perform nearest neighbor Cluster Analysis (CA) in BioDiversity Professional software (McAleece, *et al.*, 1997). Jaccard distance was used for Principal Component Analysis (PCAs) carried out by means of the PAST analysis package (Hammer, *et al.*, 2001, Vazquez, *et al.*, 2009). PCAs is a mathematical method that reduces large numbers of variables into principal components with the aim of visualising the relationships between the investigated data (Rudi, *et al.*, 2007). Analysis of Variance (ANOVA: single factor) or Student's t-test (paired two sample for means) was used to explain statistical significance and computed using OriginPro v8. (OriginLab, Northampton, MA 01060, USA).

2.7.8 Deep Amplicon Sequencing of 16S rRNA Fragment Libraries

Sequencing-by-synthesis makes use of polyphosphates (PP_i) released by the DNA polymerase during the DNA synthesis. The released PP_i is then used to produce ATP by the ATP sulfurylase in the presence of 5'phosphosulfate (APS). Finally, a charge coupled device (CCD) camera collects the light signal emitted once the ATP is used by the luciferase to convert luciferin to oxiluciferin. The light signal is proportional to the number of incorporated nucleotides in a single nucleotide flow. Deep amplicon sequencing requires special fusion primers (Figure 8). The fusion primer consists of a 20-25 bp target-specific sequence (3' end) and a 19 bp fixed sequence (Primer A or Primer B on the 5' end). The primer A and B are required to bind to the DNA capture beads utilised in the emulsion PCR:

Primer A: 5' GCCTCCCTCGCGCCATCAG 3'
Primer B: 5' GCCTTGCCAGCCCGCTCAG 3'

The sequencing key (TCAG) is used by the software for base calling and to identify valid reads.

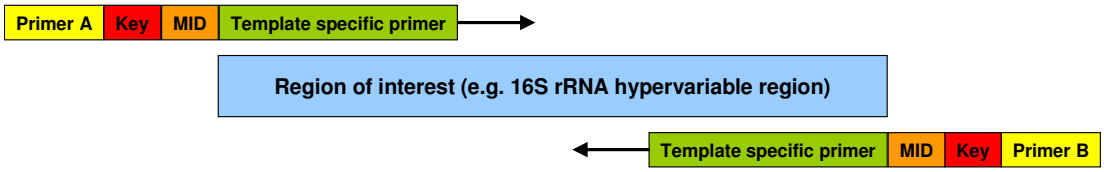


Figure 8. Schematic representation of the configuration of fusion primers (454 Sequencing Technical Bulletin No. 013-2009, www.454.com).

In addition, between the primer A or B and the template specific primer a tag (also referred to as the barcode) is integrated. Each sample (library) is tagged with a unique tag consisting of a 10 base tag referred to as the multiplex identifier (MID) and this tag is used to assign the reads to a specific amplicon library after pooling/multiplexing (Figure 9). A list of applicable MIDs is provided by Roche (454 Life Sciences enter of excellence; http://www.454.com).

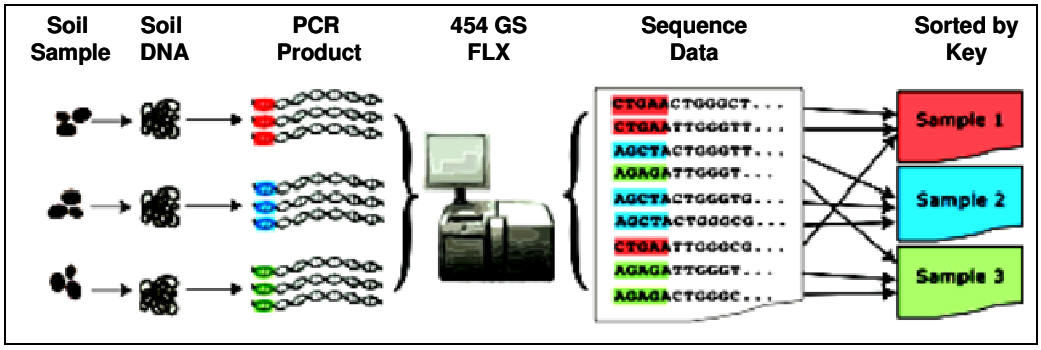


Figure 9. Schematic representation of a multiplex FLX rRNA gene sequencing procedure (source: http://pyro.cme.msu.edu/pyro/help.jsp).

2.7.8.1 Preparation of Amplicon Libraries

Amplicon libraries were generated as recommended by Roche in the 454 Life Sciences Genome Sequencer System manual and as described by other authors (Dethlefsen, *et al.*, 2008, Lauber, *et al.*, 2009). 16S rRNA fragment libraries were obtained by amplifying two partial 16S rRNA gene regions by means of the fusion primers. Fusion primers were generated from the bacterial universal primer pair

EUB8m_f and EUB338_r to amplify 16S rRNA hypervariable regions V1 and V2, whilst the hypervariable region V3 was covered by the combination of the primers EUB338_f and EUB515_r.

25 µl of Qiagen HotStarTaq Master Mix, 2 µl of each primer (10 µM) and 5-10 µl (ca. 20-30 ng) of the extracted DNA were adjusted with H_2O to a final volume of 50 µl. Cycling parameters included an initial denaturation for 15 minutes at 95 ºC, 30 cycles of 45 seconds at 94 ºC, 45 seconds at 52 ºC and 1 minute at 72 ºC, and a final extension for 10 minutes at 72 ºC. Two independent 50 µl PCR reactions were performed for each sample and the products were pooled prior to purification with the Qiagen QIAquick PCR purification kit. The purified samples were sent to Microsynth AG (9436 Balgach, Switzerland) where the samples were pooled in equimolar amounts, immobilised onto DNA capture beads, amplified by emulsion-based clonal amplification and analysed on the Roche 454 Genome Sequencer FLX system (Margulies*, et al.*, 2005).

2.7.8.2 Processing and Analysis of the Pyrosequencing Data

Signal processing was performed using the Roche 454 Genome Sequencer FLX system software. The standard PicoTiterPlate chemistry used for this study allows a read length of approx. 250 bases, according to the manufacturer's instructions (Roche, 6343 Rotkreuz, Switzerland). Low quality sequences were removed on the basis of sequence length, recognition of the tag sequence and on the sequence quality score. Sequences were analysed using the tools provided in the pyrosequencing analysis pipeline at the Ribosomal Database Project (RDP II) website (Cole*, et al.*, 2009). Firstly, to perform taxonomy-based analyses the assignment of the taxonomy was brought about using the naïve Bayesian rRNA classifier (Wang*, et al.*, 2007) with a bootstrap confidence threshold of 60% (Jones*, et al.*, 2009) which has been shown to provide fast and reliable classification of short sequence reads (Cole*, et al.*, 2009). Secondly, sequence batches from one sample sharing the same tag were aligned using the fast, secondary-structure aware "infernal" aligner (Nawrocki and Eddy, 2007) and complete clustering of the sequences was performed by means of the complete-linkage clustering method. The cluster files were used to perform taxonomy-dependent analyses by clustering the reads in OTUs used to calculate

ecological metrics such as the coverage of the libraries. Representative sequences among all investigated libraries were selected by combining all sequence reads to one single alignment, followed by the dereplication of the data based on the cluster analysis at 3% sequence divergence. Analysis of variance (ANOVA with Tukey's pairwise comparison) used to explain a statistical significance in the variation of taxonomy groups between samples was computed using OriginPro v8. (OriginLab, Northampton, MA 01060, USA).

2.8 Various Techniques for Assessing Biocide Influence on Bacteria

2.8.1 Leakage of Cellular Constituents

Bacteria were grown in Tryptic Soy Broth (TSB) at 30 ºC and 160 rpm on an orbital shaker overnight. Cells were harvested by centrifugation (5000 rcf for 10 min.), washed twice with 10 mM HEPES buffer pH 7.4 (Denyer, *et al.*, 1986) and resuspended in HEPES buffer to achieve a cell density of $5 \cdot 10^8$ to 10^9 cells ml^{-1}. The HEPES buffer-cell suspension was then supplemented with biocide, other compounds or a combination thereof and analysed at 0, 30 and 60 minutes after the respective treatments. Two biological replicates and two technical replicates of all samples were analysed.

The absorbance measurements to monitor refractive changes at 600 nm of the cell suspension and the leakage of cell constituents at 260 nm were performed using a Vaudaux-Eppendorf (Basel, Switzerland) Bio-Photometer. Prior to A_{260} analysis and determination of the potassium ion concentration, the suspension was passed through a Sartorius (Dietikon, Switzerland) 0.2 µm pore size syringe filter to remove the cells and the supernatant was immediately analysed.

The potassium ion concentration was measured by means of inductively coupled plasma optical emission spectrometry (ICP-OES) of the filtrate. Potassium ions were detected in the emission line at a wavelength 766.47 nm as described in 2.2.3.4.

2.8.2 NPN uptake assay

The 1-N-phenylnapthylamine (NPN) uptake assay was performed as described by (Helander and Mattila-Sandholm, 2000). Fluorescence was detected using a Synergy™ HT microplate reader (Biotek Instruments Inc., Winooski, VT 05404, USA). Wells were read from the bottom with a sensitivity value of 70 and filter settings were 360/40 nm for the excitation and 420/50 nm for the emission. Bacterial cells were prepared as described in section 2.8.1 and resuspended in 5 mM HEPES pH 7.2. The NPN stock solution (0.5 mM) was prepared in acetone. For each substance, 16 replicates (wells) in a 96 microtitre plate were analysed.

50 µl NPN (40 µM, diluted in 5 mM HEPES pH 7.2) and 25 µl of each substance (in HEPES buffer; 8x higher in order to achieve the desired final concentration in the well) were adjusted to a total volume of 100 µl with HEPES buffer if required. Just before measurement, 100 µl of bacterial suspension was added to the wells, the plate briefly shaken for 15 sec., and fluorescence values recorded within 5 minutes. Controls included buffer alone (200 µl), buffer (100 µl) and bacterial suspension, buffer (150 µl) and NPN (50 µl) as well as buffer (50 µl), NPN (50 µl) and bacterial suspension (100 µl). The relative fluorescence unit value (rfu) was calculated as follows: rfu = (Cells + Test Substance + NPN) - (Cells + NPN). The NPN factor was calculated as the ratio of the rfu value of the substance(s) to the rfu value of the NPN supplemented buffer background [(Buffer+NPN)-(Buffer)]. Statistically significant changes of the NPN uptake factor were determined by the student t-test (unpaired, equal variance) based on the untreated sample (buffer only).

2.8.3 Uptake of Biocides and Enhancer Compounds

Bacterial cells were prepared in HEPES buffer as described in section 2.8.1. To the cell suspension biocide and enhancer compound were added individually, or in combination. Samples were removed at 0, 30 and 60 min after the addition of the compounds and passed through a Sartorius (Dietikon, Switzerland) 0.2 µm pore size syringe filter. The cell-free supernatant was used to determine the residual formaldehyde, lithium or AMP concentrations as described in 2.2.3. Two biological replicates and two technical replicates of all samples were analysed.

CHAPTER 3

Microbial Diversity of White Mineral Dispersions

3.1 Introduction

Culture-based methods, such as viable count enumeration of bacteria on solid media, are still the most accepted method for the determination of viable bacteria in WMD. Whilst for the basic industrial monitoring at the Omya plants such methods are often sufficient to detect emerging bacterial contaminants, the traditional plating technique misses the unculturable fraction of microorganisms present in most environmental habitats. However, the enumeration of bacteria by the standard plate count method is a time-consuming process that requires 24 to 72 hours of incubation to obtain initial results. In addition prolonged incubation times are required as a consequence of variable growth conditions related to the physiological state of the cells as well as to the growth kinetics of the microbial species present in the WMD sample.

The role of individual bacteria species within a mixed population inhabiting an environmental sample such as calcium carbonate slurries can only be understood by the assessment of their *in situ* metabolic activity. It is well accepted that growth on a nutrient rich medium under laboratory conditions does not reflect metabolic activity at the time of sampling (Barer and Coates, 2003). Since environmental samples are usually composed of diverse bacterial communities, conclusions about the *in situ* community structure of active species is limited by using culture dependent methods (Williams*, et al.*, 1998). Studies comparing environmental bacterial populations characterised by cultivation methods and direct microscopic counts have lead to the conclusion that only 0.1 to 1% of the bacteria present in the environment are culturable (Amann*, et al.*, 1995). This fact is better known as the so called "great plate count anomaly" (Staley and Konopka, 1985). Other authors have suggested that maintenance of membrane potential is an essential criterion of viability, and hence physiological activity of the cells (Shapiro, 2001; Shapiro and Nebe-von-Caron, 2004). By combining flow impedance and fluorescence, the CellFacts® II (CFII) technology provides the total cell count as well as additionally information about the physiological status of the individual cells of a microbial population in real-time. The real-time growth kinetic data acquired by CFII makes it possible to draw conclusions about the microbial load and the physiological activity of the cells *in situ*, however, more detailed monitoring of the bacterial population in calcium carbonate slurries is warranted (Schwarzentruber, 2002).

To date a vast assortment of culture-independent molecular techniques are available to quantify microbial diversity *in-situ* by using the 16S rRNA gene. The 16S rRNA gene remains the most used molecular marker for the phylogenetic characterisation of bacteria (Woese, 1987): (i) The 16S rRNA gene is ubiquitous in the prokaryotes, (ii) in comparison to the 5S and 23S, the 16S rRNA gene is sufficiently long (approx. 1500 bp) to visualise phylogenetic correlations and (iii) the presence of hypervariable and constant regions permit the alignment and the design of either universal or taxon-specific probes. The most used method to study microbial diversity in the environment is unquestionably sequence analysis of 16S rRNA gene clone libraries. Extensive databases of 16S rRNA sequences are available on the web: The Ribosomal Database Project (RDP) (Olsen*, et al.*, 1992), the Green Genes Database (De Santis*, et al.*, 2006) or the ARB Silva Database (Pruesse*, et al.*, 2007) are the most popular.

Nevertheless, because the construction of clone libraries is an expensive, laborious and time-consuming method, fingerprinting analysis methods such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) have been shown to be suitable for rapid profiling of mixed bacterial populations (Liesack and Dunfield, 2004). PCR amplification of the 16S rRNA gene by means of universal primers from mixed bacterial populations to generate clone libraries, in T-RFLP fingerprinting analysis or to carry out other molecular down stream analyses is subjected to bias occurring during the template amplification by PCR. Firstly, bias can occur during the isolation of the investigated nucleic acids and secondly, during the subsequent PCR these biases can be summarised in preferential, unspecific or inhibited amplification of certain 16S rRNA gene templates (Hartmann and Widmer, 2008). Other artefacts occurring during the amplification of DNA templates by PCR are errors due to the inaccuracy of the DNA polymerase enzyme and the formation of chimeric PCR products (Thompson*, et al.*, 2002). Further bias are encountered due to the preferential amplification and thus more frequent ligation into cloning vectors of certain amplicons, it can be assumed that cloning will not necessarily reflect the original species ratio of the analysed microbial communities (Orcutt*, et al.*, 2009). The reliability of T-RFLP profiles to elucidate effectively the community composition and abundance ratios of mixed bacterial populations is affected by variations most notably when detecting low-abundance taxa (Kitts, 2001). Additional

pitfalls are encountered in relation to the digestion of the amplified products. Incomplete digestion of the amplified products and partially single-stranded DNA generating pseudo terminal restriction fragments can lead to an alteration of the actual diversity (Egert and Friedrich, 2003, Egert and Friedrich, 2005). Moreover, discrepancies in the migration of T-RFs can occur due to the length of the analysed fragments, run to run variation, differential migration of internal ladder, use of various fluorescent dyes and not at least because of the secondary structure of the analysed fragments (Stres, 2006). Therefore, the relative abundance of T-RFs is not an absolute reflection of the actual abundance of single species but is an estimation of the operational taxonomic unit (OTU) proportion within a bacterial community and is nevertheless useful to assess the temporal and spatial shifts in microbial community structures (Schutte*, et al.*, 2008).

To characterise the diversity of microbial communities the terms phylotypes or operational taxonomic units are used in general (Bent and Forney, 2008). Due to the bias mentioned above, an additional issue to be faced when using molecular techniques is the fact that the conclusions drawn are strongly dependent on the similarity cut-offs of the OTUs. The definition of a bacterial species based on the sequence of the 16S rRNA gene is problematic, consequently OTUs have arbitrarily been defined to be different species when the difference in sequence composition is more than 3% (Stackebrandt and Goebel, 1994). When analysing phylogenetic relationships by means of 16S rRNA gene sequences gathered from clone libraries, the calculation of diversity or similarity indices as well as coverage measures is popularly. Nevertheless, the relative diversity can only be compared if similar methods were used within a study and it has to be kept in mind that diversity indices reflect the relative diversity influenced by all methodological bias rather than the absolute diversity of microbial communities (Röling and Head, 2005). Differing from the above is the DNA fingerprinting approach where the number of present species and the abundance of the species are considered to be characteristic of the bacterial community diversity. As a result, the information provided by T-RFLP profiles is the presence and predominance of a certain species rather than the phylogenetic classification. In T-RFLP analysis the term operational taxonomic unit (OTU) is defined by the length of restriction fragments or even by a range of fragment lengths and for that reason one OTU may be specific for a genus, a species

or even a strain (Dunbar *et al.* 2001; Kitts 2001). Finally, samples run under the same PCR conditions are probably affected equally by any PCR biases that occur and therefore taking into account presence and abundance of OTUs are the basis for the comparison of microbial community fingerprints (Egert, *et al.*, 2004).

Recently, a breakthrough in sequence analysis was the introduction of tag-coded pyrosequencing to carry out deep amplicon sequencing of mixed bacterial 16S rRNA gene pools (Sogin *et al.* 2006; Shendure and Ji 2008). The limitations encountered by sequencing clone libraries due to the direct impact of the sample size on the number of detected species are overcome by the massively-parallel DNA sequencing of short hypervariable regions of the 16S rRNA gene to characterise microbial populations (Huse, *et al.*, 2008). Deep amplicon sequencing does not require a cloning of the 16S rRNA gene fragments prior to the sequencing analysis, thus offers a high-throughput tool to acquire thousands of sequences in parallel. The analysis of such large amounts of data consists of the estimation of the taxonomic richness in term of OTUs at 3% divergence rather than assigning the taxonomy based on the affiliation of sequences in a phylogenetic tree including reference taxa. In addition, deep amplicon sequencing is less expensive in term of costs per base and to date read lengths of nearly 400 bp allows a deeper analysis of microbial structure compared with Sanger sequencing (Tringe and Hugenholtz, 2008). However, the classification accuracy of bacterial taxa and the resolution of diversity in term of OTUs on the basis of short pyrosequencing reads of the 16S rRNA gene depends on the length and on the taxonomic cut-off (usually > 97% similarity = identical OTUs) (Youssef, *et al.*, 2009). Several studies have reported the sufficient accuracy of short pyrosequencing reads of the 16S rRNA gene for the classification of bacteria down to the family or even genus level (Liu, *et al.*, 2007, Wang, *et al.*, 2007) and the hypervariable regions of the 16S rRNA gene V1+V2 and V3 have been reported to be the most successful to identify bacteria down to the genus level (Hamp *et al.* 2009; Petrosino *et al.* 2009). By means of this technique the bacterial diversity in various environments such as seawater (Sogin, *et al.*, 2006), oral cavity (Huse, *et al.*, 2008), human gut (Dethlefsen, *et al.*, 2008, Zhang, *et al.*, 2009), deep mines (Edwards, *et al.*, 2006) and soil (Acosta-Martínez, *et al.*, 2008, Jones, *et al.*, 2009, Lauber, *et al.*, 2009, Roesch, *et al.*, 2007) has been explored. The data revealed a greater taxonomic richness that has been reported previously by Sanger sequencing

and can be readily applied to explore changes in microbial communities over space and time. Difference among microbial communities between environments have been demonstrated using several phylogenetic tests such as hierarchical clustering and/or principal coordinates analysis (PCAs) (Huber*, et al.*, 2009, Roh*, et al.*, 2009).

3.1.1 Aims and Objectives

The aims of the followings experiments were:

- To characterise the *in-situ* microbial diversity and to visualise the phylogenetic relationship of WMD inhabiting bacteria by molecular techniques.
- To study the seasonal and spatial succession of microbial communities at various Omya plants and in different calcium carbonate products.
- To investigate the diversity of viable, compromised and dead bacteria in WMD.

The CFII instrument was used to determine the number of bacterial cells and to assess the physiological state of bacterial populations occurring in calcium carbonate slurries in real-time. A further goal of this thesis is to present detailed insights into the biodiversity and succession of microbial communities inhabiting various WMD manufactured at a choice of geographically distributed Omya plants. This research data in combination with microbial identification and physiological status will provide important insights about the microbes populating a particulate matrix such as WMD. Above all, the knowledge about bacterial species domination and the species succession in such an environmental niche will lead to a better understanding of the behaviour of mixed populations after biocide challenge. For this purpose the disturbance response and the population distribution in WMD production lines and storage tanks before and after biocide dosage was investigated. Finally, 16S cDNA from rRNA, flow cytometry in combination with FACS and DNA-intercalating dyes such as propidium monoazide (PMA), were used to discriminate the physiological state of cells within a bacterial community, to characterise the biodiversity of viable, compromised and dead cells in biocide preserved calcium carbonate slurries.

The molecular techniques used in this study to characterise the microbial communities in calcium carbonate slurries are summarised in Figure 10.

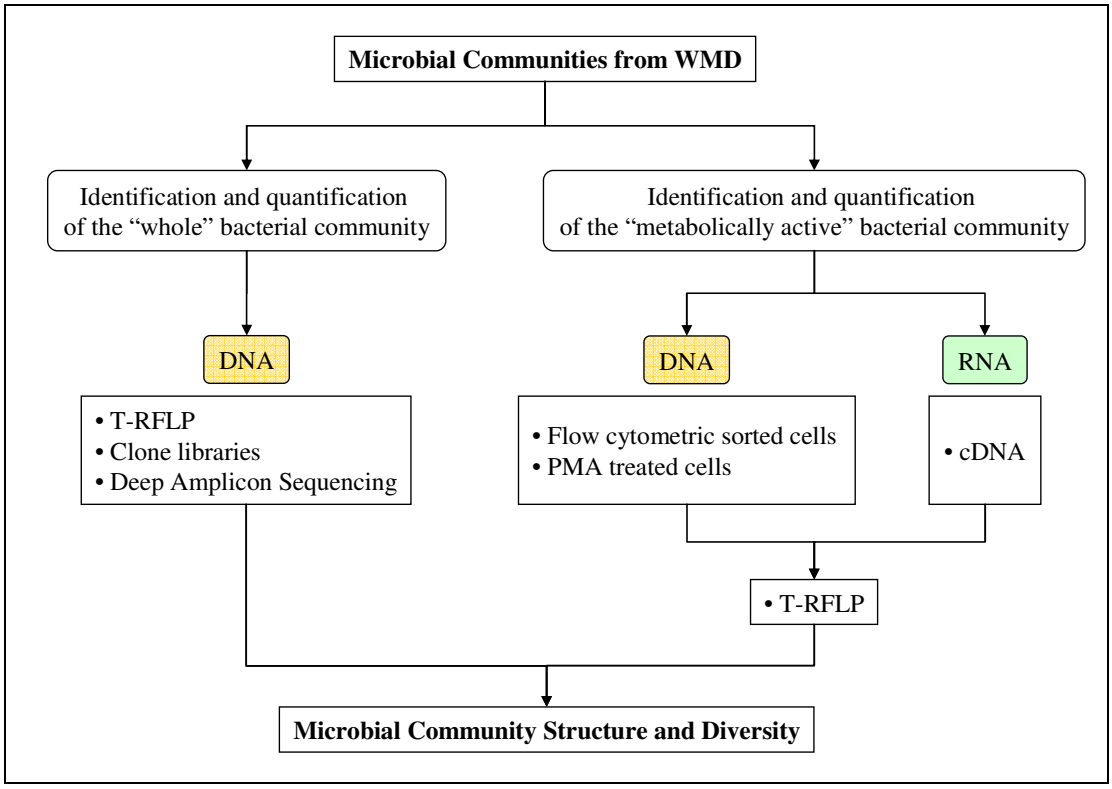


Figure 10. Molecular techniques used for the characterisation of microbial communities in white mineral dispersions.

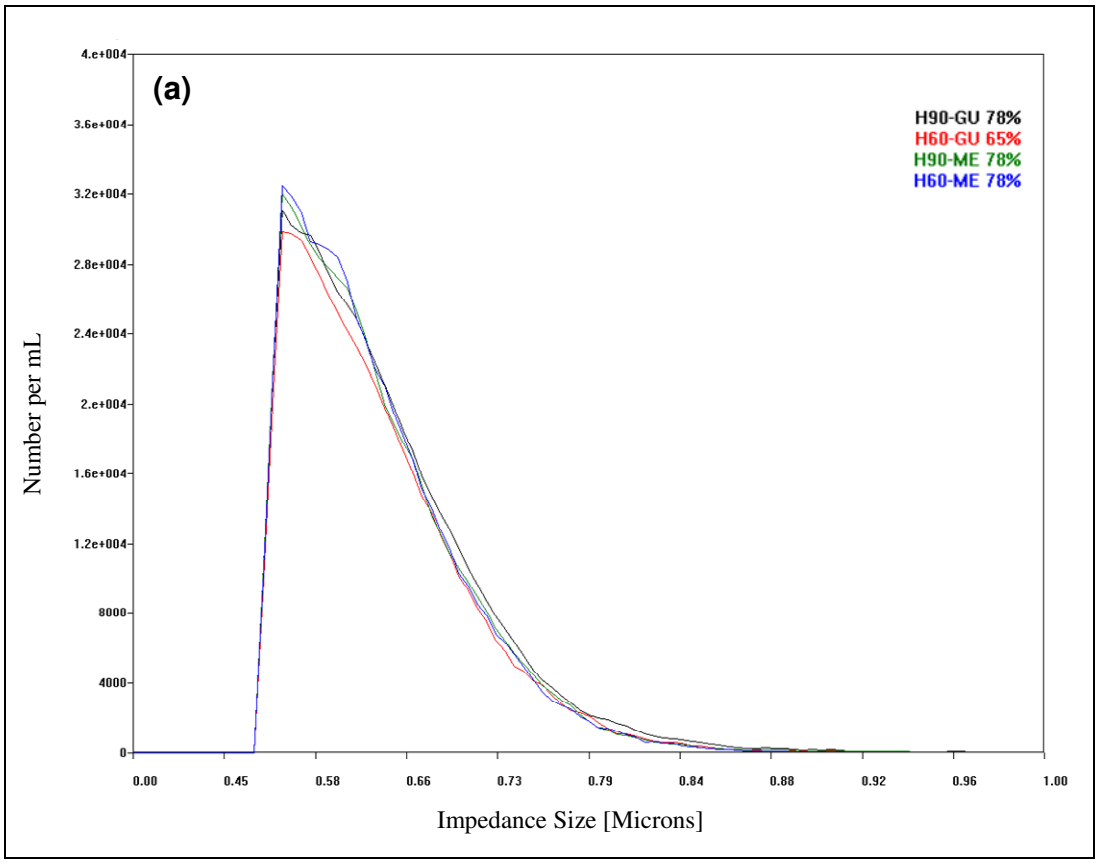
3.2 Results and Discussion

3.2.1 Microbial Characterisation of WMD by CellFacts II®

Electrical impedance sensitive counters (Coulter counters) have been used to count bacteria for a long time (Shapiro, 2000). By combining flow impedance and fluorescence, the CFII technology provides additional information about the physiological status of the individual cells of a microbial population in real-time and with high sensitivity. The CFII device was used to investigate the average microbial activity in calcium carbonate slurry samples collected from the various Omya sites included in the biodiversity studies. By means of this technology the cellular profiles and the fluorescence of each sample was recorded.

3.2.1.1 Evaluation of CellFacts II® Data

When analysing calcium carbonate slurry by means of the CFII technology two key outcomes are valuable for the assessment of the microbial load. The first parameter involves the total particle count. With the optimised sample preparation to separate the microbial cells from the calcium carbonate matrix (Schwarzentruber, 2003b) slurries contain a fraction of small particles < 1 µm and a large peak is typically obtained in the size range from 0 to 0.8 µm (Figure 11 and Figure 12). Moreover, Figure 11 shows a slurry product which was sterilised by steam heat in order to lyse the bacterial cell and to visualise the particle background typical of a particulate matrix such as calcium carbonate slurry.



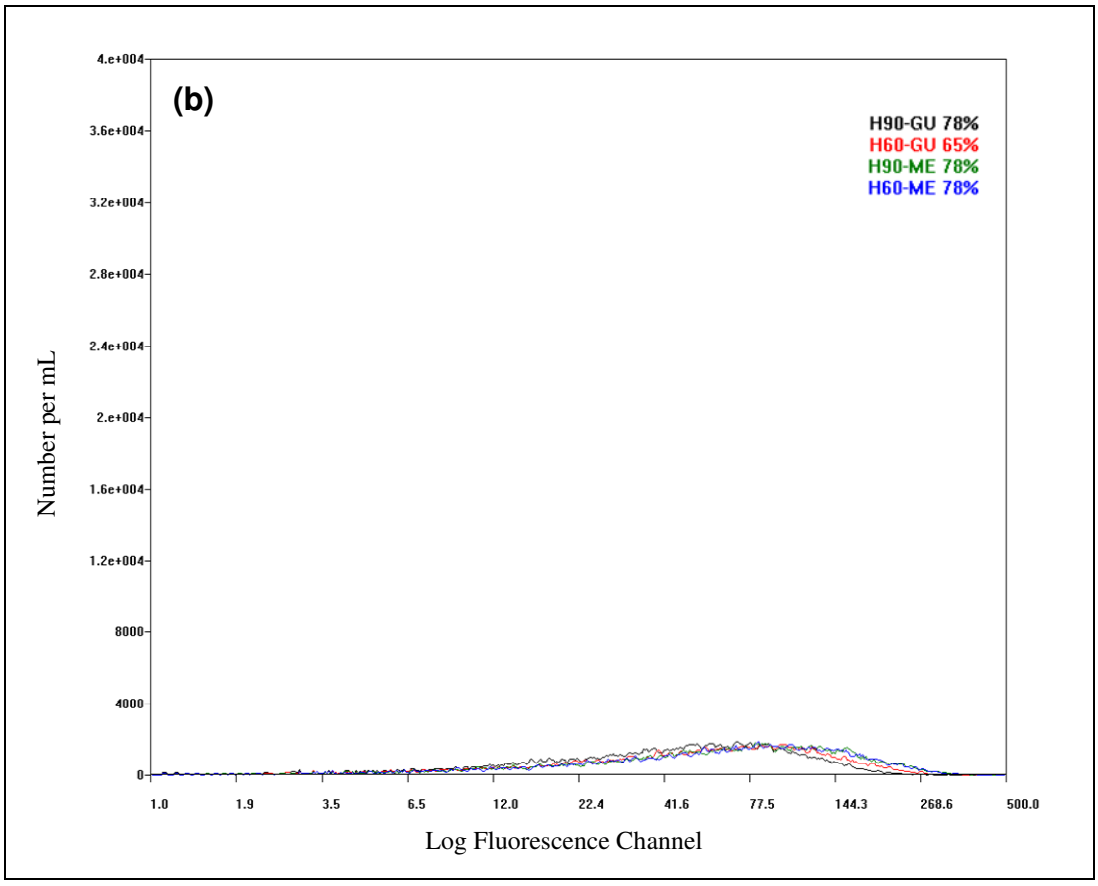


Figure 11. (a) Particle size distributions and (b) fluorescence intensity profiles of steam heat sterilized calcium carbonate slurries.

By means of the particle size distribution bacterial cells that fall within the background are not distinguishable from the residual calcium carbonate particles. In comparison, particles above this region can be attributed to bacterial cells and are indicative for the total cell count (TCC ml^{-1}) of calcium carbonate slurries (Schwarzentruber, 2002). In addition, the CFII device provides the fluorescence intensity of the fluorescent-dye stained bacterial cells. The fluorescence intensity resulting from the cell-permeant SYTO62 is useful to discriminate between carbonate particles and bacterial cells, even though a certain background noise is present in the fluorescence channels (Figure 11), the carbonate particles are not stained by the applied dyes. The fluorescence resulting from $DiSC_3(5)$ is fundamental to estimating the membrane potential of bacterial cells and hence to estimate the mean physiological activity of the bacterial population.

Figure 12 illustrates the particle size distribution and the fluorescence intensity profile of bacterial cells growing in calcium carbonate slurry. Sterile slurry (start t0) was inoculated with contaminated slurry and both total cell count and fluorescence were monitored by CFII over a period of 170 h. From the CFII profiles an increase in the TCC ml^{-1} was evident. Within the bacterial size range an accumulation of biomass occurred during the period of analysis. The fluorescence profile showed a significant increase in the membrane potential hence an increase of the physiological activity during the exponential growth phase.

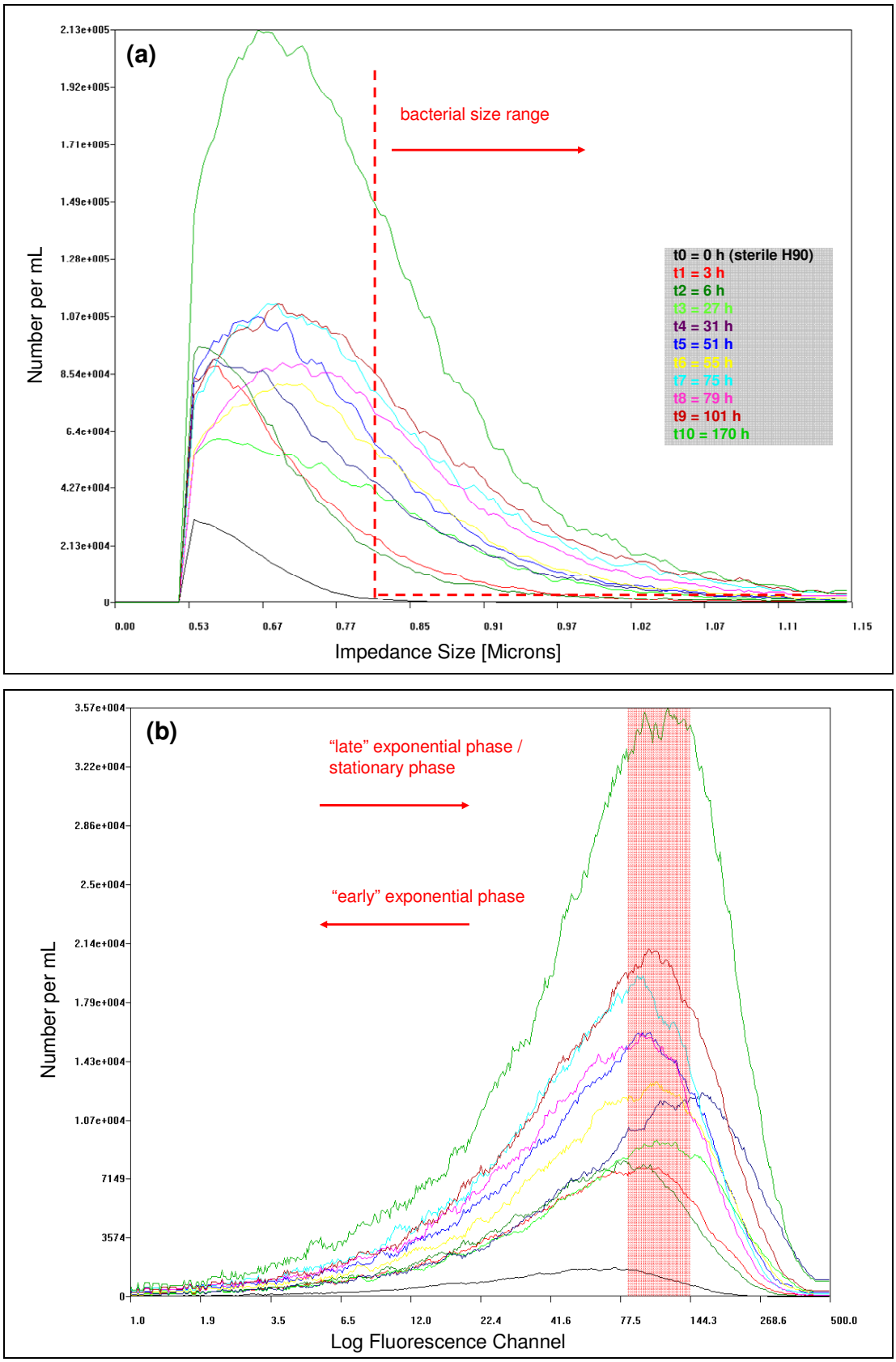


Figure 12. (a) typical particle size distribution and (b) fluorescence intensity profiles of WMD bacteria measured by CFII. The fluorescence band in (b) represents the area of transition between early log phase and stationary phase cells.

During the first 31 hours of growth as the cells progressed into the exponential phase the membrane potential peaked to a maximum value as a consequence of the high physiological activity during growth (Figure 13). The TCC increased exponentially and a growth rate μ of 0.0136 h^{-1} was calculated. This corresponds to a doubling time of 51 hours ($d = ln(2)/\mu$). As evident from Figure 13 after 50 hours of growth the average fluorescence dropped back again as a consequence of growth cessation. As the slurry culture progressed into the stationary phase the average fluorescence remained high compared to the average fluorescence at the beginning of the growth curve. The decrease of the fluorescence per cell observed over time resulted from starvation of cells proceeding into the stationary phase. It has been shown that these changes in membrane potential are characteristic for the cell cycle where the maximum metabolic activity is achieved during the period prior to the mid-exponential phase (Monfort and Baleux, 1996).

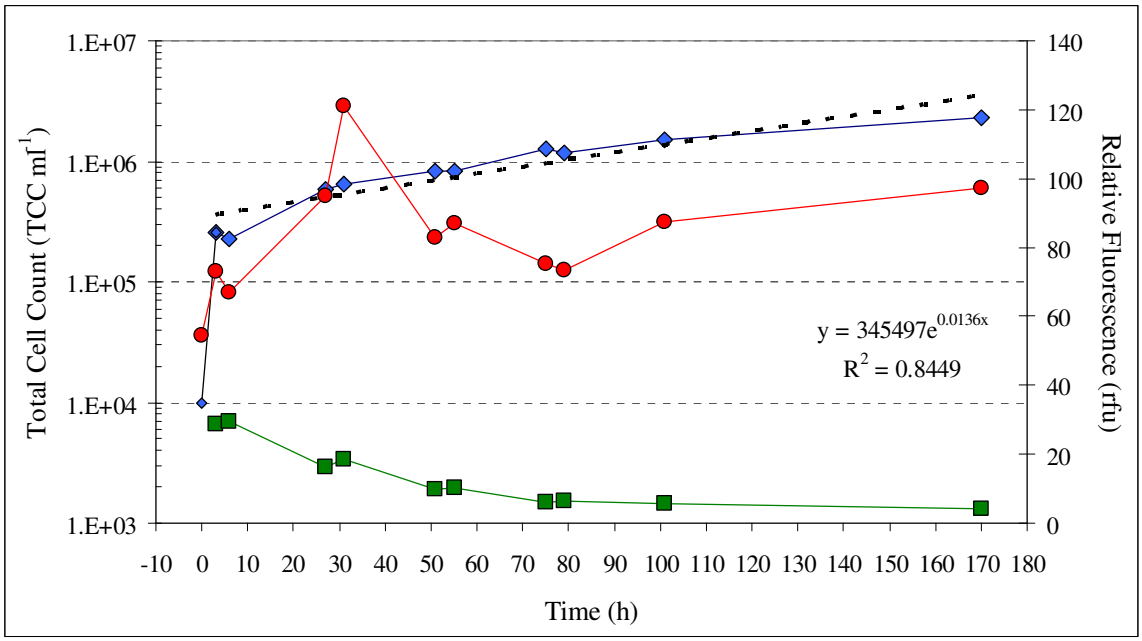


Figure 13. Course of total cell count (♦), average fluorescence (●) and relative fluorescence per cell (■) of bacteria growing in calcium carbonate slurry. The relative fluorescence per cell was calculated as follows: *average rfu / TCC*. The value was rescaled by multiplication with 10^5 in order to fit into the scale of the average fluorescence. Dotted line shows linear trend of ln(TCC ml^{-1}).

As demonstrated by the above data the average fluorescence intensity increased in correlation with the increasing number of cells and their physiological activity. These

results are in accordance with Schwarzentruber (2003b), who reported constant cell count and a significant increase of the average fluorescence, as a result of enhanced physiological activity, 24 hours after the addition of an insufficient amount of biocide to calcium carbonate slurries. The correlation between the membrane potential and the fluorescence intensity is accentuated when the membrane potential of WMD cells was dissipated by the addition of the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Figure 14).

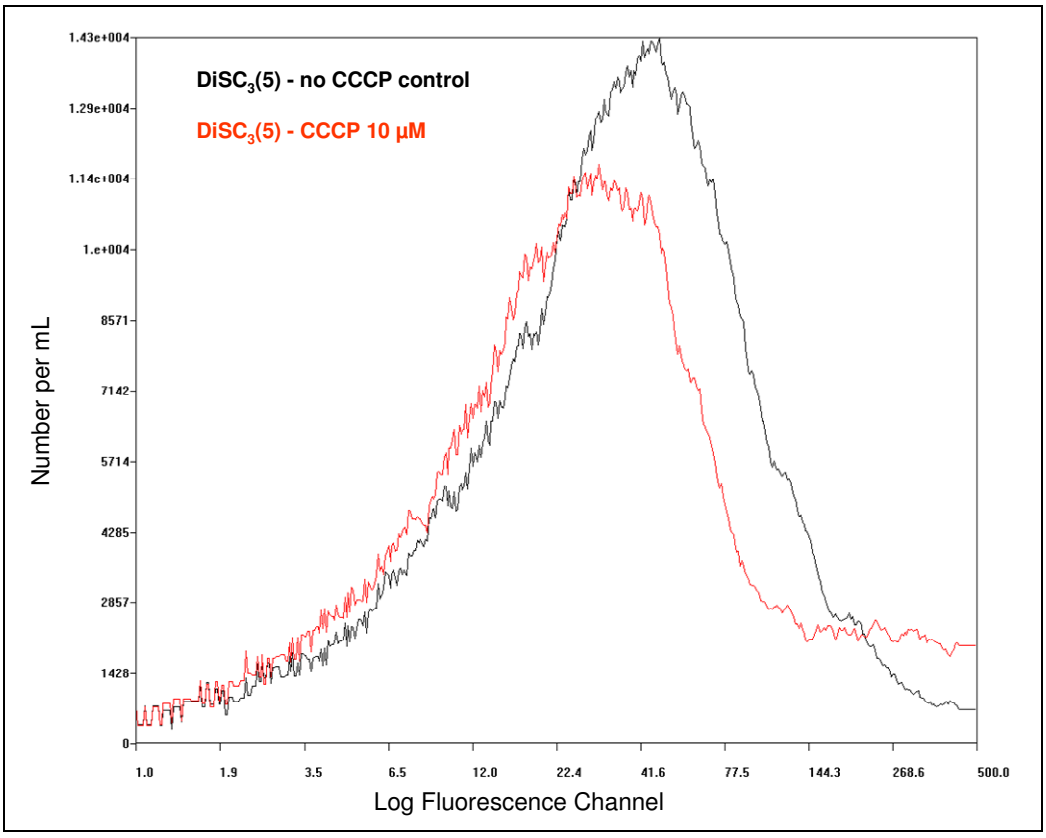


Figure 14. CFII fluorescence intensity profiles of untreated (control) and CCCP treated WMD bacterial cells.

5 minutes after the treatment of the WMD cells with CCCP a significant decrease of the fluorescence was seen compared to untreated control cells. The membrane potential of microorganism is maintained by the extrusion of H^+ ions via the H^+-ATPase or the electron transfer chain (Breeuwer and Abee, 2000). CCCP treatment causes a depolarisation of the cell membrane and the membrane potential generally collapses because of the absence of the proton gradient across the membrane (Novo *et al.* 1999; Novo *et al.* 2000).

3.2.1.2 Microbial Activity Determined by CellFacts II®

The microbial contamination of the samples analysed in the following sections were firstly characterised by means of CFII. The analysis of the results was performed in two ways: (i) according to the analytical rules applied to the CFII data algorithm, which has been described by Schwarzentruber (2003b) and further improved and maintained by CellFacts Instruments and (ii) using the CellFacts II® Viewer software. By means of the algorithm an estimation of the total viable count (TVC) is made based on the average total cell count (TCC) and by cluster analysis of the raw fluorescence data of individual representative cells clusters with related fluorescence values. In contrast, the CellFacts II® Viewer software enables manual integration of the data and the setting of regions of interest in the impedance (particle count) or fluorescence channels. Fluorescence values can be calculated over all 64,000 channels available by CFII (i.e. size range from 0.5 to 12 µm). The value from the algorithm can be interpreted as follows:

- Positive samples: TVC $ml^{-1} > 1.0 \cdot 10^4$ and fluorescence > 10 rfu
- Sample at risk: TVC $ml^{-1} > 1.0 \cdot 10^4$ and fluorescence < 10 rfu
- Negative samples: Where none of the above described rules are applicable

Of all calcium carbonate slurries examined in the following sections the total cell count and the average fluorescence for all sampling times are given in Table 7 and Table 8.

Table 7. Microbial characterisation by means of CFII of the calcium carbonate slurry products sampled in autumn 2007 (Q407 = October 2007).

Period of analysis	Product ID	Total Viable Count (ml^{-1})	Activity (rel. fluo)	Total Cell Count (ml^{-1}) (0.8 to 1.5 µm)	Activity (rel. fluo) (Channel 0 to 1000)
		Algorithm		*CellFacts II viewer*	
Q407 no biocide [a)]	H90-ME	below	-1.07	4.4E+03	71.59
	CC60-ME	6.5E+03	2.56	1.0E+04	88.54
	H60-ME	below	2.95	6.9E+03	73.36
	H90-GU	below	1.62	5.7E+03	64.13
	CC60-GU	6.3E+04	23.12	2.2E+05	105.38
	H60-GU	3.0E+03	14.77	1.8E+04	118.52
Q407	H90-ME	below	1.86	7.6E+03	73.19
	CC60-ME	below	0.58	2.2E+04	60.37
	H60-ME	2.5E+04	14.63	9.7E+04	88.52
	H90-GU	below	1.62	5.9E+03	76.99
	CC60-GU	1.3E+04	19.58	5.2E+04	112.76
	H60-GU	1.7E+05	42.19	2.8E+05	129.75

Positive samples Negative samples

Below = below the algorithm detection limit threshold ($1 \cdot 10^3$ cells ml^{-1})

a) biocide-free samples taken before the addition of biocide

In the calcium carbonate slurries sampled in Q407 (autumn 2007) significant microbial contamination was determined in the product CC60-GU in both the production pipeline (no biocide) as well as in the storage tank (with biocide). Similarly, the coarse product H60 showed a significant microbial load in the samples retained out of the preserved tank from both investigated production sites.

Table 8. Microbial characterisation by means of CFII of the calcium carbonate slurry products sampled from winter 2008 (Q108) to autumn 2008 (Q408).

Period of analysis	Product ID	Total Viable Count (ml^{-1})	Activity (rel. fluo)	Total Cell Count (ml^{-1}) (0.8 to 1.5 µm)	Activity (rel. fluo) (Channel 0 to 1000)
		Algorithm		*CellFacts II viewer*	
Q108 no biocide [a)]	H90-ME	1.1E+03	3.09	1.0E+05	68.09
	CC60-ME	1.2E+06	18.89	1.3E+06	80.77
	H60-ME	1.2E+04	8.99	3.3E+05	61.86
	H90-GU	3.3E+06	10.93	4.5E+06	117.44
	CC60-GU	8.8E+05	14.66	6.6E+05	97.66
	H60-GU	1.9E+06	13.16	1.8E+06	123.90
Q108	H90-ME	below	-0.95	4.5E+04	73.19
	CC60-ME	5.0E+03	1.66	7.1E+04	67.13
	H60-ME	below	1.03	4.5E+04	61.39
	H90-GU	below	2.23	3.5E+04	77.67
	CC60-GU	6.0E+06	38.38	1.9E+06	117.57
	H60-GU	1.3E+05	6.11	8.5E+04	143.21
	H90-AV	below	-1.61	6.7E+04	75.68
	H60-AV	below	2.74	5.5E+04	76.65
	Syncarb-GO	1.1E+05	1.51	3.4E+06	68.56
Q208	H90-ME	below	3.27	4.3E+04	77.86
	CC60-ME	6.0E+04	11.43	9.7E+04	85.87
	H60-ME	below	0.07	2.9E+04	54.51
	H90-GU	7.0E+03	-1	4.6E+04	55.68
	CC60-GU	4.8E+03	-0.35	5.5E+06	66.51
	H60-GU	1.5E+05	9.26	5.8E+04	87.08
	H90-AV	1.9E+04	2.58	5.6E+04	83.06
	H60-AV	below	1.28	3.9E+04	76.68
	Syncarb-GO	2.8E+05	12.07	2.3E+05	88.71
Q308	H90-ME	below	4.61	3.0E+04	61.09
	CC60-ME	below	2.61	3.3E+04	63.24
	H60-ME	below	2.31	2.5E+04	53.40
	H90-GU	below	0.64	2.4E+04	67.29
	CC60-GU	4.0E+05	2.94	2.5E+06	60.99
	H60-GU	5.5E+03	2.64	2.9E+04	75.73
	H90-AV	1.1E+06	28.11	3.3E+05	95.35
	H60-AV	below	1.22	3.6E+04	60.27
	Syncarb-GO	3.3E+06	23.85	1.4E+06	131.16
Q408	H90-ME	below	1.93	3.3E+04	37.00
	CC60-ME	below	1.52	1.8E+04	43.68
	H60-ME	below	2.06	2.6E+04	52.07
	H90-GU	1.1E+03	4.74	3.7E+04	57.05
	CC60-GU	2.8E+03	0.42	4.7E+06	63.54
	H60-GU	4.8E+04	3.34	3.7E+04	83.66
	H90-AV	2.9E+06	34.07	1.0E+06	106.65
	H60-AV	below	2.08	3.0E+04	62.87
	Syncarb-GO	1.0E+07	41.24	3.4E+06	134.17

Positive samples Samples at risk Negative samples

Below = below the algorithm detection limit threshold ($1 \cdot 10^3$ cells ml^{-1})

a) biocide-free samples taken before the addition of biocide

The products sampled in Q108 prior to the addition of biocide revealed high total viable count and an increased level of microbial activity. This is not surprising since the manufacture of calcium carbonate slurry is performed in a non sterile "open system" environment and the recirculation of process water as well as the inherent microbial load coming from the raw materials is a potential source of contamination. Despite the high temperatures achieved during the slurry production process through the wet grinding step (up to 90 ºC or 110 ºC for coarse or fine products respectively), the total cell count and the physiological activity suggests that bacterial contamination is not eliminated and a fraction of the bacterial population is able to recover once the product cools. Moreover, at several production sites prior to the dosage of biocide the product is cooled in so called flash cooler devices (40-80 ºC) and these devices have been found to be one of the main contamination source of WMD (Schwarzentruber, 2003b). Other authors have also reported high bacterial counts in white mineral slurry used in the paper mills to formulate paper pigment coatings (Vaisanen*, et al.*, 1998).

The majority of the samples taken from the biocide treated storage tanks revealed an overall low contamination level when analysed using the TVC algorithm of the CFII. However, taking a closer look at the particle count between 0.8 and 1.5 µm disclosed the presence of particles within the bacterial size range. The mean fluorescence of the particles calculated by means of the CellFacts II® viewer software is typically greater than those calculated by the algorithm because the dynamic range (channels) considered for the calculation is different (1,000 vs. 64,000 channels). However, the values are in correlation and the assessment of the physiological activity is comparable for both evaluation approaches. To date the CellFacts II® software is not able to calculate the percentage of cells causing the fluorescence signal. This information would have been important to discriminate samples with a high content of dead biomass from samples with a low concentration of highly active cells, respectively. The integration of such a percentage evaluation in the CellFacts II® software is subject of future development at CellFacts Instruments research facilities.

3.2.2 Biodiversity of Culturable Bacteria in WMD

Nowadays, total viable count determined by the plate count method is the method of choice to characterise the culturable fraction of bacteria in calcium carbonate slurry. Plate count agar (PCA) commonly utilised to isolate water, wastewater, food and dairy microorganisms, is used as a non-selective medium. By means of this medium a preliminary isolation of mesophilic aerobic bacteria inhabiting calcium carbonate slurry can be done. When incubating the plates for a long time (up to 7 days) an increase in the phenotypes in respect to the morphology of the colonies on the plates is observed and provides a first indication for the species diversity of culturable bacteria in WMD.

From H90-AV calcium carbonate slurry (from the piping system without biocide and from the storage tank with biocide) using PCA plates, bacterial isolates with diverse morphologies, i.e. size, shape, colour and opacity were isolated over a time period of 12 days and identified by partially sequencing the 16S rRNA gene (Table 9). Moreover, the T-RF size of the 16S rRNA gene was determined by T-RFLP analysis or *in silico* by means of the gathered sequences.

Table 9. Identification of colonies isolated from calcium carbonate slurries by means of plate count agar. Yellow coloured boxes are species that represent <10%, orange coloured boxes 10% to 30% and red coloured >30% of the abundance in each sample.

Species	**Identity (%)**	**Occurence (%)**		**T-RFs (bp)**	
		H90-AV w/o biocide	H90-AV with biocide	*in silico*	measured
Alishewanella sp.	97	10	0	477	479
Flavobacterium sp.	99	3	0	89	81
Hydrogenophaga sp.	97	18	0	488	489
Pannonibacter phragmitetus	99	18	0	437	433
Pseudomonas mendocina	99	41	61	490	489
Pseudomonas stutzeri	99	10	21	490	489
Pseudomonas pseudoalcaligenes	98	0	18	490	489

These results are limited by the small sample size, however, the unpreserved slurry sample taken directly after the production process (without biocide) showed a broader biodiversity compared to the biocide treated sample from the tank. In the biocide supplemented sample an evident shift of the culturable bacterial fraction

toward the genus of *Pseudomonas* was seen. In both samples the majority of bacterial colonies growing on PCA were assigned to the genus *Pseudomonas* and varied from 51% in the biocide-free sample to 100% in the biocide treated sample, respectively. In the biocide-free samples members of the *Gammaproteobacteria*, *Flavobacteria*, *Betaproteobacteria* and *Alphaproteobacteria* were also found. The differentiation of related species, as exemplified by the species belonging to the genus *Pseudomonas*, was not possible by means of the T-RF length. This fact relates to a number of constrains given for T-RFLP analysis, including multiple species allocation to the same OTU because of equal T-RF lengths. These data, however, provide support for the hypothesis that in biocide-free samples multiple bacterial species are able to coexist and to form colonies when environmental circumstances are favourable (no nutrient limitation) such as on agar-based medium. On the other hand, there is an obvious reduction of diversity caused by the addition of biocide. In this specific case, the culturable bacterial population was reduced to the occurrence of *Pseudomonas* sp., suggesting that these bacterial species were able to recover from the toxic activity of the biocide or even were resistant to the biocide. Moreover, colonies differing in shape and size were frequently shown to be the same bacterial species, hence highlighting the fact that cells of the same species which were hit by the biocide recovered on the nutrient agar heterogeneously. The parameters influencing the action of biocidal compounds are discussed in section 4.2.1. The phylogenetic affiliation of cultured bacteria and phylotypes retrieved from the clone libraries are shown in Figure 28 of section 3.2.4.1.

Several limitations should be pointed out in the presented data. First of all, a significant disadvantage of the isolation of WMD bacteria on PCA by traditional plating methods is the underestimation of the microbial diversity due to the selective conditions created by the medium. As mentioned above the culturability of environmental bacteria has been estimated to be less than 1% and in other studies investigating soil samples particularly, a vast diversity of microorganisms was confirmed by culture-independent methods (Ritz, 2007). Secondly, a further crucial factor influencing growth on solid media is the actual physiological state of the individual species within a population which can influence the recovery of dormant cells or cells which have been physiologically compromised, e.g. because of substrate-accelerated death (Kell*, et al.*, 1998). During the manufacture of calcium

carbonate slurry the bacterial cells are subject to different forms of metabolic perturbation such as temperature variations, water availability and chemical influences mainly driven by the toxicity of the biocides. It has been described that antimicrobial treated cells can enter into a dormant (non-dividing) state and survive in the form of persistent cells (Lewis, 2007). Therefore, in WMD the presence of dormant and compromised cells has to be considered. Inevitably, the elucidation of the microbial diversity of WMD can not only be deduced from the culturable fraction, as it represents a simplification of the species occurrence. The implementation of culture-independent molecular techniques will open new perspectives in the elucidation of microbial diversity of calcium carbonate slurries.

3.2.3 Culture-independent Molecular Characterisation of WMD

3.2.3.1 Sampling sites – Spatial and Temporal Analysis

The calcium carbonate samples used in this study were collected from various Omya plants in Europe. Plants processing natural ground calcium carbonate (GCC) from marble and synthetic precipitated calcium carbonate (PCC) have been considered in this study. The analysed GCC products were sampled from three major Omya production plants: Gummern in Austria, Hustadmarmor in Norway and Avenza in Italy. The PCC was obtained from Golling in Austria. During the time period of one year, on a quarterly base, samples were collected and analysed by T-RFLP. Table 10 summarises the details of the sampling sites and the analysed samples.

Table 10. Sample sites and sample details of used calcium carbonate slurries.

Plant	Code	Lat. and Long.	Product	Period of Analysis				
				Q407	*Q108*	*Q208*	*Q308*	*Q408*
Gummern	ATGU	N 46° 38' 60"	H90-GU 78%	x [a)]	x [a)]	x	x	x
"GCC"		E 13° 46' 48"	H60-GU 65%	x [a)]	x [a)]	x	x	x
			CC60SW-GU 72%	x [a)]	x [a)]	x	x	x
Hustamarmor	NOME	N 62° 50' 24"	H90-ME 78%	x [a)]	x [a)]	x	x	x
"GCC"		E 7° 6' 36"	H60-Me 78%	x [a)]	x [a)]	x	x	x
			CC60-ME 72%	x [a)]	x [a)]	x	x	x
Avenza	ITAV	N 44° 2' 24"	H90-AV 78%		x	x	x	x
"GCC"		E 10° 4' 12"	H60-AV 78%		x	x	x	x
Golling "PCC"	ATGO	N 47° 34' 12" E 13° 10' 12"	Syncarb-GO 52%		x	x	x	x

a) samples available from the piping system (without biocide) and from the tank (with biocide)

Q407: October 2007 (Autumn07)
Q108: January 2008 (Winter08)
Q208: April 2008 (Spring08)
Q308: July 2008 (Summer08)
Q408: October 2008 (Autumn08)

3.2.3.2 Biocide Perturbation of the Microbial Population of WMD

In the manufacturing process of calcium carbonate slurries the storage tanks form the end of the production chain and due to the prevalent favourable temperatures (30-40 ºC) and the occasionally long retention periods they are generally susceptible to microbial growth. Therefore, the preservation of calcium carbonate slurry is brought about by the addition of biocide either just before delivering the product into the storage tank or directly into the storage tanks.

The influence on the microbial population structure after the addition of biocide to calcium carbonate samples was investigated via T-RFLP analysis and both the occurrence and the relative abundance of the determined OTUs were used to compare the profiles. The succession of OTUs after biocide addition to various calcium carbonate slurry products is illustrated in the histograms in Figure 15.

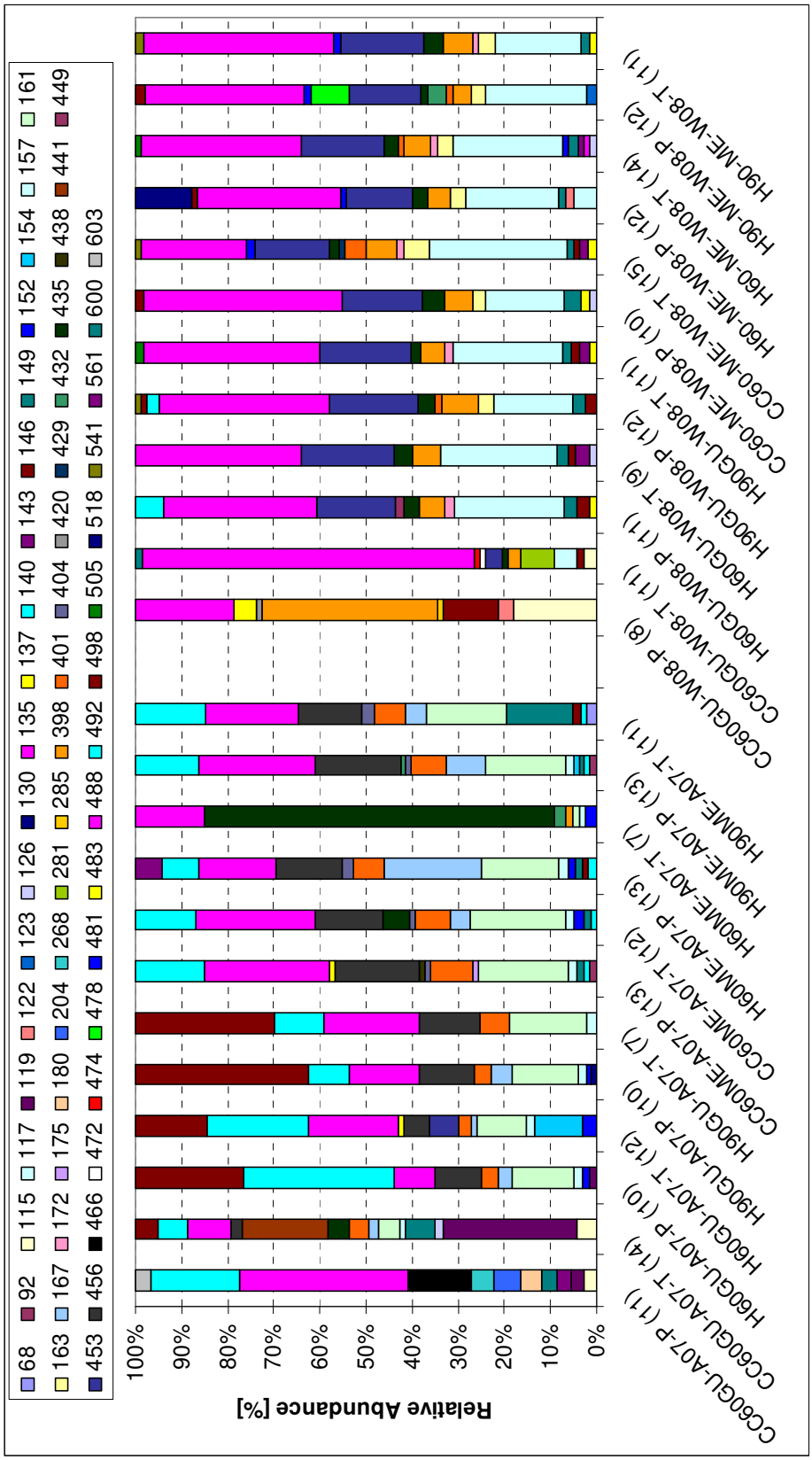


Figure 15. Relative abundance of OTUs determined in different calcium carbonate slurries sampled in autumn 2007 (A07) and winter 2008 (W08). The samples were collected either before biocide addition in the piping system (P) or after preservation in the storage tank (T). Numbers in brackets represent the number of detected OTUs.

In all samples from the Norwegian plant a total number of 39 distinct OTUs were retrieved in the fragment range between 68 bp and 561 bp. In comparison, in the samples from the Austrian plant a total number of 40 distinct OTUs ranging from 115 bp to 603 bp were recovered. The overall most common OTU was determined to be fragment 488 bp (29% over all samples) which was present in all analysed samples (Figure 16). Based on the fact that the accuracy in size of identical T-RFs can vary among different runs about 1-2 bp (Osborn *et al.* 2000; Egert *et al.* 2004; Liesack and Dunfield 2004; Schutte *et al.* 2008) and according to the data from section 3.2.2 it can be assumed that the 488 bp OTU probably primarily represented the genus *Pseudomonas*. In this context it is necessary to highlight that due to the limited resolution of the T-RFLP methodology a single T-RF in the following analysis corresponded to one OTU and an unambiguous T-RF represents more than one phylotypes having identical T-RF length but differing in the gene sequence (Liu *et al.* 1997; Liesack and Dunfield 2004; Schutte *et al.* 2008).

Moreover, from the histogram in Figure 15 it was apparent that after the addition of biocide there was a rearrangement of the abundance ratios of distinct OTUs rather than the alteration of the OTU quantity. Even though the differences in OTU numbers per sample between the two sample groups where not significant ($p > 0.05$, t-test: paired two samples for means), the quantity of determined OTUs in the biocide-free samples from the piping system (OTUs 46) was greater than in the biocide supplemented samples from the storage tanks (37 OTUs). This is in line with other studies generally reporting low diversity and a high dominance of single bacterial species in perturbed environments (Denaro *et al.* 2005). A closer examination of the incidence of OTUs commonly present in the samples with and without biocides revealed that the profiles were characterised by the presence of 28 concomitantly occurring OTUs (Figure 16) which were equivalent. However, analysis of variance (ANOVA) used to compare the frequency of OTUs in the two sample groups did not show any statistical significance either for all identified OTUs or for OTUs commonly present ($p > 0.05$). In the sample without biocide 18 unique OTUs were found whereas in the samples supplemented with biocide 9 unique OTUs only were detected (Figure 17). ANOVA confirmed statistical significance of variance for the incidence of distinctive OTUs (singletons) in the sample without biocide ($p < 0.05$). Therefore, the differences in the microbial populations between

the two sample groups, without and with biocide, were mainly driven by unique T-RFs (singletons) found in either sample group.

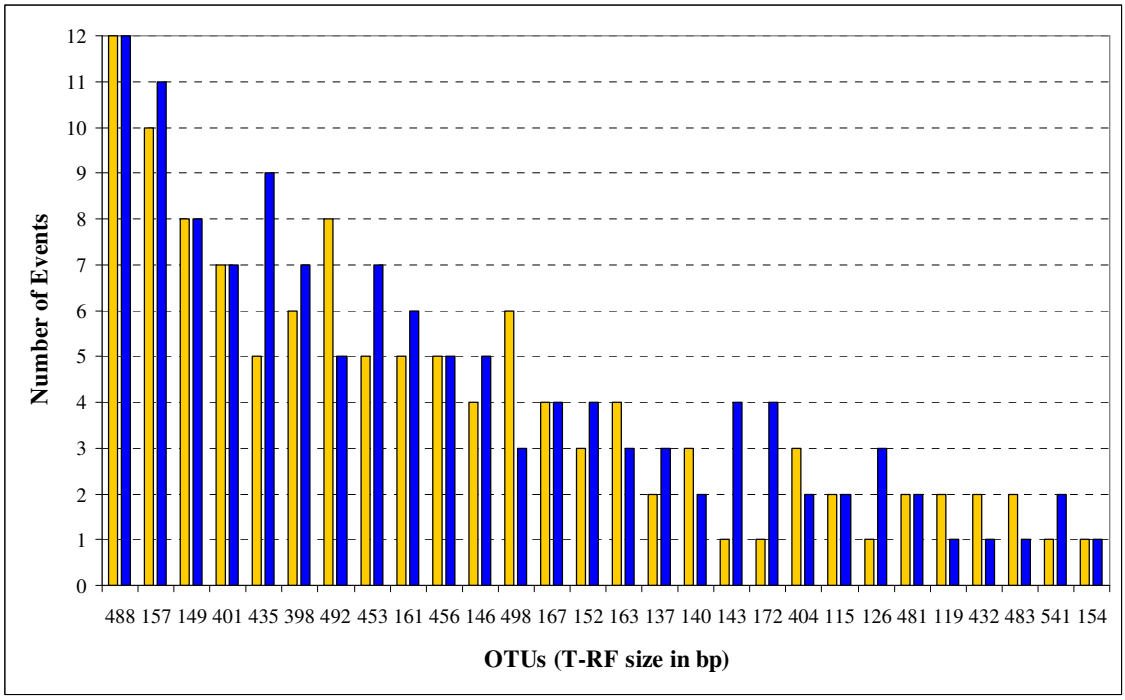


Figure 16. Ranked event plot of OTUs commonly present both in the samples collected before biocide addition in the piping system (■) and after preservation in the storage tank (■) in autumn 2007 and winter 2008.

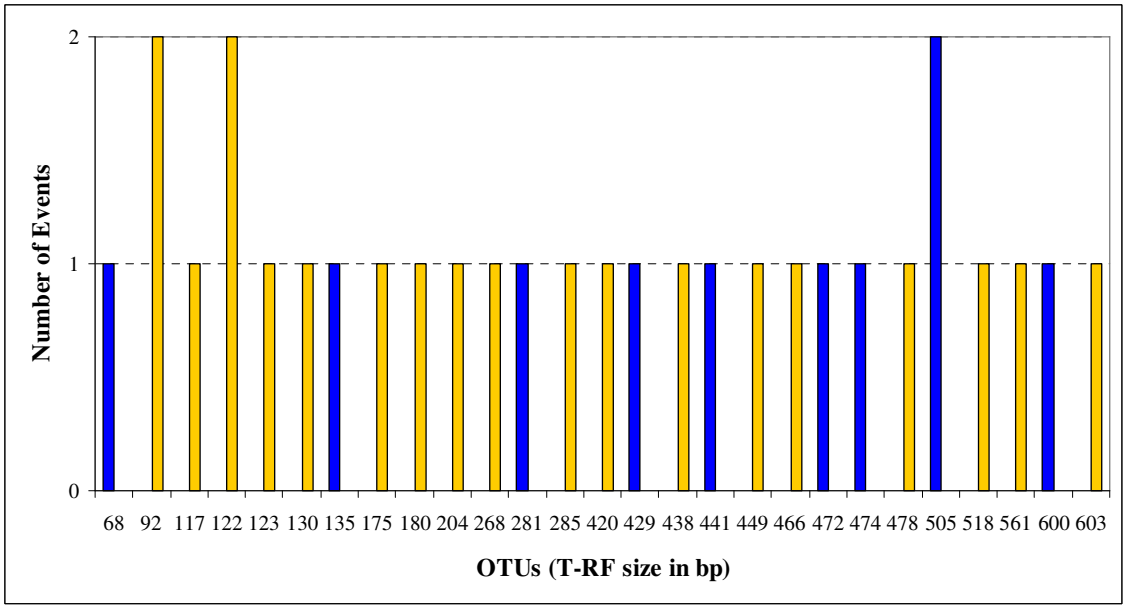


Figure 17. Ranked event plot of unique OTUs detected both in the samples collected before biocide addition in the piping system (■) and after preservation in the storage tank (■) in autumn 2007 and winter 2008.

In order to evaluate the relationship between the bacterial communities found at the various production plants in calcium carbonate slurries containing either biocide or not Cluster Analysis (CA) and Principal Component Analysis (PCAs) were carried out. Cluster analysis of all samples was performed with the Bray-Curtis distance which is sensitive for abundance data (Figure 18).

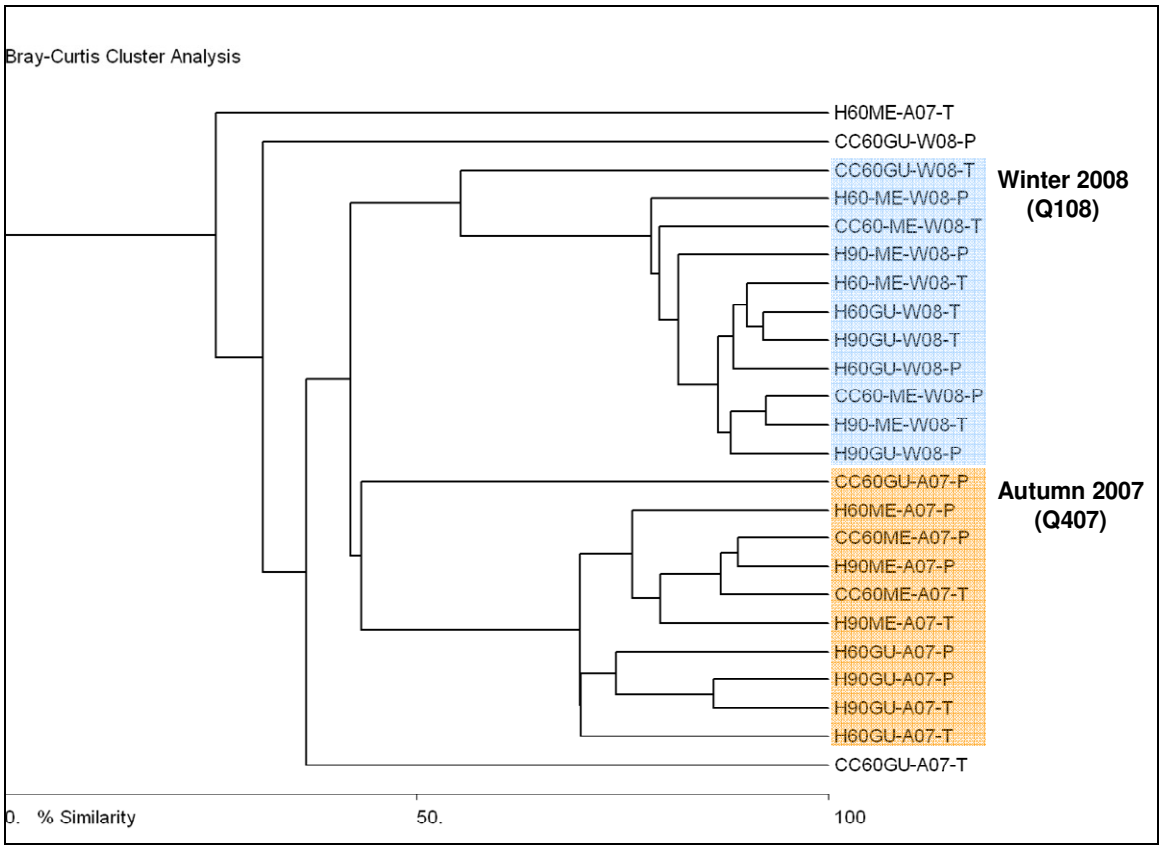


Figure 18. Cluster analysis based on the Bray-Curtis distance of OTUs determined in calcium carbonate slurries sampled in autumn 2007 (A07) and winter 2008 (W08). The samples were collected either before biocide addition in the piping system (P) or after preservation in the storage tank (T).

The cluster profiles showed a clear separation of two distinct clusters of the samples from the same sampling period Q407 (autumn 2007) and Q108 (winter 2008). Furthermore, samples from the sampling time Q407 (autumn 2007) showed the profiles from the two different Omya sites forming single sub-clusters whereas samples from Q108 (Winter 2008) were poorly separated in relation to the sampling location. Nevertheless, an unambiguous overall separation of the samples obtained either before biocide addition in the piping system (P) or after preservation in the storage tank (T) was not recognised. In some cases there was a clustering of the same slurry product type independently of the treatment with biocide whereas in other

cases the samples either with or without biocide were clearly related to each other. Five samples (H60ME-A07-T and all CC60GU samples from both periods) did not fit into the described clusters and constituted outliers. These samples showed either a low number or high number of OTUs with one or more predominant OTUs accounting for an imbalance of the abundance ratios within the bacterial population.

These findings are corroborated by multivariate analysis of the T-RFLP data from both analysis periods. By the reduction of the T-RFLP data to presence/absence matrix by means of the Jaccard distance samples were subjected to Principal Component Analysis (PCAs) (Figure 19). Component 1 (x-axis) accounted for 32% of the variance and disclosed a seasonal-dependent separation of the samples drawn either in Q407 (autumn 2007) or in Q108 (winter 2008). The second component (y-axis) accounted for 10% of the variance and indicated increasing similarity of the samples toward a value of 0. Furthermore, bacterial communities originating from the same calcium carbonate manufacturing plant grouped together along component 2 and indicated the similarity of the bacterial population structure within a specific production site. Additionally, the PCAs figure suggested that the Q108 (winter 2008) bacterial populations were more divergent either for the samples retained from the piping system as well as for the samples drawn from the storage tank.

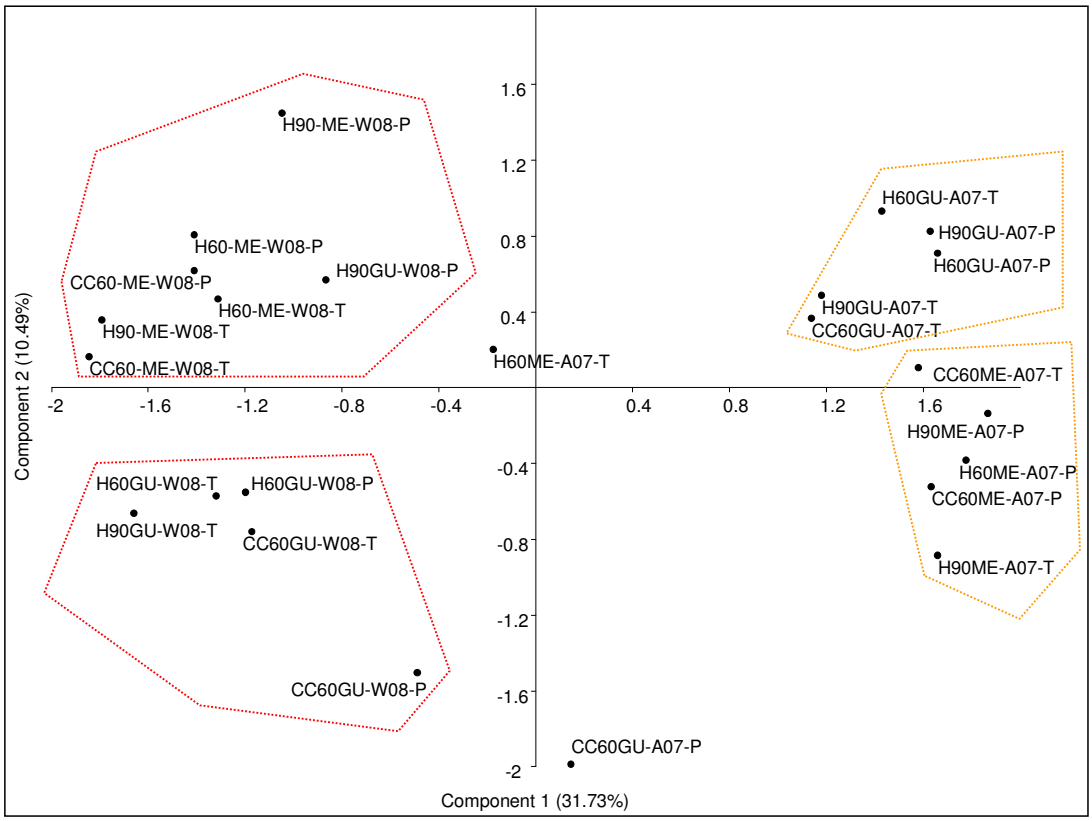


Figure 19. Principal Component Analysis based on the Jaccard distance of OTUs determined in calcium carbonate slurries sampled in autumn 2007 (A07) and winter 2008 (W08). The samples were collected either before biocide addition in the piping system (P) or after preservation in the storage tank (T).

Finally, relating the abundance data of certain OTUs to the total cell count determined by means of the CFII device showed that heavily contaminated samples (> 10^6 TCC ml^{-1} and fluorescence > 100 rfu) tended towards a narrow diversity because single dominant species had taken over and prevailed. In such cases, the relative abundance of the dominant species assessed by T-RFLP ranged up to 76% of the entire microbial community (e.g. H60ME-A07-T or CC60GU-W08-T). This fact might also be attributed, in part, to the failure of the biocide system applied to preserve the product in the storage tank thus promoting the proliferation of biocide-adapted or even biocide-resistant bacteria species. On the contrary, samples with lower total cell count (< 10^5 TCC ml^{-1} and fluorescence < 100 rfu) contained evenly distributed phylotypes.

3.2.3.3 Spatial and Temporal Analysis of Microbial Communities in WMD

During a period of one year (2008) calcium carbonate slurry samples, GCC and PCC, taken every quarter were subjected to T-RFLP analysis with the intention of investigating the occurrence of microbial community patterns. Overall, 56 OTUs were determined in all analysed samples and indicated an extensive microbial diversity in calcium carbonate slurries. Operational taxonomical units occurring at least a total of four times across all analysed samples are visualised in Figure 20. The fragment with the length 488 bp was retrieved in all analysed calcium carbonate slurry products at all sampling times. As mentioned before this fragment putatively originates from species belonging to the genus *Pseudomonas*. The OTUs with the fragment length of 157 bp and 398 bp were frequently found in the preceding analyses (Figure 16) but were not retrieved at each sampling time. Analysis of variance (ANOVA with Tukey's pairwise comparison) used to compare the occurrence of OTUs at different sampling times disclosed a significant variance between the samples from Winter 08 and Summer 08 ($p < 0.05$). Among all other sampling time points no significant variance of the occurring OTUs was observed.

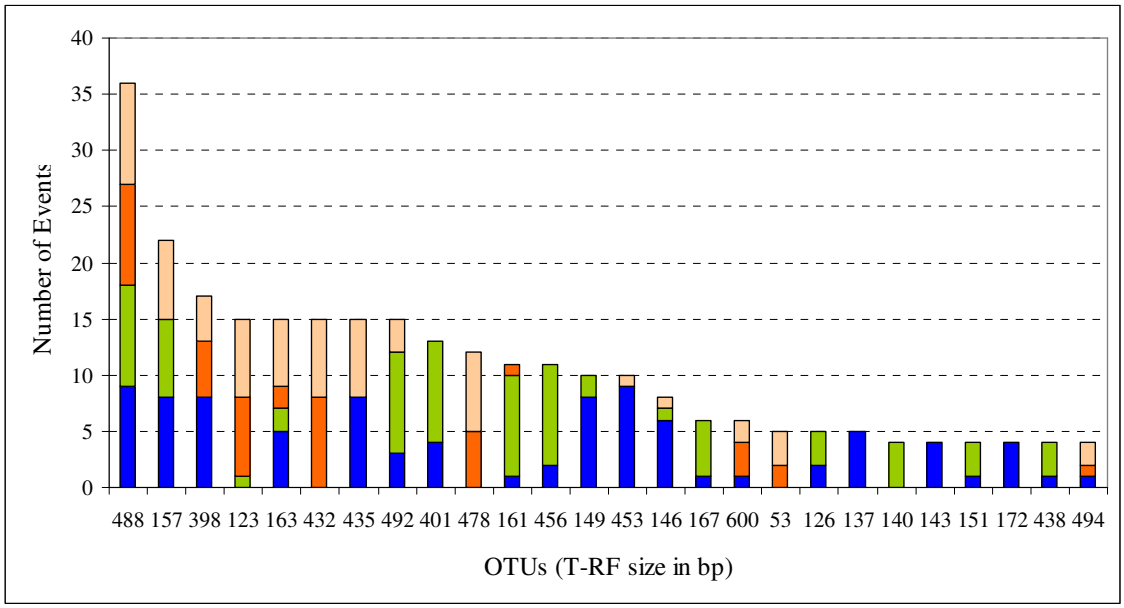


Figure 20. Ranked event plot of OTUs determined by T-RFLP in samples retained in winter 08 (■), spring 08 (■), summer 08 (■) and autumn 08 (■) from four Omya plants.

The relationship between the bacterial communities found in calcium carbonate slurries from different production plants were evaluated by means of Cluster Analysis (CA) and Principal Component Analysis (PCAs). Cluster analysis was carried out with the Bray-Curtis distance which is sensitive for abundance data (Figure 21). From the Cluster Analysis it was apparent that there was the formation of three distinct clusters. Two distinct clusters were formed by the samples collected in Winter 08 and Spring 08 with a minimum similarity within each group of 80% and 65%, respectively. The third cluster included all samples collected in summer 08 and autumn 08. Relevant similarity in bacterial community structure between the summer and autumn samples can be inferred, since no evident grouping in relation to the sampling period was resolved by CA. The synthetic calcium carbonate slurry Syncarb-GO (Sync) represented an outlier with a similarity of less then 10% in Summer 08 compared to the other samples and the placement of the Winter 08 sample into the spring cluster. Overall, the spring samples formed the more distinct cluster and were separated from the other seasons sharing a similarity below 50%.

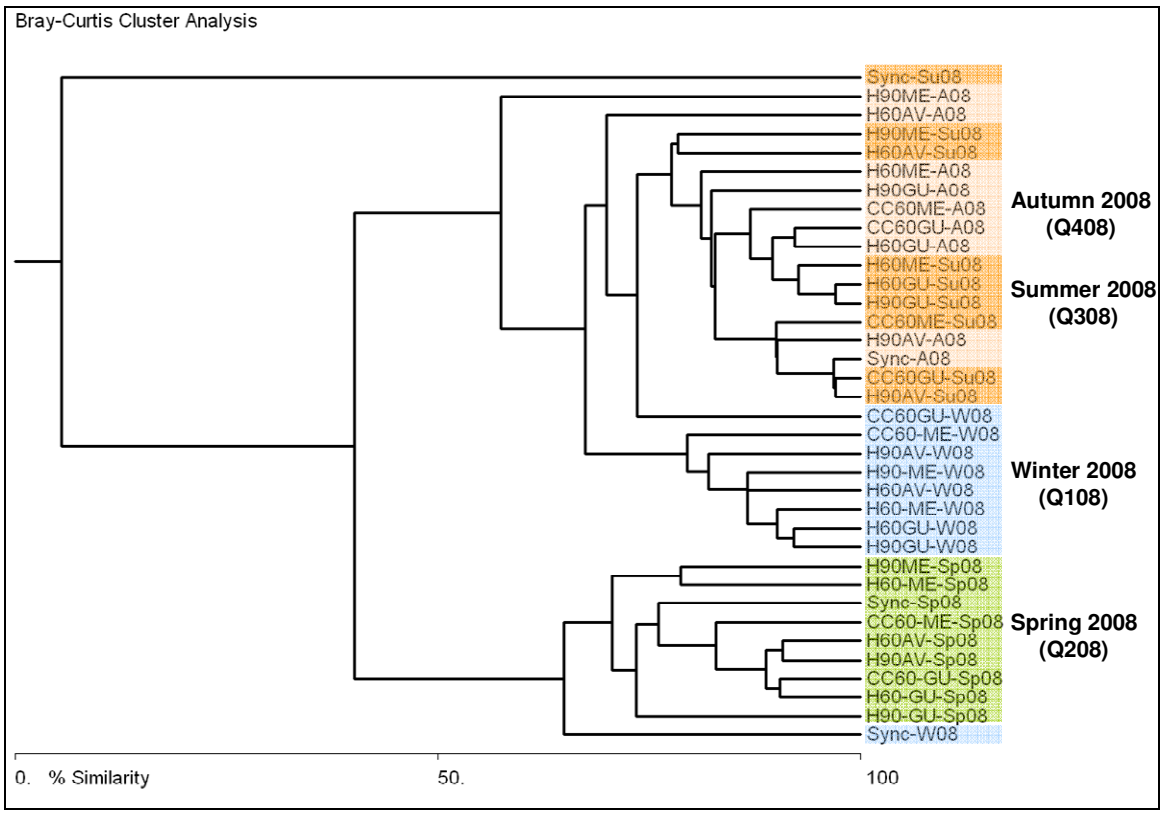


Figure 21. Cluster analysis based on the Bray-Curtis distance of OTUs determined in calcium carbonate slurries sampled quarterly in 2008.

These findings are emphasised by the principal component analysis of the T-RFLP abundance data reduced to presence/absence matrix by means of the Jaccard distance (Figure 22). Component 1 (x-axis) explained 25 % of the variance and samples were separated along this axis in relation to the sampling season uncovering a seasonal dependent shift of the bacterial community of calcium carbonate slurries. Component 2 (y-axis) explained 19% of the variance and along this axis samples were differentiated according to their similarity which increased toward a y-value of 0.

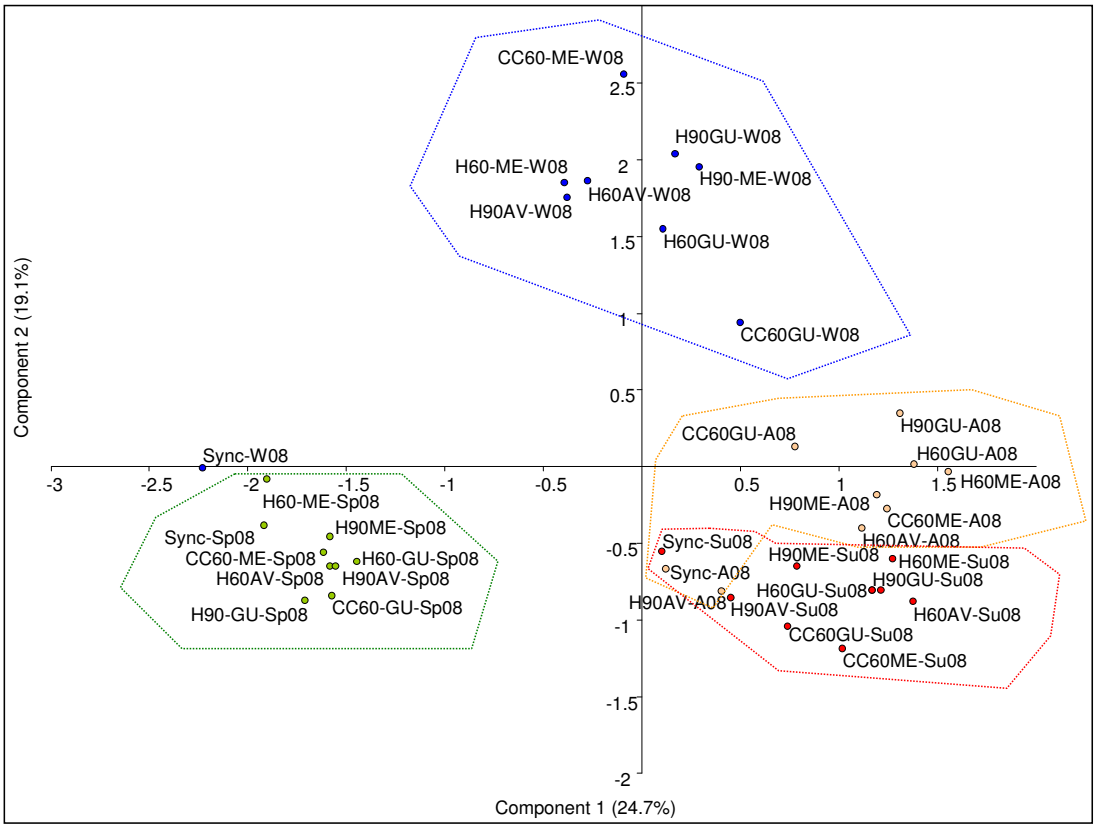


Figure 22. Principal Component Analysis based on the Jaccard distance of OTUs determined in calcium carbonate slurries sampled quarterly in 2008.

The summer and autumn samples clustered close together, although two separate groups along the Y-axis were formed. In addition, PCAs appeared to show an anticlockwise movement of the cluster patterns for successional seasons. According to the supposed axes interpretation, the transition of the bacterial population structure of calcium carbonate slurries in spring, summer and autumn implicates less variances compared to the alteration of the bacterial community structure in the winter season.

The prominent shift of the bacterial population in the winter season may be temperature driven and is more likely to result from faster cooling of the calcium carbonate slurry after the wet grinding stage and lower temperatures in the calcium carbonate slurry storage tanks. Presumably, abiotic factors such as temperature are influencing both the microbial community structure present in the raw material used to process calcium carbonate slurries (e.g., process water and crude marble) and the microbial diversity present in the plant facilities. Other studies investigating the dynamics of microbial populations in soil (Smit *et al.* 2001; Wolsing and Priemé 2004) or indoor environments (Rintala*, et al.*, 2008) have reported seasonal fluctuations in bacterial community structure.

3.2.3.4 T-RFLP Analysis of WMD Bacterial rRNA

T-RFLP analysis is typically performed at the DNA level i.e. no information can be concluded about the function and metabolic activity of the investigated bacterial communities. Several authors have suggested that the RNA content of bacterial cells shows close correlation with the physiological activity of the cells given that the content of ribosomes is increased in metabolic active cells compared to the ribosome content of stationary phase or even quiescent cells (Cenciarini-Borde*, et al.*, 2009, Ludemann*, et al.*, 2000, Mengoni*, et al.*, 2005, Williams*, et al.*, 1998). The fraction of active microbial cells can be estimated by analysing microbial communities by utilising 16S rRNA gene cDNA reverse-transcribed from 16S rRNA instead of DNA to perform T-RFLP analysis (RT-T-RFLP). It has been suggested that analysing microbial communities based on the reverse transcription of 16S rRNA provides the link between community structure and metabolic activity (Nocker and Camper, 2009, Nocker*, et al.*, 2007b).

T-RFLP analysis using either DNA or cDNA was carried out of three analogous calcium carbonate slurry products (Hydrocarb 90) sampled in autumn 2008 (Q408) at the Omya plant in Norway, Austria and Italy. Table 11 illustrates the categorisation of the T-RFLP occurrence and abundance data in the investigated samples. 21 distinct T-RFs were retrieved from both DNA and cDNA analysis. Whilst 9 T-RFs (43%) were detected exclusively in the DNA samples and 5 T-RFs (24%) were exclusively cDNA-derived, 7 T-RFs (33%) were shared between the

DNA and cDNA samples. This result are comparable with previous studies comparing 16S rDNA clone libraries generated either from DNA or cDNA isolated from soil which noted an intersection of 29% of the analysed sequences (Nogales, *et al.*, 2001). Moreover, the number of OTUs detected in respect to the utilised nucleic acids suggested that the DNA-derived profiles (16 OTUs) were more complex than the cDNA-derived profiles (12 OTUs).

Considerable reallocation of the microbial population centre was observed among all samples when the occurrence and the abundance of the found OTUs were compared. In the profiles generated from the DNA, the most prevalent T-RF, 488 bp, accounted for at least 30% of the abundance in all three samples. On the contrary, of the cDNA-generated profiles T-RF 488 bp was only found to be dominant in a single sample (H90-AV). In particular, the dominance shifted from T-RF 488 bp to T-RF 157 bp in samples H90-GU and H90-ME when cDNA was used instead of DNA for the T-RFLP analysis.

Table 11. Categorisation of DNA-based and cDNA-based T-RFLP data from calcium carbonate samples retained in autumn 2008 (Q408).

T-RF size (bp)	**H90-GU**	**H90-ME**	**H90-AV**	**H90-GU**	**H90-ME**	**H90-AV**
	T-RFLP based on DNA			T-RFLP based on RNA (cDNA)		
51				12.6	9.3	8.0
53	1.2					
76		39.9				
81				13.6	9.0	3.2
101				8.3	6.0	0.8
123	12.8	1.2			1.2	
146		1.2		23.3	26.7	3.2
149				7.4	4.3	1.6
157	9.6	3.8		32.0	38.4	8.3
163	1.5					
172				0.0	1.3	
398	1.7			1.3	1.4	
432	13.6	8.0				
435	1.2	1.9				
453	2.2					
462	1.2	1.7		1.6	2.5	
478	1.9	1.7	9.8			
488	49.2	39.2	88.6			73.4
492	2.7					
494	1.1	1.3				
600			1.6			1.5

Yellow coloured boxes are T-RFs that represent <10%, orange coloured boxes 10% to 30% and red coloured >30% of the abundance in each sample. Number in the boxes represents the percental abundance of each fragment within the analysed sample.

Based on the fact that the RNA content reflects the metabolic activity of bacteria, these findings support the assumption that T-RFLP analysis of samples H90-GU and H90-ME, utilising DNA as the source of the analysis, accentuated the presence of metabolic inactive (or even dead) phylotypes with a T-RF length of 488 bp. In comparison, the predominance of this phylotype in the sample H90-AV, independently of the used nucleic acids for the T-RFLP analysis, indicated a high metabolic activity of this bacterial community member. In a similar fashion, the dominance of the 157 bp T-RF based on the analysis of cDNA of the samples H90-GU and H90-ME demonstrated the presence of this phylotype in a metabolic activity state at the sampling time.

Additionally, as evident from the results, the presence of T-RFs from DNA and cDNA analysis only partially overlapped. In this context it is necessary to highlight the limitations of interpreting the results because of the additional bias introduced by the reverse transcription of DNA to cDNA i.e. additional biases introduced due to nucleic acid extraction and PCR amplification of the 16S gene of mixed bacterial populations (Mengoni*, et al.*, 2005). The lack of certain T-RFs originates from the preferential amplification of abundant bacterial species, hence rare species can be missed within a heterogeneous microbial community (Kitts, 2001, Liesack and Dunfield, 2004, Orcutt*, et al.*, 2009, Suzuki and Giovannoni, 1996).

Cluster Analysis showed a distinct clustering of the cDNA-derived profiles suggesting that the metabolically active community members in all three analysed calcium carbonate slurry products were similar (Figure 23). The lowest level of similarity of the cDNA-derived profiles was 60% and the highest 80%. The sample H90-AV-DNA represented an outlier within the DNA-derived profiles because in this sample only 3 OTUs were retrieved and a disproportionately high abundance of the T-RF 488 bp was detected. Again, such narrowing of T-RF diversity and increase in abundance of single T-RFs may result from the preferential amplification of DNA from overrepresented bacterial species or from the dominance of a single species.

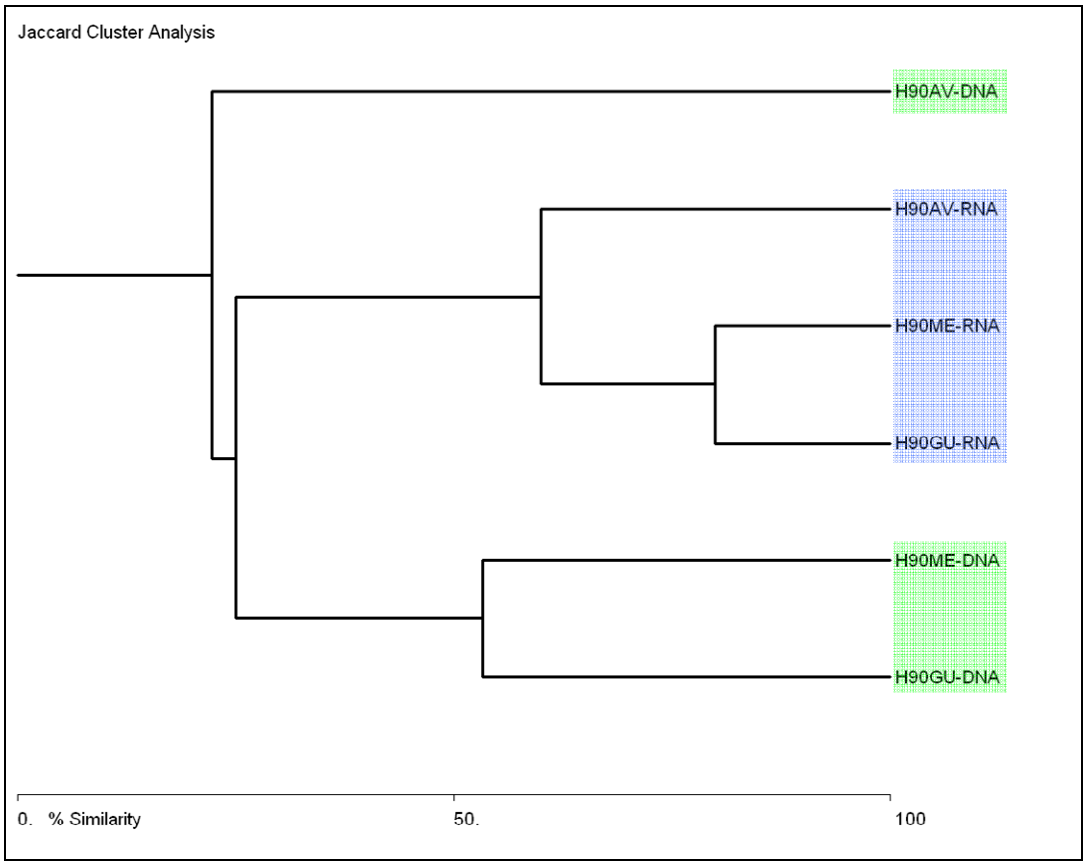


Figure 23. Cluster analysis based on the Jaccard distance of OTUs determined by T-RFLP analysis of DNA or RNA (cDNA) retrieved from calcium carbonate slurries sampled in autumn 2008 (Q408).

Taking a closer look at the total cell counts and at the physiological activity data gathered by means of the CFII device strengthens the assumption that sample H90-AV was dominated by the species with the T-RF 488 bp. This was the only sample with a total cell count higher than 10^4 ml^{-1} and a relative fluorescence above 60 rfu ($1 \cdot 10^6$ TCC ml^{-1} and 107 rfu). On the other hand in this sample the T-RF with a length of 488 bp occurred with an abundance of over 70% both in the DNA-based and in the cDNA-based T-RFLP profiles, this indicated the presence of this OTUs in fact in the active physiological condition. In the following section the differentiation of metabolically active and inactive members of the bacterial populations found in calcium carbonate slurries was further accomplished using flow cytometry combined with Fluorescence Activated Cell Sorting (FACS).

3.2.3.5 T-RFLP Analysis of WMD Bacteria Sorted by FACS

In general the analyses of microorganisms embraces the assumption of dealing with homogenous populations and the studies outcomes are expressed to be average values of bulk measurements (Muller and Davey, 2009). Instead, single cell measurements can reflect the authentic heterogeneity of microbial populations (Nebe-von-Caron, *et al.*, 2000). Analogous to CellFacts II®, Flow Cytometry (FCM) combines the measurement of size, shape and fluorescence of bacterial cells, hence allowing multiparameter analysis of single bacterial cell (Bergquist, *et al.*, 2009). A vast range of fluorescent dyes are available on the market targeting physical and biological cellular entities in order to make quantifiable cellular characteristics. Even though viability is still defined as demonstrable reproductive growth, with FCM, parameters such as metabolic activity, membrane integrity and membrane permeability are analysed to characterise the physiological state and to describe the viability of bacterial cells (Nebe-von-Caron, *et al.*, 2000). The two fluorescent dyes, propidium iodide (PI) and SYTO9 (S9), have been extensively used to differentiate between bacteria with intact and damaged cytoplasmic membranes (Berney, *et al.*, 2007). Therefore, an approach to resolve the correlation between microbial population structure and activity is the analysis of individual cells within microbial communities with apparent differing membrane integrities: (i) "viable" and active cells with intact membrane, (ii) "dead" and inactive cells permeant to PI and (iii) compromised cells referred to as "injured" (Ben Amor, *et al.*, 2005, Gregori, *et al.*, 2003). However, the uptake of PI strongly depends of the physiological state on the cells and even though under certain conditions transient PI uptake by bacterial cells has been observed, none of the staining techniques ensures the assessment of viability (Shi, *et al.*, 2007). Nevertheless, the combination of metabolic activity measurement coupled to flow cytometric cell sorting and down-stream molecular techniques offers enormous potential for the investigation of the biodiversity of mixed microbial communities.

Of three calcium carbonate slurries sampled in autumn 2008 (Q408) at the Omya plant in Austria (PCC), Germany and Italy (both GCC) T-RFLP analysis was carried out from the whole bacterial population as well as from flow cytometry sorted subpopulations (live, dead and compromised). The analysis of WMD bacteria

by flow cytometry, however poses a challenge to the methodology used in the separation of the bacterial cells from the calcium carbonate matrix. Bacterial cells were pooled from quadruple Nycodenz isolations in order to increase the density of the cells prior to FCM analysis. This was essential since the counting error ($n^{0.5}$) only moves below 1% if above 5000 events are investigated (Nebe-von-Caron, *et al.*, 2000), and secondly because a certain concentration of cells is needed for down-stream nucleic acid isolation of sorted cells. Table 12 illustrates the number of sorted cells in each subpopulation. The highest concentration of cells in the "live" region, namely 76% of the total number of events, was recovered in the PCC sample. In contrast to this, comparable percental quantities of cells in the "dead" region (44% to 53%) and "compromised" region (24 to 25%) were observed in the H90 and H60 samples.

Table 12. Details of FCM analysis and sorted WMD microbial populations.

Sample ID	**No. of Events on the FACS (%)**			
	Live	**Dead**	**Compromised**	***Total***
Syncarb-GO	406364 (76%)	106630 (20%)	20035 (4%)	533'029
H90-AV	17895 (32%)	24589 (44%)	12999 (24%)	55'483
H60w-BG	23489 (22%)	55673 (53%)	26093 (25%)	105'255

Figure 24 shows the cytogram of green fluorescence (FL1) versus red fluorescence (FL3) for the bacterial cells retrieved from calcium carbonate and stained with PI and SYTO9. Cut off boundaries to separate the noise signal from the bacterial signal were defined by gating on the forward and side scatter. The cells were sorted based on the position on the fluorescence scatter by defining the region of interest (live, dead and compromised). Typically for simultaneous staining with PI and SYTO9 there is a split of the bacterial population into three bacterial clusters reproducing green fluorescent particles representing "live" cells, red fluorescent particles representing "dead" cells and intermediate particles constituting "compromised" cells with transiently permeable membranes to PI.

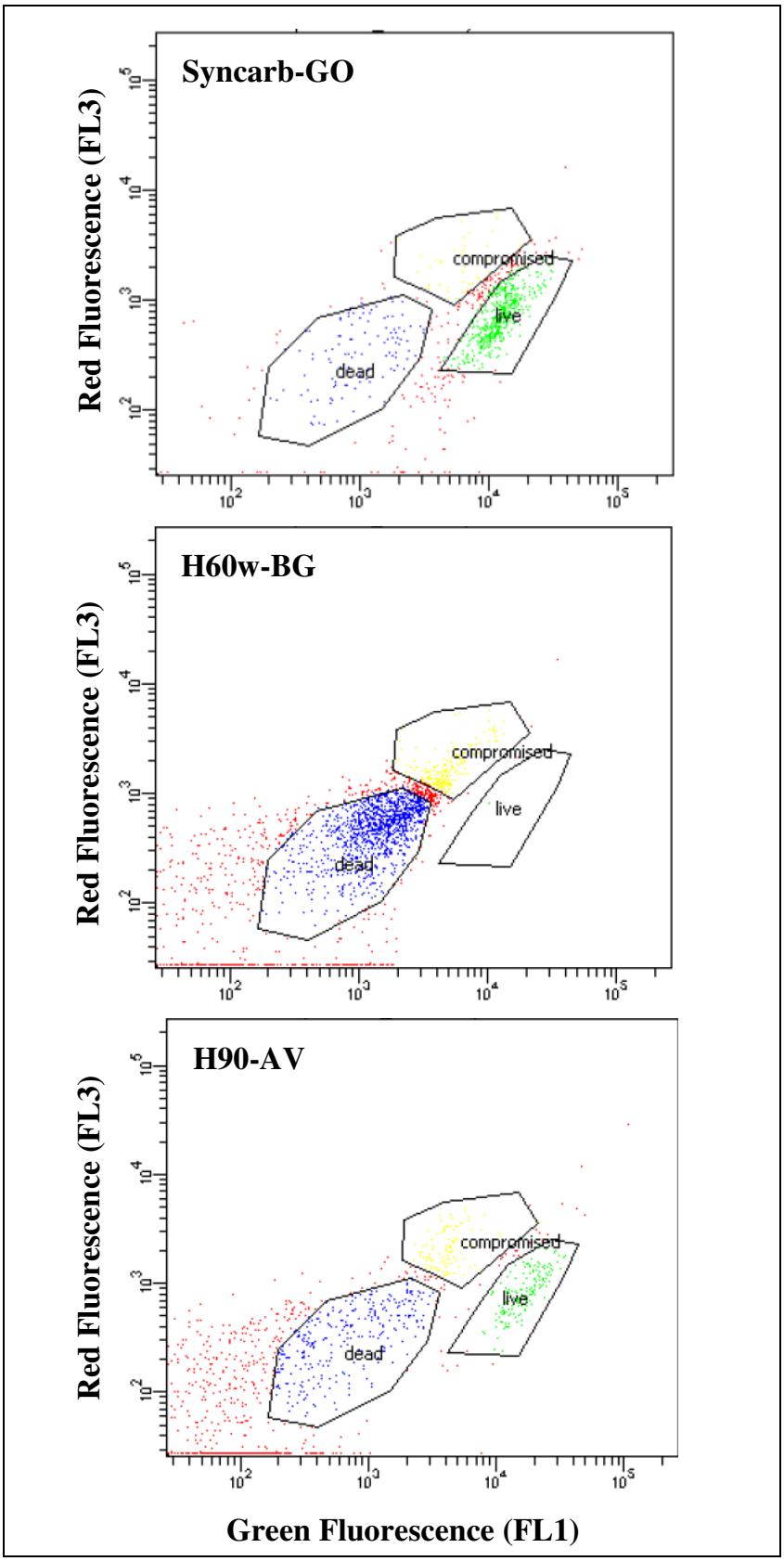


Figure 24. Flow cytometric analysis of bacterial cells extracted from calcium carbonate slurries sampled in autumn 2008 (Q408). "Live", "Dead" and "Compromised" cells are delimited by regions of interest on each cytogram.

Nevertheless, the state of the cytoplamic membrane of these intermediate states has been affiliated to the degree of membrane damage because of the interaction of low PI concentration and SYTO9 in such cells emitting both green and red fluorescence (Ben Amor*, et al.*, 2005).

Furthermore, as evident from the cytograms in Figure 24 the bacterial populations inhabiting calcium carbonate slurries are heterogeneous and for the samples containing a high amount of dead biomass, H90-AV and H60w-BG, particles were visible in the lower left corner which may be cell debris.

Categorisation of the T-RFLP analysis of the total bacterial population revealed distinct profiles compared to those obtained for the sorted viable, dead and compromised cells (Table 13). In each of the analysed calcium carbonate samples the number of occurring OTUs from the total bacterial population was less compared to the number of OTUs found in the three subpopulations. Above all, in the Syncarb-GO sample 11 OTUs were retrieved in the dead subpopulation whereas by using the entire microbial population only a single OTU was retrieved indicating the predominance of viable species with a common T-RF length. These findings differ from data obtained for subpopulations of faecal microbiota analysed by denaturating gradient gel electrophoresis (DGGE) disclosing in all subpopulations a less complex community (Ben Amor*, et al.*, 2005). The differences in the T-RF profiles between the total bacterial community and the sorted subpopulations can be attributed to the elevated quantity of dead biomass in the analysed samples. In fact, the prevalent T-RF of each total community profile was found in the "dead" subpopulation. As mentioned in section 3.2.3.4 the amplification of T-RFs from underrepresented species is biased by the presence of dominating species. Furthermore, the samples Syncarb-GO and H90-AV, showed a depleted bacterial diversity based on the T-RFLP data from the total bacterial community. The presence of common T-RFs in different subpopulations confirmed the physiological heterogeneity of the bacterial cells in calcium carbonate slurries.

Table 13. Categorisation of the T-RFLP data from FACS sorted WMD bacterial cells.

T-RF size (bp)	Syncarb-GO Q408				H90-AV Q408				H60-BG Q408			
	T	L	C	D	T	L	C	D	T	L	C	D
53				1.0								
57											4.3	
68												1.0
115				1.5					71.4		24.9	56.4
130										1.1		
132											7.0	
135				1.0						2.0		
137				1.0								
143				1.0					5.8		49.2	27.6
146			1.0				1.0	1.0	14.3	10.0		
149			1.3	2.1			1.6	1.9		7.0		1.9
154									2.2		2.0	
157				2.6			1.3	1.7		8.4		2.0
161										1.9		
163			31.6	3.7				1.4		2.3	2.6	1.2
175									3.6		1.1	
207									2.6			
268										1.8		
297				2.6								
301										2.5		
398										1.0		
403										2.3		
432								1.0		2.0		
435										2.7		1.0
438								1.0		3.1	2.6	3.1
451										2.0		
462										2.8		
478					9.8		1.2	19.8				
485								1.8				
488	100	97.3	64.8	81.0	88.6	99.1	93.4	70.8		47.9	4.4	5.9
492										1.0		
600		1.5		1.4	1.6	1.0	1.5					

Total cells (T), Live cells (L), Compromised/Injured cells (C) and Dead cells (D).Yellow coloured boxes are T-RFs that represent <10%, orange coloured boxes 10% to 30% and red coloured >30% of the abundance in each sample. Number in the boxes represents the percental abundance of each fragment within the analysed sample.

Of the sample H90-AV, T-RFLP profiles based on both cDNA and flow cytometry cell sorted subpopulations were available. Owing to the overlap of the found OTUs, correlation between profiles generated from cDNA and sorted cell analysis was confirmed (Table 14). None of the T-RFs found in the cDNA-based profile were shared with OTUs of the dead cell pool exclusively.

Table 14. Comparison of the T-RFLP profiles generated from cDNA and FACS sorted WMD bacterial cells.

T-RF size (bp)	H90-AV Q408				
	T	L	C	D	cDNA
53					8.0
81					3.2
101					0.8
146			1.0	1.0	3.2
149			1.6	1.9	1.6
157			1.3	1.7	8.3
163				1.4	
432				1.0	
438				1.0	
478	9.8		1.2	19.8	
485				1.8	
488	88.6	99.1	93.4	70.8	73.4
600	1.6	1.0	1.5		1.5

Total cells (T), Live cells (L), Compromised/Injured cells (C), Dead cells (D) and profile generated from total cells RNA (cDNA). Yellow coloured boxes are T-RFs that represent <10%, orange coloured boxes 10% to 30% and red coloured >30% of the abundance in each sample.

These data provide an indication for the assessment of active bacterial members within mixed populations by performing RNA-based T-RFLP analysis. However, cDNA-generated T-RFLP analyses not only retrieved active cells but at least recognised compromised cells which per definition are categorised to be rather "dead" than active (Nebe-von-Caron*, et al.*, 2000). A possible explanation for the detection of compromised cells via cDNA-based T-RFLP is the residual amount of rRNA contained by the compromised cells which cannot be omitted during nucleic acid extraction (Röling and Head, 2005).

To conclude, the T-RFLP profiles of bacterial cells obtained by sorting subpopulations according to their membrane integrity can overlap due to the intended and defined specification of the boundaries enclosing populations with certain characteristics. For that reason the T-RFLP profiles of flow cytometry sorted subpopulations and from the unsorted populations are to a limited extent congruent. Boundary definitions between live, compromised and dead cells cannot be defined definitively and therefore differentiation between bacteria with intact and compromised cytoplamic membrane has to be regarded as an intermediate transition (Barer and Harwood, 1999, Kell*, et al.*, 1998).

3.2.3.6 Propidium Monoazide Viable Cell Enrichment

For many years the discrimination of viable cells in environmental samples by means of molecular techniques has posed a challenge to environmental microbiologist. A drawback when analysing environmental microbial populations applying DNA-based techniques is that the elucidated community structures do not reflect the proportion of live cells. Several studies have shown that bacterial DNA can persist in the environment for a long time after cell death (Cenciarini-Borde*, et al.*, 2009, Nocker*, et al.*, 2007a). Therefore, it can be assumed that a certain portion of the microbial community composition elucidated via DNA-based analysis originates from dead cells and this makes microbial risk evaluation even more difficult. Moreover, the effectiveness of disinfection or preservation treatments such as in practice the application of biocide to preserve calcium carbonate slurries cannot be evaluated by means of molecular techniques. The use of cDNA reverse-transcribed from rRNA and fluorescence-activated cell sorting after staining the cell with viability tracking dyes (e.g. PI/SYTO9) have been used to estimate the fraction of metabolically active cells in environmental samples (Kramer*, et al.*, 2009, Nocker*, et al.*, 2007a) and were applied to calcium carbonate samples in the preceding sections of this study.

Novel approaches making use of PCR amplification inhibition of DNA originating from cells with compromised cell membranes, which per definition are inactive or even dead, by the DNA-intercalating dye propidium monoazide (PMA) have been described (Kramer*, et al.*, 2009, Nocker*, et al.*, 2007a). The chemical structure of PMA is identical to PI and likewise PMA is impermeant to cells with intact cell membranes, hence PMA has been shown to be of use for the discrimination of live/dead cells (Nocker*, et al.*, 2007a). Once the cell membrane becomes damaged as a consequence of external impacts (e.g. biocide treatment) PMA penetrates the cell membrane and intercalates with the DNA of compromised cells. Upon exposure to light, the PMA azide group covalently binds to the DNA and consequently the PCR amplification of such DNA is completely inhibited. Therefore, analysing PMA treated DNA from bacterial populations reflects the structure of metabolically active/viable cells. PMA-PCR is in fact an effective tool to discriminate between live and dead cells in both gram-positive and gram-negative bacteria (Cenciarini-Borde*, et al.*, 2009). The preferential detection of metabolically

active bacteria in distinction to “growth enrichment” has been termed as “molecular enrichment” (Nocker and Camper, 2009). However, in this context it is important to highlight that the PMA approaches can only detect compromised cells based on changes in the membrane integrity and the fact that a bacterial cell holds an integral membrane is not proof of activity in respect to substrate uptake, biotransformation or respiration (Nocker*, et al.*, 2007a).

The objective of the following analyses was to evaluate the applicability of PMA to assess the population structure for metabolically active cells isolated from calcium carbonate slurries. Additionally, the antimicrobial efficacy of biocide in calcium carbonate slurries was monitored by determining the slurry bacterial fraction enduring a single biocide challenge. Hydrocarb 60 and Hydrocarb 90 calcium carbonate slurries from the Italian plant in Avenza (AV) with high total cell count and metabolic activity as determined by CFII (> 10^6 TCC ml^{-1} and fluorescence > 200 rfu) were analysed. The T-RFLP profiles of both native and biocide treated samples were determined directly or upon treating the cells with PMA. Bacterial cells were isolated from calcium carbonate slurries by means of the Nycodenz method prior to the treatment with PMA. The electropherograms in Figure 25 visualise the PCR product yields of 16S rRNA gene analysis of DNA isolated from calcium carbonate bacterial cells. The electropherograms in the first two rows suggested that a high yield of PCR product is achieved when analysing both cells from native slurries and cells from biocide-free slurries incubated at 30ºC for 24 h. Taking a closer look at the differences in the peak heights revealed a rise of the amplicon concentration in PMA treated cells (H60 overall). A possible explanation for these differences is the reduction of amplifiable DNA template due to the partial inhibition of the DNA pool originating from compromised bacteria by PMA and consequently an increase in the amplicon yield due to the lower template competition in the PCR reaction. On the contrary, the two lower electropherograms emphasise that in biocide treated samples PMA treatment of the bacterial cells led to a significant reduction of PCR product yield. It can be suggested that the membrane of biocide treated cells had been compromised as a result of the biocide activity and therefore the DNA of these cells was almost totally inhibited by PMA. However, bacterial species able to persist were present in the biocide treated sample since a small amount of PCR product was amplified after PMA treatment.

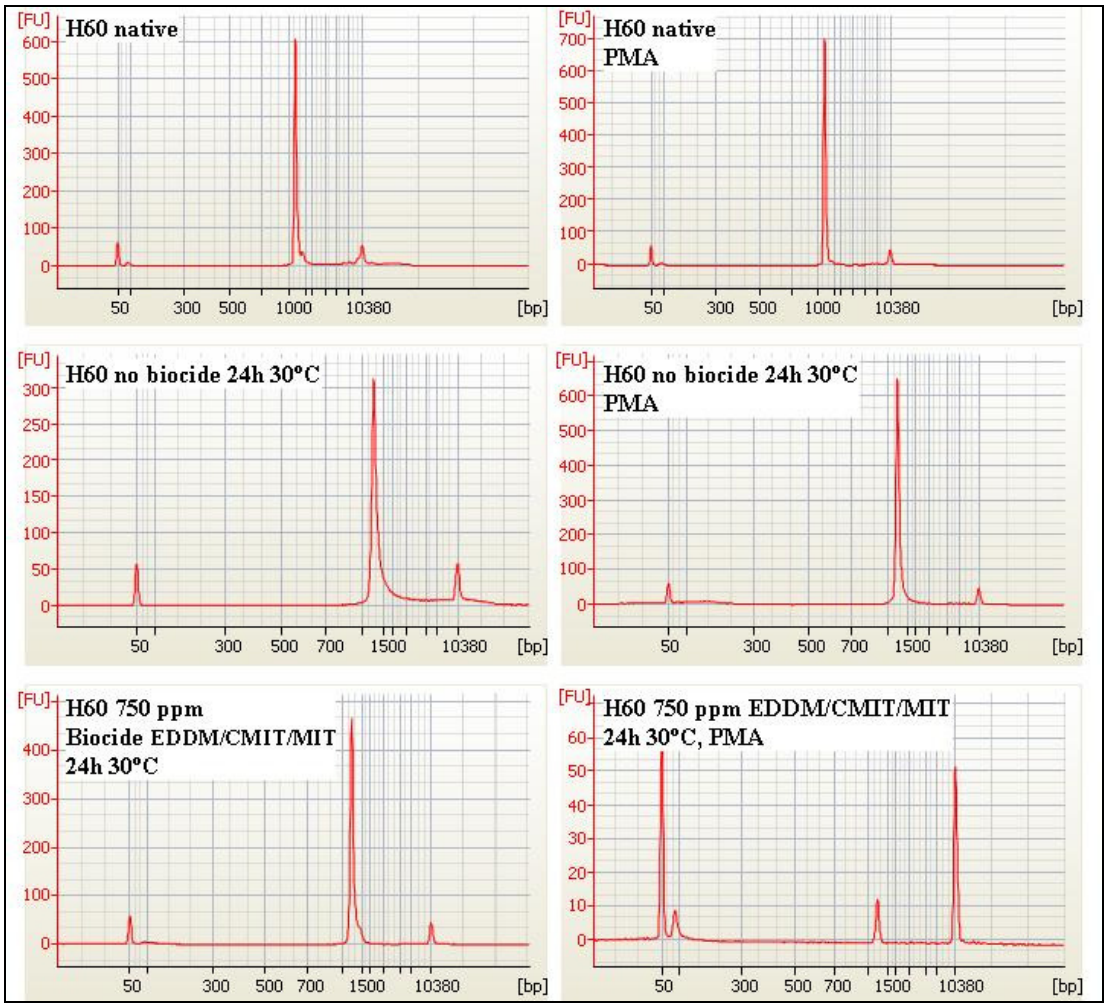


Figure 25. Effect of PMA on the 16S rRNA gene PCR analysis of bacterial communities retrieved from calcium carbonate slurries. Electropherograms were recorded by means of the Agilent Bioanalyzer. Peaks at 50 bp and 10,380 bp represent the lower and upper marker and were used to align the samples. Y-axis is displayed in relative fluorescence units (FU). Biocide concentration ppm (c/l).

T-RFLP profiles obtained from the 16S rRNA gene PMA-PCR products described above are shown in Figure 26. In respect to the number of OTUs the T-RFLP profiles observed in the native samples and in the samples incubated at 30ºC for 24 h were similar. Apparently, incubation at 30 ºC favoured the proliferation of some bacterial species and as a result the bacterial population structure changed at the expenses of rare species. As mentioned above, PMA treatment lead to a reduction of the amplifiable DNA pool, thus to a loss of OTUs. The treatment with PMA accentuated the relative abundance of probably metabolic active cells with PMA impermeant cell membranes.

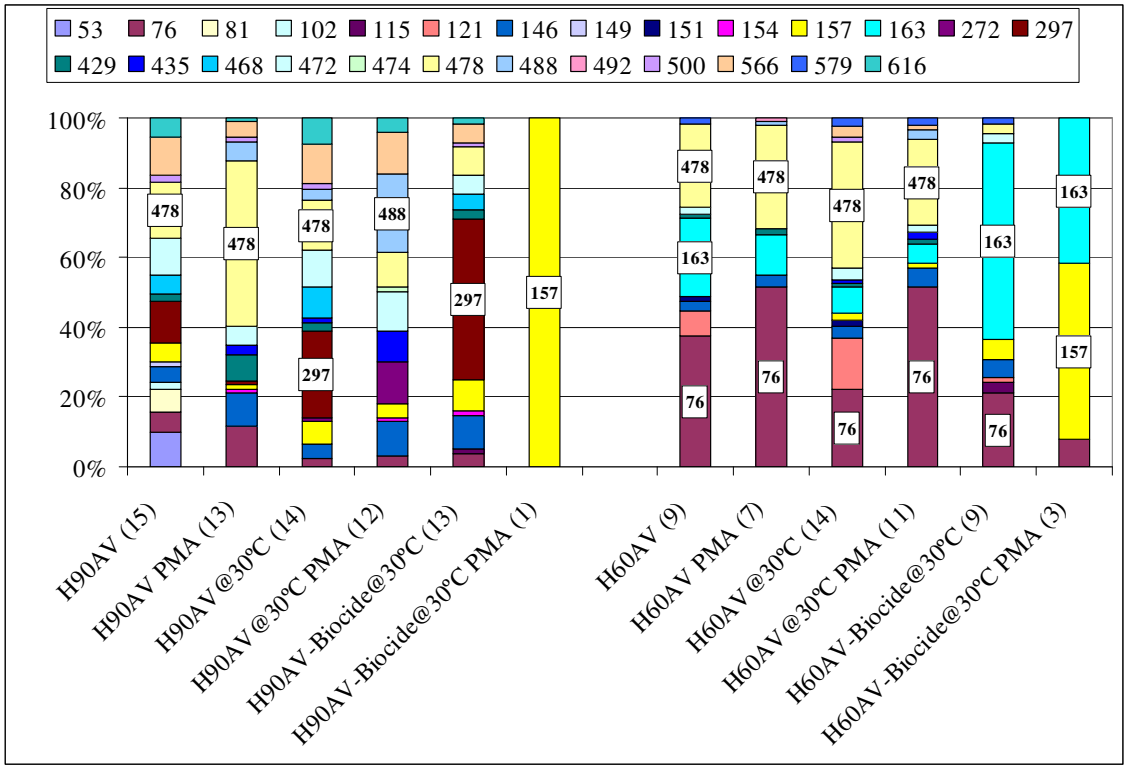


Figure 26. T-RFLP profiles of 16S rRNA gene PMA-PCR products from calcium carbonate slurries H90-AV and H60-AV samples, which were supplemented either with or without biocide (750 ppm c/l EDDM/CMIT/MIT formulation). Samples were analysed after treating the cells either with or without PMA prior to extraction and amplification of DNA. Numbers in brackets represent the number of determined OTUs.

In the native H90 sample the OTU with a T-RF length of 478 bp was found to be the dominant live species with an intact cell membrane, whereas in H60 the OTUs 76 bp and 478 bp accounted for the majority of PMA-impermeant cells (i.e. live cells). A significant increase in the occurrence of OTU 488 bp (putative *Pseudomonas* sp.) in the PMA treated sample was observed in H90 slurry after incubation at 30 ºC. In contrast, the dominating species in H60 slurry after incubation at 30 ºC were still OTU 76 bp and 478 bp. Biocide supplemented samples were strongly affected in respect to the T-RFLP profiles of PMA treated cells. Overall, there was a remarkable reduction of the number of detected T-RFs in the biocide supplemented treatments after exposing the cells to PMA. In H90 slurry a single OTU with a T-RF length of 157 bp was detected whilst in H60 slurry three OTUs, namely 76, 157 and 163 bp, were retrieved. These results indicated that the membrane of these bacterial species remained impermeable to PMA even after a challenge with biocide. Therefore, it can

be assumed that these bacteria species survived the biocide challenge and are able to recover once the activity of the biocide is depleted. Nevertheless, in the biocide treated samples no viable count was detected on solid media (PCA) suggesting that the bacterial species found to possess an intact cell membrane due to the exclusion of PMA might entered a dormant state referred to as "Active-But-Non-Culturable" (ABNC) (Kell*, et al.*, 1998, Nocker and Camper, 2009, Nocker*, et al.*, 2007a).

Cluster analysis using the Jaccard distance, taking into account the presence or absence of distinct OTUs, shed light on the relationship between the samples after the treatment with PMA (Figure 27). The PMA treated samples after the biocide challenge of both products as well as all the other samples of the respective products H90 and H60 formed three distinct clusters.

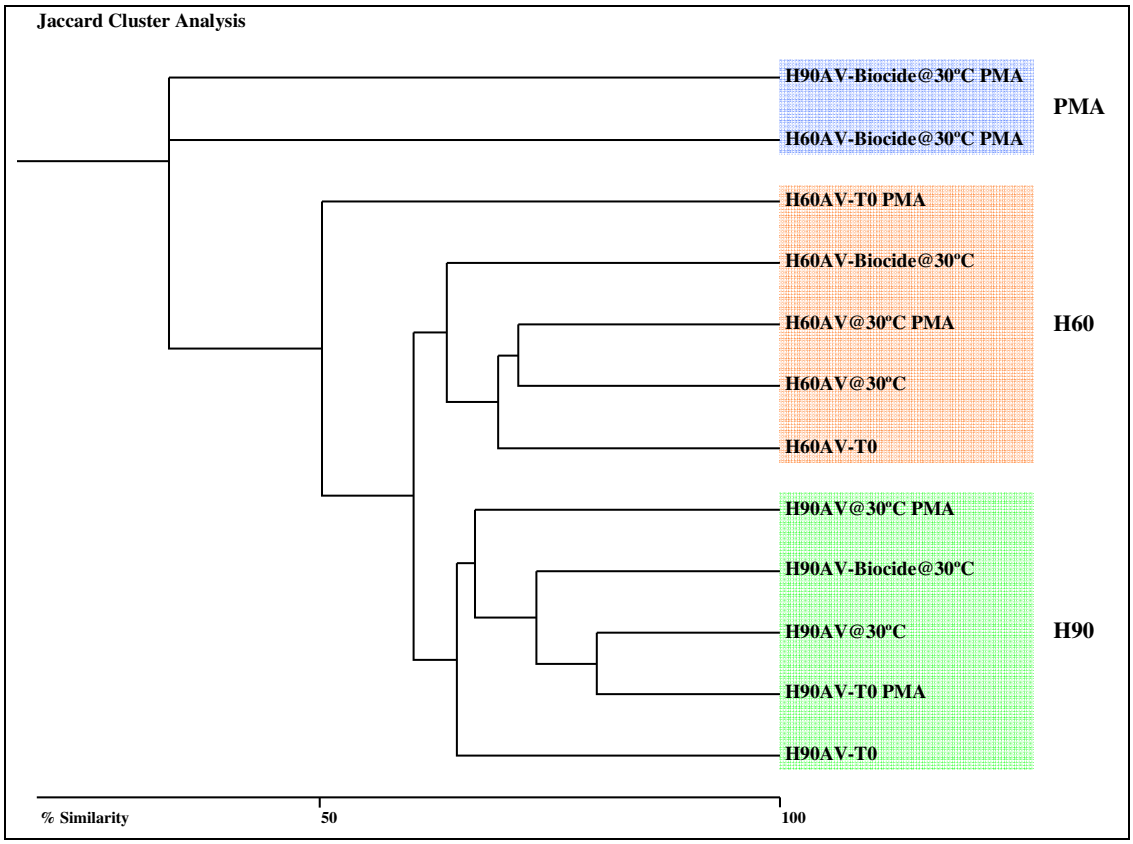


Figure 27. Cluster analysis based on the Jaccard distance of OTUs determined in biocide-free or biocide supplemented calcium carbonate slurry samples after PMA-PCR. Samples were either subject to PMA treatment or not. T0 = native samples; Biocide = treated with 750 ppm (c/l) EDDM/CMIT/MIT biocide for 24 h; @30 ºC = incubated for 24 h at 30 ºC.

The PMA treated samples after the biocide challenge segregated from the rest of the samples as a result of the narrowed diversity of bacterial cells impermeable to PMA. In contrast to the native H60 product (H60AV-T0), the incubation of the product at 30 ºC lead to an increase in the similarity (71%) between the PMA treated and non-PMA-treated sample probably originating from the enrichment of OTUs with PMA-impermeable membranes (i.e. live cells). In the H90 product the greatest similarity (80%) was reported for the native H90 sample treated with PMA and the non-PMA-treated incubated H90 sample indicating a preferential detection of OTUs with intact membranes after incubation even though the PMA treatment was not performed. The abundance ratios suggested that such patterns may result from growth induction due to the incubation at 30 ºC and therefore the dominance of individual OTUs leading to a detection failure of low-abundance OTUs. According to the T-RFLP profiles in Figure 26 there was a significant alteration in the abundance proportions within the bacterial population as well as an increase of specific OTUs in the incubated non-PMA-treated sample at the expense of other OTUs represented in the native PMA-treated sample. In this specific case, the abundance of OTU 478 bp in the native PMA-treated H90 sample decreased from 48% to 14% whereas OTU 297 bp increased from 1% to 25%.

In adopting the PMA approach to calcium carbonate slurry bacteria similar limitations were encountered in relation to the preferential PCR amplification of highly abundant templates limiting the resolution of the T-RFLP methodology as described in the preceding sections. In addition, it was observed that low abundance OTUs detected in PMA-treated samples frequently were not detected in the related non-PMA-treated sample, thus these low-abundance OTUs were only detectable once the pool of amplifiable DNA had been reduced by the performance of PMA.

3.2.4 Phylogenetic Analysis of WMD Bacteria

The composition of microbial communities and the phylogenetic relationship of the species inhabiting calcium carbonate slurries were further investigated using 16S rRNA gene sequencing. In a first attempt, 16S clone libraries were constructed to examine the microbial diversity of calcium carbonate slurries prior to and after the addition of biocide. Biocide-free samples were collected after the slurry grinding procedure whereas biocide containing samples were retrieved from the storage tank. However, Sanger-based sequencing is expensive and time consuming and above all to achieve significant species coverage and resolution the analysis of hundreds of clones is needed (Janssen, 2006). These constraints were resolved by adopting tag-encoded deep amplicon sequencing on a large set of calcium carbonate slurry samples to estimate the diversity of microbial communities inhabiting such particular matrices.

3.2.4.1 Clone Libraries

From the calcium carbonate product H90-ME sampled in winter 2008 (Q108) both with and without biocide two clone libraries were generated. A total number of 96 clones, 48 clones per library, were obtained and an average 16S rRNA gene length of 507 bp was sequenced. After excluding low quality samples from the analysis, using 3% sequence distance as a criterion to define OTUs, 39 OTUs were detected among 87 analysed sequences. Individual examination of the samples without and with biocide revealed 23 and 20 OTUs, respectively. The phylogenetic classification and the number of representative sequences per bacterial division, as well as T-RF values obtained from the MICA database (Shyu*, et al.*, 2007), are shown in Table 15.

Table 15. Phylogenetic breakdown of 16S rRNA gene sequences retrieved from clone libraries obtained from H90-ME (Q108) with and without biocide.

		No. of clones observed in each sample[b]			T-RF (bp)[c]
Taxonomic affilation	Closest relative[a]	Total	without biocide	with biocide	*in-silico*
Cyanobacteria		4			
	Uncultured cyanobacterium		0	4	
Actinobacteria		14			
	Brevibacterium sp.		1	0	81-82/145-167
	Leifsonia sp.		0	1	68/163/165/281
	Microbacterium oxydans		0	2	279-282
	Propionibacterium acnes		5	1	165
	unclassified Actinomycetales		0	4	
Bacteroidetes		10			
Sphingobacteria	*Pedobacter* sp.		8	1	434/540
	Unclassified Sphingobacteriales		1	0	
Proteobacteria		52			
Gammaproteobacteria	*Moraxella osloensis*	2	0	1	159
	Unclassified Gammaproteobacteria		1	0	
Alphaproteobacteria	*Afipia* sp.	8	0	3	152/197
	Bradyrhizobium sp.		0	1	152
	Caulobacter fusiformis		2	0	150
	Unclassified Bradyrhizobiaceae		1	0	152
	Unclassified Alphaproteobacteria		1	0	
Betaproteobacteria	*Ralstonia insidiosa*	39	5	10	86/430
	Leptothrix sp.		1	0	135/486
	Unclassified Incertae sedis 5		1	2	
	Bordetella sp.		6	3	
	Achromobacter xylosoxidans		2	4	490
	Unclassified Alcaligenaceae		2	2	
	Unclassified Burkholderiales		1	0	
	Unclassified Proteobacteria		3	0	

a. Closest relative according to the RDPII databases (Naïve Bayesian rRNA Classifier Version 2.0, July 2007)
b. Assignment confidence threshold 80% (bootstrap confidence)
c. Values from database MiCA: Virtual Digest (ISPaR) (Shyu *et al.* , 2007)

The recovered phylotypes were affiliated to four distinct phyla: *Cyanobacteria, Actinobacteria, Bacteroidetes and Proteobacteria.* Overall, these bacterial lineages are typically found in 16S rRNA gene clone libraries assembled from both soil and freshwater sediments (Jackson and Weeks, 2008, Van Elsan*, et al.*, 2007). Phylotypes related to the phylum of *Cyanobacteria* were only found in the biocide supplemented sample taken from the storage tank. However, the majority of the phylotypes, namely 60%, were affiliated with the *Proteobacteria*, whereof 45% belonged to the class of *Betaproteobacteria*. Across all identified phylotypes, the bacterial species *Pedobacter* sp., *Bordetella* sp. and *Propionibacterium* sp. were

found to be dominant in the biocide-free samples, whereas in the biocide containing sample *Ralstonia* sp. were the most common found species. Similar findings were reported for biocide treated cave halls where bacteria of the genus *Ralstonia* and *Pseudomonas* have been found to predominate and had replaced the native bacterial population as a result of the biocide treatment (Bastian*, et al.*, 2009).

Further two calcium carbonate slurry products, namely H90-GU and H60-GU sampled in winter 2008 at the Austrian plant, were analysed by 16S rRNA gene sequences retrieved from clone libraries. Both samples were drawn from the storage tank and contained biocide at the sampling time point. Table 16 summarises the phylogenetic classification and the number of representative sequences per bacterial division as well as the T-RF values obtained from the analysed clones and retrieved from the MICA database. Overall 86 clones were examined (average sequence length 606 bp) and 26 OTUs were found by applying 3% sequence divergence to define distinct species. Analysing the individual samples returned 14 OTUs for the H90-GU sample and 17 OTUs for the H60-GU sample. The phylogenetic affiliations of the phylotypes detected in these samples are consistent with those reported for the sample H90-ME above. Phylotypes belonging to the phyla *Cyanobacteria, Actinobacteria, Bacteroidetes* and *Proteobacteria* were found to be dominant. Additionally, in the H60-GU samples phylotypes affiliated to the phylum *Firmicutes* and to the class of *Gammaproteobacteria* occurred with a frequency less than 2%. Similar to the H90-ME samples, most of the found phylotypes were covered by the *Proteobacteria* (87%), whereof the *Betaproteobacteria* accounted for 60% and *Alphaproteobacteria* for 22% of the analysed sequences. Comparing the two clone libraries according to the Naïve Bayesian Classifier (Wang*, et al.*, 2007) revealed that the frequency of members of the *Betaproteobacteria* in sample H60-GU was significantly different ($p < 0.01$). Phylotypes with high sequence similarity to the species *Ralstonia* sp., *Achromobacter* sp. and *Mesorhizobium* sp. were found to be the most abundant in the *Betaproteobacteria* class. In agreement with the findings of the H90-ME clone library, the overall most abundant phylotypes was affiliated to *Ralstonia* species. The species belonging to the genus *Ralstonia*, the most prominent represented by the species *Ralstonia solanacearum* and previously named *Pseudomonas solanacearum*, is an aerobic, non-sporing, gram-negative motile rod bacterium with a polar flagellar tuft (Garrity*, et al.*, 2005). These bacteria in general

inhabit soil, water (irrigation and wash water) plants and crops (mainly potato and tomato) causing substantial economic damage (Castillo and Greenberg, 2007).

Table 16. Phylogenetic breakdown of 16S rRNA gene sequences retrieved from clone libraries obtained from H90 and H60 in winter 2008 (Q108) from the Austrian plant.

		No. of clones observed in each sample[b]			T-RF (bp)[c]	
Taxonomic affilation	Closest relative[a]	Total	H90-GU	H60-GU	*in-silico*	*measured*
Firmicutes		2				
Bacilli	*Flackmia* sp.		0	1		559
Clostridia	unclassified Clostridiales		0	1		
Actinobacteria		2				
	Rhodococcus sp.		0	1	159	160
	Brachybacterium sp.		1	0	165	165
Cyanobacteria		1				
	Streptophyta sp.		1	0		
Bacteroidetes		5				
Sphingobacteria	*Sphingobacterium* sp.		0	2	142	139
	Pedobacter sp.		0	2	434	437
	unclassified Sphingobacteriales		1	0	93	88
Proteobacteria		75				
Gammaproteobacteria	*Stenotrophomonas* sp.	1	0	1	452/464/498	
Alphaproteobacteria	*Mesorhizobium* sp.	19	5	2	128	124
	Afipia sp.		1	0	152/197	191
	Unclassified Bradyrhizobiaceae		5	5	152	151
	Unclassified Alphaproteobacteria		0	1		
Deltaproteobacteria	*Bdellovibrio sp.*	4	2	0		689
	unclassified Cystobacterineae		1	0	157	157
	unclassified Myxococcales		1	0		
Betaproteobacteria	*Achromobacter* sp.	51	2	13*	490	491
	Bordetella sp.		2	0	490	
	Pelomonas sp.		2	0		
	Ralstonia sp.		13	9	430	438
	Unclassified Alcaligenaceae		3	3		
	unclassified Incertae sedis 5		2	1		
	unclassified Burkholderiaceae		1	0		

a. Closest relative according to the RDPII databases (Naive Bayesian rRNA Classifier Version 2.0, July 2007)
b. Assignment confidence threshold 80% (bootstrap confidence)
c. Values from database MiCA: Virtual Digest (ISPaR) (Shyu *et al.* , 2007)
* = significantly different at 0.01
Grey shaded boxes are phylotypes detected in the T-RFLP profiles also (± 2 bp)

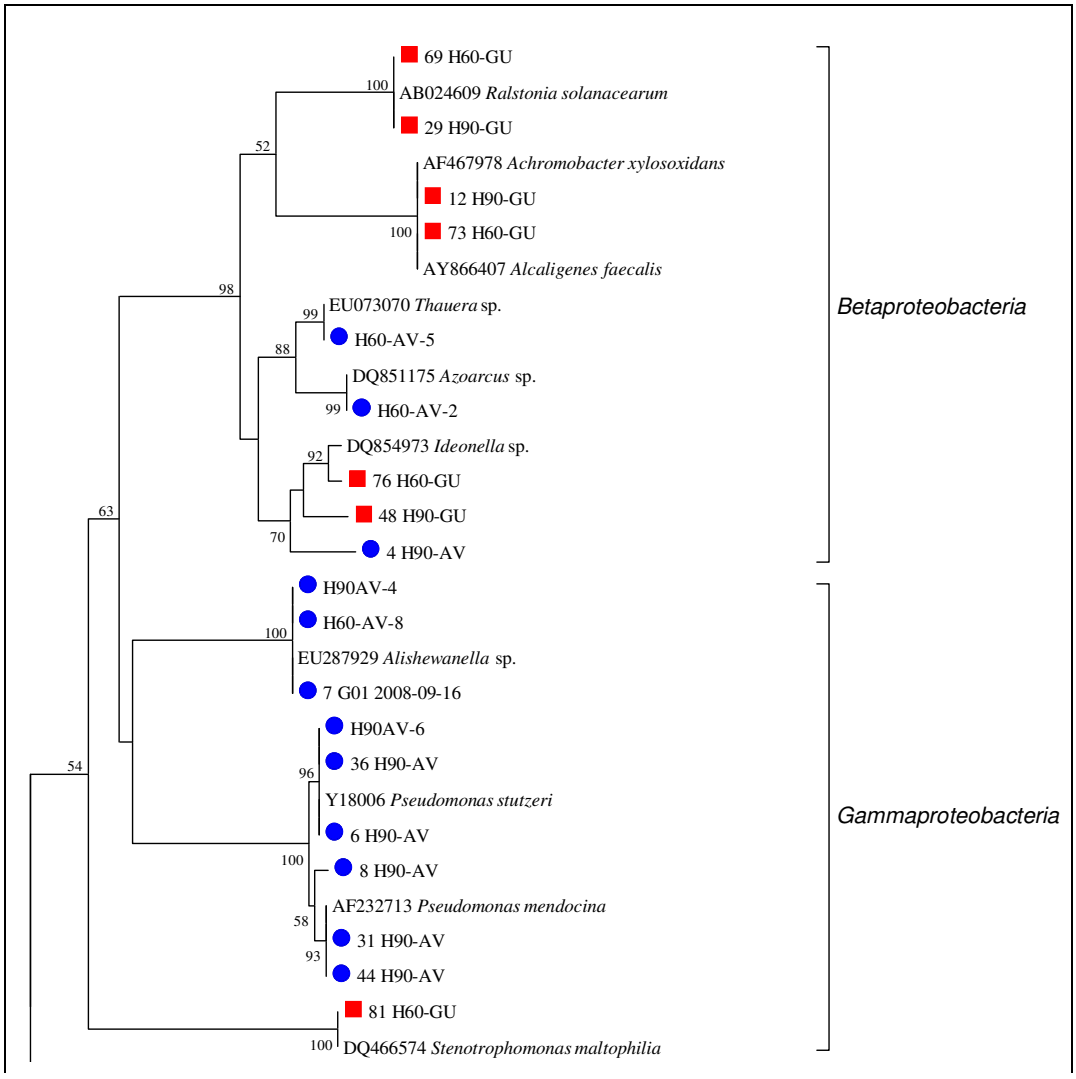
A number of selected clones from the clone library generated from samples H90 and H60 in Q108 were subject to T-RFLP analysis in order to investigate the relationship between individual phylotypes retrieved both in the T-RFLP profiles of the whole microbial community and from the clone libraries. The phylotypes determined in the T-RFLP profiles of the samples H90-GU and H60-GU in winter 2008 (Q108)

corresponded with a rate of 60% to those identified in the clone libraries of the same samples. Independently of the analysis method, the phylotypes observed in the T-RFLP profiles and in the clone libraries where comparable with those occurring with the overall highest abundance. However, the relative abundance of phylotypes determined by T-RFLP varied significantly from those identified in the clone library. A recent study by Orcutt *et al.* (2009) comparing the diversity of microbial seafloor basalt by 16S rRNA sequencing and T-RFLP showed that about 50% of the species were missed by T-RFLP but the dominant species were identified by both methods. The differences in relative abundance of distinct phylotypes may result from the lack of resolution of low-abundance taxa by the T-RFLP method due to the normalisation and cut-off baseline settings. On the other hand, the preferential ligation of abundant 16S rRNA gene amplicons into the cloning vector, the random selection and the small sample size of the analysed clones are further constraints affecting the relative abundance of OTUs assessed in the clone library.

Of several identified phylotypes *in silico* prediction of the TRFs length was generated using the Virtual Digest tool (ISPas) from the MICA database (Shyu*, et al.*, 2007). Close correlation of the predicted and measured T-RF lengths of the clones as well as of the putative corresponding T-RFs determined from the T-RFLP profiles of the whole microbial community was observed. Although for some of the observed T-RFs the difference in length reached up to ± 4 base pairs. The difference in size of expected and measured T-RFs is a common observed fact making a comparison challenging (Kaplan and Kitts, 2003).

The phylogenetic relationship of representative 16S rRNA gene sequences from both calcium carbonate slurry bacterial isolates (H90-AV, H60-AV and H90-ME) and sequences retrieved from the clone library from DNA extracted *in situ* from calcium carbonate slurries are shown in Figure 28. Seven clusters representing distinct bacterial classes were inferred from the phylogenetic tree: *Actinobacteria*, *Bacilli*, *Bacteroidetes*, *Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria*. Bacteria affiliated to the class *Gammaproteobacteria* (mainly *Pseudomonas* spp.) were almost exclusively found in the sequences gathered from the bacterial isolates (50%), whereas most of the sequences affiliated to the class *Actinobacteria*, *Bacteroidetes* and *Deltaproteobacteria* were derived from the clone libraries (48%). The dominance of calcium carbonate slurry bacterial isolates in the class

Gammaproteobacteria and in some extent to the class *Betaproteobacteria* led to the conclusion that these bacteria are favoured in the transition from the environment niche created in calcium carbonate slurries to the artificial nutrient media such as agar plates. Moreover, these species dominate the culturable bacterial community fraction of calcium carbonate slurries. Surprisingly, even though the sample size taken under consideration for the analysis of culturable bacteria was small, in coarse calcium carbonate slurries such as Hydrocarb 60 a minor incidence of culturable bacterial species occurred compared to the fine grained slurry Hydrocarb 90. In contrast, the data from the clone libraries suggested that the biodiversity of both H90 and H60 calcium carbonate slurries were comparable since sequences from both products were found in each bacterial class.



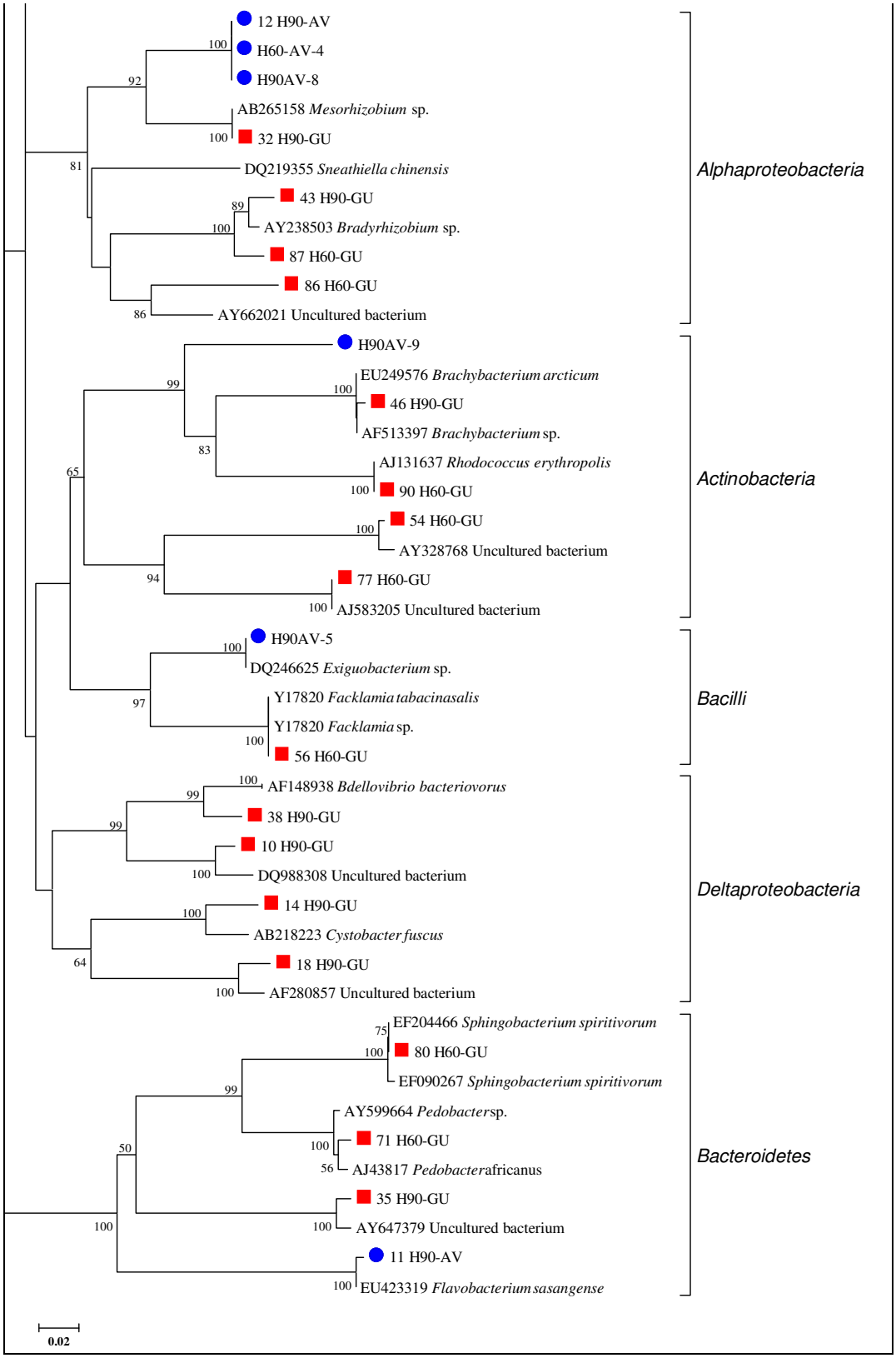


Figure 28. Phylogenetic tree constructed from partial 16S rRNA gene sequences for cultured colonies (●) and clone libraries from DNA extracted *in situ* from H90-GU and H60-GU calcium carbonate slurries (■). Method: Neighbour-joining; Model: Maximum Composite Likelihood. Phylogeny test: bootstrap (1000 replicates), values > 50 are shown. The scale bar represents 0.02 substitutions per nucleotide position.

3.2.4.2 Deep Amplicon Sequencing

To gain deeper insights about the microbial diversity of calcium carbonate slurries, 12 samples were investigated by parallel DNA pyrosequencing. All slurry products H90 and H60 sampled in spring and autumn 2008 at the plants in Italy, Austria and Norway were analysed. In order to achieve a better coverage percentage and consistent classification of the 16S rRNA gene sequences into the taxonomical hierarchy of the RDPII classifier, the 16S rRNA hypervariable region V1+V2 as well as the region V3 were sequenced in parallel. From these 24 libraries a total of 82,231 reads were obtained. Table 17 shows the number of OTUs and the coverage of the libraries at different similarity cut-offs. The chao1-estimator for richness was used to estimate the total OTU richness and to calculate the percentage coverage (Hill*, et al.*, 2003).

Table 17. OTUs and species richness estimated at different similarity cut-offs.

		Cluster distance (%)								
		0.03			0.06			0.1		
Product ID	No. of reads	OTUs	Chao1	Coverage Chao (%)	OTUs	Chao1	Coverage Chao (%)	OTUs	Chao1	Coverage Chao (%)
H90AV-Sp08	6882	996	1022	97	742	746	99	518	519	100
H60AV-Sp08	5873	774	797	97	562	566	99	410	411	100
H90AV-A08	5875	197	214	92	109	111	98	79	80	99
H60AV-A08	8395	604	616	98	425	427	100	307	307	100
H90ME-Sp08	5398	713	736	97	516	521	99	371	373	99
H60ME-Sp08	6348	735	751	98	554	558	99	399	401	100
H90ME-A08	6117	453	476	95	290	294	99	186	187	99
H60ME-A08	7955	599	614	98	411	412	100	278	278	100
H90GU-Sp08	6573	967	987	98	697	700	100	504	505	100
H60GU-Sp08	7107	918	941	98	664	667	100	478	479	100
H90GU-A08	7959	572	580	99	387	388	100	275	275	100
H60GU-A08	7749	669	679	99	445	447	100	280	280	100

At a cluster distance of 3%, all of the analysed samples showed a percental coverage of the libraries ranging from 92% to 99%. Defining OTUs at 6% divergence even improved the coverage of the libraries to greater than 98%. These data indicated that the number of analysed pyrosequencing reads was representative and allowed estimation of the total microbial diversity of these samples. Overall, the calcium carbonate slurry samples retained in spring 2008 revealed a higher number of phylotypes compared to the analogous samples from autumn 2008. The chao1

estimator for richness estimated the greatest biodiversity in the spring 2008 sample H90-AV (996 OTUs), whereas the lowest richness was estimated in the same sample in autumn 2008 (197 OTUs). Analysing all samples collectively as a single library returned 5,515 phylotypes at 3% sequence divergence which were assigned at the bacterial family level. The high number of phylotypes assessed by parallel pyrosequencing demonstrated than the biodiversity in calcium carbonate slurry is greater than expected. These results are in line with recently published studies showing that the species richness of soils (Roesch *et al.* 2007; Jones *et al.* 2009), human gut (Dethlefsen, *et al.*, 2008, Zhang, *et al.*, 2009) and oral cavity (Keijser, *et al.*, 2008) as well as deep mine (Edwards, *et al.*, 2006) and deep sea (Sogin, *et al.*, 2006) is at least one order of magnitude greater than 16S rRNA gene clone libraries have ever disclosed before.

The phylogenetic classification of sequences from H90 and H60 calcium carbonate slurries sampled in Italy (AV), Norway (ME) and Austria (GU) in spring and autumn 2008 is summarised in Figure 29.

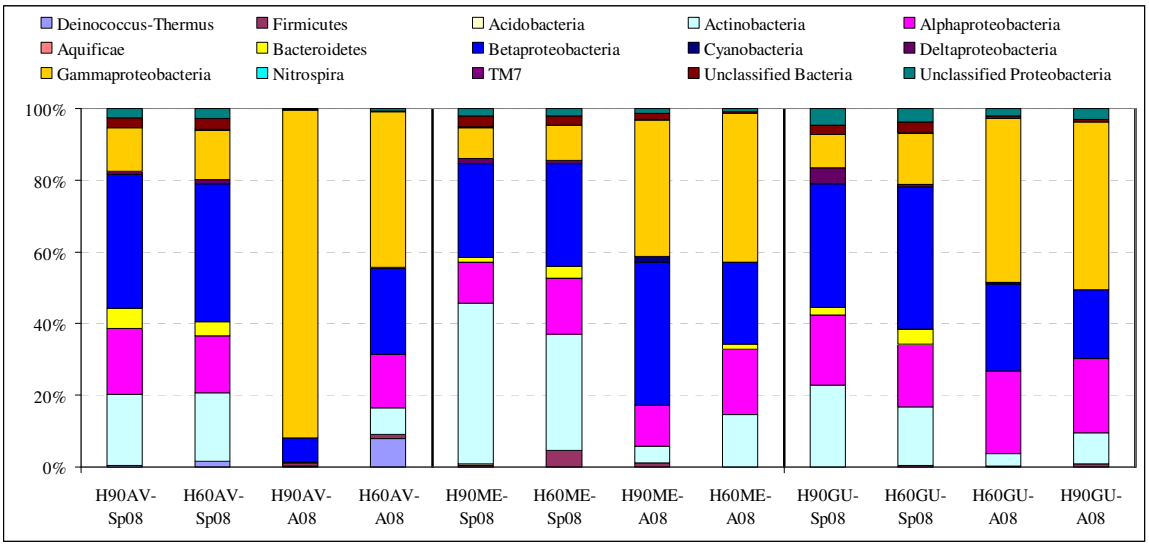


Figure 29. Relative abundance and phylogenetic classification of pyrosequencing reads (V1+V2+V3) into bacteria classes determined using the Ribosomal Database Project (RDPII) classifier (http://rdp.cme.msu.edu/) from H90 and H60 slurries sampled in spring and autumn 2008 at various plants.

The majority of the sequences were affiliated to the class *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria*. Except for the *Gammaproteobacteria* these data are in line with the findings reported by the 16S rRNA clone libraries in section 3.2.4.1. By using the traditional clone library sequencing methodology, the *Gammaproteobacteria* were completely underestimated and only identified to be dominant when calcium carbonate slurry bacterial isolates were investigated. Moreover, the composition across the samples was apparently variable between the seasons rather than between the different products (H90 and H60). Products from the same calcium carbonate manufacturing plant were inhabited by similar bacterial communities in terms of bacterial class occurrence. An exception was sample H90-AV-A08 which was mostly dominated by bacteria belonging to the class *Gammaproteobacteria* (mainly *Stenotrophomonas* spp.). The high total cell number as well as the high fluorescence value of this sample determined by CFII indicated that this sample was highly contaminated with metabolically active cells and most probably a single species dominated. This domination typically results in a reduction of the biodiversity. Similar domination patterns have been observed by using T-RFLP to characterise microbial communities as shown in section 3.2.3.2 (Figure 15). Occasionally, single bacterial species are able to come through because the environmental conditions are temporarily favourable to this specific species or because these bacteria had been able to resist a biocide challenge and recovered once the biocide activity declined.

The cluster analysis shown in Figure 30 confirmed the seasonal dynamics of bacterial communities inhabiting calcium carbonate slurries. These findings are comparable to the findings observed in the T-RFLP analysis of the samples in section 3.2.3.3 with the exception of sample H90-AV-A08, samples of the same season formed distinct clusters with a similarity of greater than 50%. In addition, samples from the same plant clustered close together within the season, indicating that the contamination is plant and season specific rather than product specific.

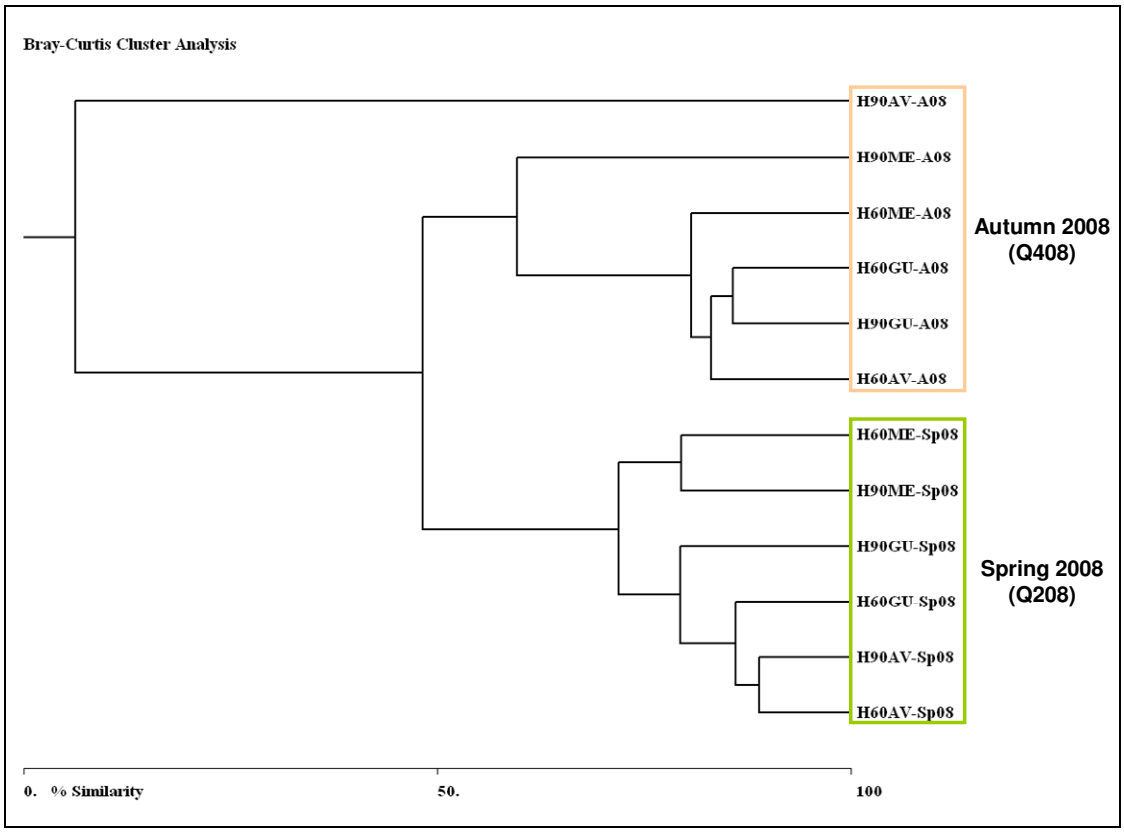


Figure 30. Cluster analysis based on the Bray-Curtis distance of OTUs abundance (3% divergence) determined from 16S rRNA deep amplicon sequencing reads of calcium carbonate slurries sampled in spring (Sp08) and autumn (A08) 2008.

The taxonomic breakdown and the relative abundance of the bacterial families revealed further insights about the microbial communities populating calcium carbonate slurries. An extensive analysis of the relative abundance of the bacterial families present in calcium carbonate slurries across all analysed samples of both seasons revealed that *Gammaproteobacteria*, *Betaproteobacteria*, *Actinobacteria* and *Alphaproteobacteria* were the most abundant classes found to occur in calcium carbonate slurries (Figure 31). Earlier studies investigating bacteria associated with paper mill raw materials have reported comparable bacterial dominance in white mineral pigments (Kolari, *et al.*, 2003, Vaisanen, *et al.*, 1998). *Gammaproteobacteria* occurred at a comparable rate as *Betaproteobacteria* (28-32%) whilst the incidence of *Actinobacteria* and *Alphaproteobacteria* was similar (15-16%). Nevertheless, the most abundant bacteria in calcium carbonate slurries belong to the class *Betaproteobacteria* in relation to the median value which is more robust to outliers than the average value. The other bacterial classes were present at a frequency of less than 2.5%.

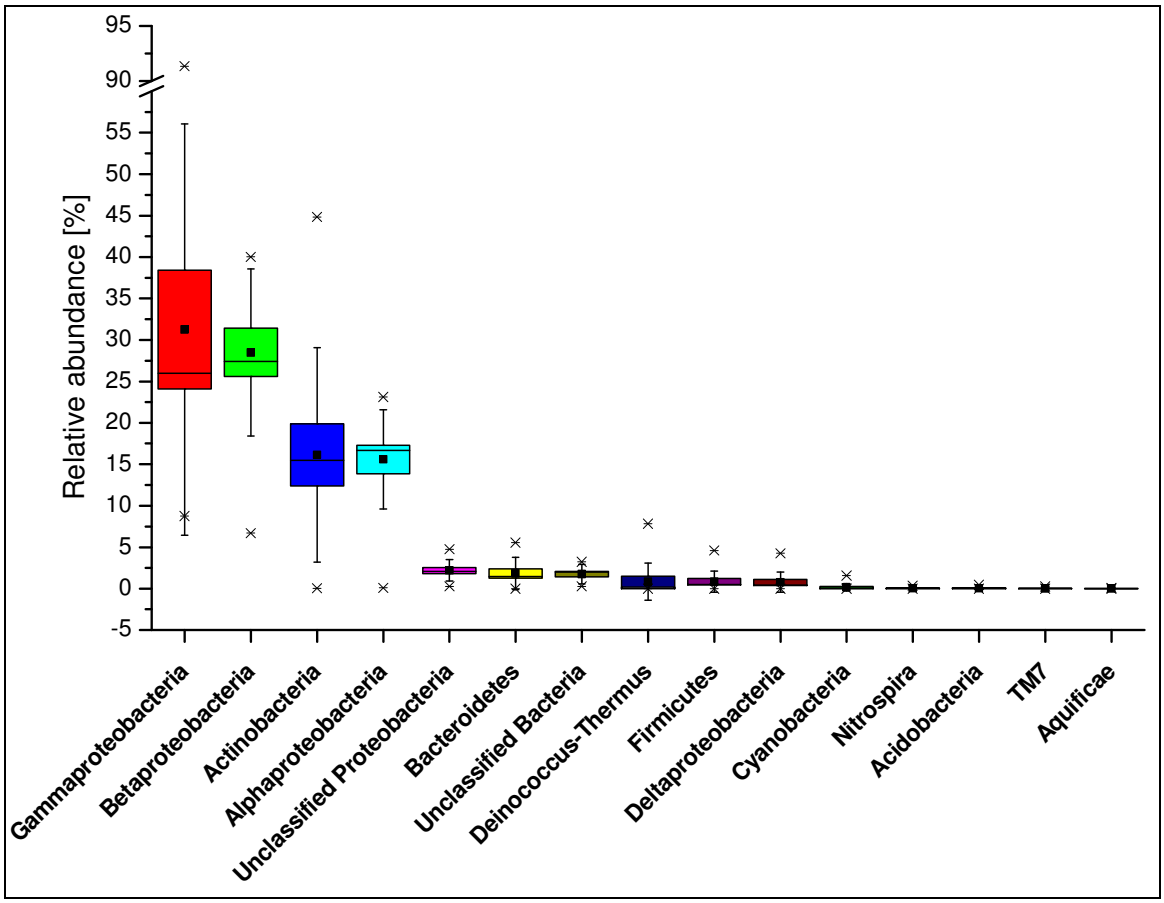


Figure 31. Relative abundance box plot of bacterial classes and phyla found in calcium carbonate slurries sampled in spring and autumn 2008. The number of analysed samples were n = 12. The mean value (■) and the standard deviation (y-error bars) are plotted. The boxes represent the median ± 1 standard error and outliers are represented by (x).

The picture is completely different when analysing the single seasons individually. Figure 32 illustrates the seasonal comparison of the relative abundance of bacterial classes and phyla identified in calcium carbonate slurries sampled in spring or autumn 2008. In the spring 2008 samples, bacteria from the class *Betaproteobacteria* were the most abundant, followed by the *Actinobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*. In contrast, the relative abundance of *Gammaproteobacteria* increased toward the autumn at the expense of *Betaproteobacteria*, *Actinobacteria* and *Alphaproteobacteria*. The *Betaproteobacteria* peaked in the spring samples and *Bacteroidetes* as well as *Deltaproteobacteria* were exclusively present in spring. The seasonal variation of the proportional changes of the bacterial classes is related to the seasonal specific bacteria occurring at the WMD production facilities and most probably originates from the raw material such as the marble and the process water.

On the other hand, the similarity in bacterial community composition of calcium carbonate slurries determined from the same plant is related also to the biocide in-use for preservation.

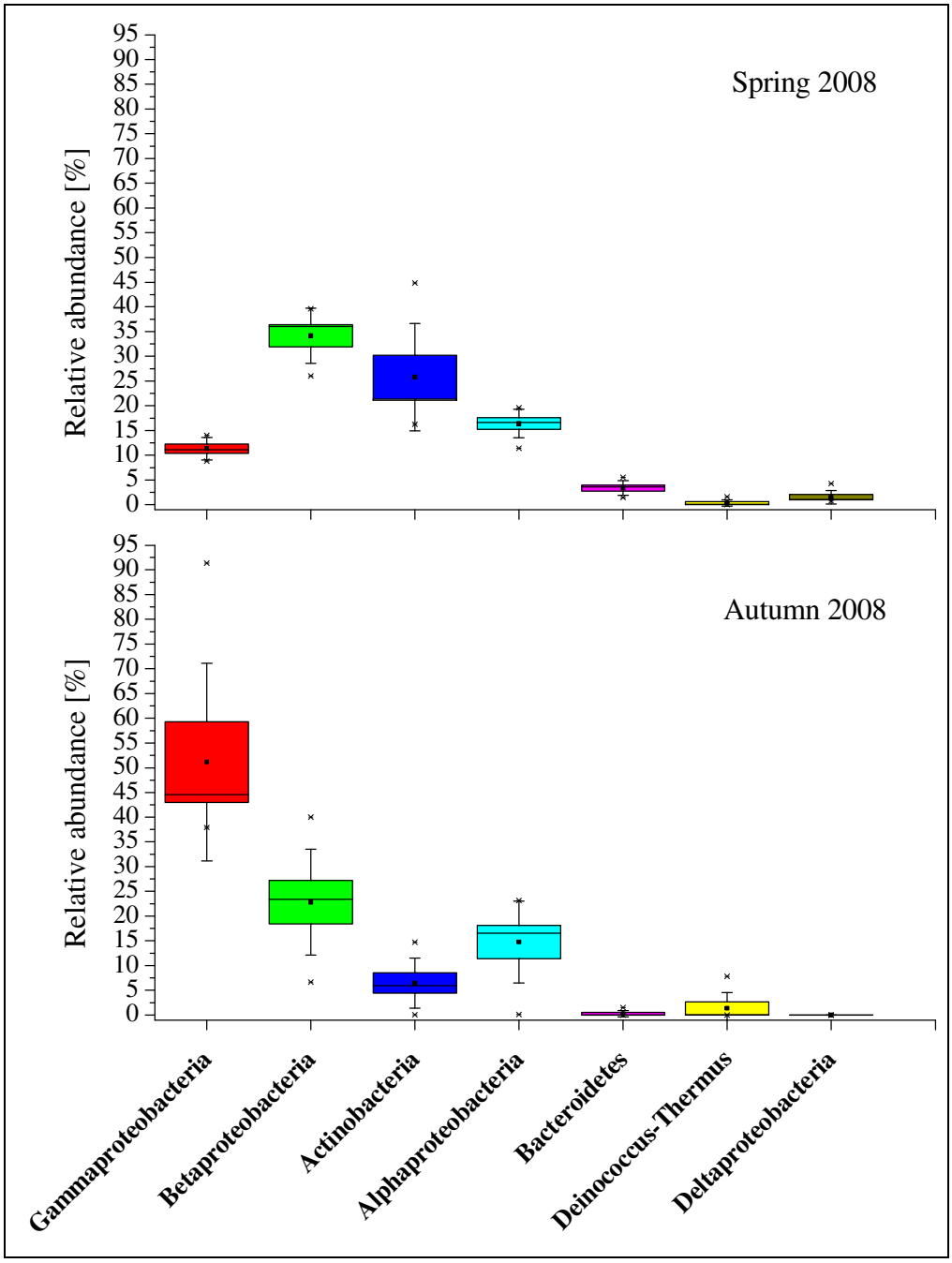


Figure 32. Seasonal comparison of the relative abundance box plot of bacterial classes and phyla found in calcium carbonate slurries sampled in spring or autumn 2008. The number of analysed samples were n = 6 for each season. The mean value (■) and the standard deviation (y-error bars) are plotted. The boxes represent the median ± 1 standard error and outliers are represented by (x).

Analysis of variance (ANOVA with Tukey's pairwise comparison) used to compare the occurrence of bacterial classes in spring and in autumn disclosed a significant variance for the classes *Gammaproteobacteria*, *Betaproteobacteria* and *Actinobacteria* ($p < 0.05$).

In the context of this study and by means of the deep amplicon sequencing technology attempts were made to characterise the bacterial species inhabiting calcium carbonate slurries in greater detail at the family or even genus level. Taking a closer look at the phylogeny of the gathered sequences revealed the complexity of microbial diversity. Defining phylotypes to be different at 3% sequence divergence across all analysed samples returned a number of 6,233 distinct phylotypes, 5,515 of which were assigned by the RDPII classifier to a bacterial family and genus (Table 18). A more conservative approach defining phylotypes to be different at 6% divergence yielded 4,165 distinct phylotypes.

Table 19 lists the most abundant genera assigned by the RDPII classifier and found in calcium carbonate slurries at a relative abundance of at least 1%. These genera represented 50.5% of the total relative abundance, thus it can be assumed that there are several very low abundance reads, particularly singletons, which may be artefacts. A fraction of the overall identified genera, namely 25%, was also detected in both the clone libraries and the bacterial calcium carbonate slurry isolates. Bacterial diversity estimated by means of deep amplicon sequencing has disclosed bacterial genera from several phyla and families. Apparently, calcium carbonate slurry is inhabited by both gram-positive and gram-negative bacteria, even though gram-negative bacteria are more resistant to the antimicrobial efficacy of biocides. Most of the identified bacteria are environmental bacteria typically able to survive under challenging environmental conditions such as formed in calcium carbonate slurries. The high pH, the low nutrient availability and the antimicrobial pressure due to the preservation of calcium carbonate slurry with biocides generate an environmental niche selecting bacterial species with high adaptation abilities. Prediction of the T-RFs using the Virtual Digest tool (ISPas) *in silico* analysis from the MICA database demonstrated that the prevalent OTUs found with T-RFLP analysis were recovered by the deep amplicon sequencing analysis also (Table 19). Close correlation of the T-RFLP length predicted for the prevalent genera and the T-RFs determined for the T-RFLP profiles of the whole microbial community was

observed when defining similarity at ± 2 bp. The most abundant observed OTU with T-RF length 488 bp can be assigned to bacterial species belonging to the families of the *Alcaligenaceae*, *Comamonadaceae* and *Pseudomonadaceae*. In this context it is important to point out that for the evaluation the samples analysed by means of the deep amplicon sequencing were treated to be a single library. Therefore conclusion about product specific or production plant specific occurrence of certain bacterial species is limited. However, taking a closer look to the dominant bacterial genera summarised in Figure 19 disclose interesting trends. Product specific contamination, which means related to the coarse product Hydrocarb 60 or fine product Hydrocarb 90, was not recognised. In contrast bacteria of the genus *Thermus* exclusively occurred in the plant in Italy (Avenza) and therefore it can be assumed that they are plant specific. The bacteria related to the genera *Caulobacter*, *Brevundimonas*, *Asticcacaulis* and *Bradyrhizobium* were only found in the spring samples whereas those related to the genera *Massilia* and *Alishewanella* were only found in the autumn samples. Because of the many variables such as in-use biocide, tank condition and level at the sampling time, degree of contamination (domination of individual species), freshness of the product and many others general conclusions about the specific occurrence of bacterial species in calcium carbonate slurries are limited. Finally, it can be concluded that microbial populations inhabiting calcium carbonate slurries are subjected to seasonal fluctuations in relation to the above mentioned variables.

Table 18. Classification into phyla, classes and families as well as number of OTUs identified across all 12 analysed calcium carbonate slurry samples. The classification within the various bacterial families and genera was determined at 3% sequence.

Class - Family	Genus
Aquificae	
Aquificaceae	*Hydrogenobacter*
Nitrospira	
Nitrospiraceae	*Nitrospira*
Bacilli	
Aerococcaceae	*Flackmia*
Carnobacteriaceae	*Alkalibacterium*
Streptococcaceae	*Streptococcus*
Staphylococcaceae	*Staphylococcus*
Paenibacillaceae	*Paenibacillus*
Bacillaceae	*Paraliobacillus*
	Bacillus
	Caldalkalibacillus
	Ureibacillus
	Anoxybacillus
Clostridia	
Incertae Sedis XIV	*Anaerobranca*
Clostridiaceae	*Geosporobacter*
	Alkaliphilus
Incertae Sedis XI	*Peptoniphilus*
Peptostreptococcaceae	*Sporacetigenium*
Veillonellaceae	*Veillonella*
Deinococci	
Thermaceae	*Thermus*
Actinobacteria	
Acidimicrobiaceae	*Ferrithrix*
Catenulisporaceae	*Catenulispora*
Micromonosporaceae	*Catelliglobosispora*
Actinomycetaceae	*Actinomyces*
Propionibacteriaceae	*Microlunatus*
	Brooklawnia
	Propionibacterium
Pseudonocardiaceae	*Pseudonocardia*
Actinosynnemataceae	*Lentzea*
Micrococcaceae	*Rothia*
	Nesterenkonia
Microbacteriaceae	*Leifsonia*
	Microterricola
	Zimmermannella
	Microbacterium
Dietziaceae	*Dietzia*
Corynebacteriaceae	*Corynebacterium*
Nocardiaceae	*Rhodococcus*
Flavobacteria	
Flavobacteriaceae	*Sejongia*
Sphingobacteria	
Sphingobacteriaceae	*Pedobacter*
	Sphingobacterium
Chitinophagaceae	*Parasegetibacter*
	Terrimonas
	Lacibacter
	Sediminibacterium
	Filimonas
Alphaproteobacteria	
Rickettsiaceae	*Orientia*
Rhodospirillaceae	*Oceanibaculum*
Hyphomonadaceae	*Maribaculum*
Caulobacteraceae	*Brevundimonas*
	Phenylobacterium
	Asticcacaulis
	Caulobacter
Rhodobacteraceae	*Amaricoccus*
	Paracoccus
	Rhodobacter
	Labrenzia
	Roseibium
	Pannonibacter
Erythrobacteraceae	*Porphyrobacter*
Sphingomonadaceae	*Novosphingobium*
	Sphingopyxis
	Sphingosinicella
	Zymomonas
	Sphingobium
	Sphingomonas
Bartonellaceae	*Bartonella*
Methylobacteriaceae	*Methylobacterium*
Hyphomicrobiaceae	*Rhodoplanes*
	Hyphomicrobium
	Filomicrobium
Beijerinckiaceae	*Methylovirgula*
	Chelatococcus
	Methylocapsa
Xanthobacteraceae	*Xanthobacter*
	Azorhizobium
Brucellaceae	*Daeguia*
	Pseudochrobactrum
	Ochrobactrum
Bradyrhizobiaceae	*Nitrobacter*
	Balneimonas
	Afipia
	Bosea
	Agromonas
	Blastobacter
	Bradyrhizobium

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Class - Family	Genus
continued from the previous page	
Phyllobacteriaceae	*Mesorhizobium*
	Phyllobacterium
	Nitratireductor
	Hoeflea
	Defluvibacter
Rhizobiaceae	*Kaistia*
	Ensifer
	Sinorhizobium
	Rhizobium
Betaproteobacteria	
Methylophilaceae	*Methylotenera*
	Methylophilus
Neisseriaceae	*Simonsiella*
	Microvirgula
Rhodocyclaceae	*Thauera*
	Azoarcus
	Methyloversatilis
Burkholderiales	*Oxalobacteraceae*
	Herminiimonas
	Naxibacter
	Massilia
	Duganella
Burkholderiaceae	*Burkholderia*
	Limnobacter
	Ralstonia
Alcaligenaceae	*Alcaligenes*
	Bordetella
	Pigmentiphaga
	Azohydromonas
	Achromobacter
Comamonadaceae	*Diaphorobacter*
	Variovorax
	Tepidicella
	Macromonas
	Brachymonas
	Malikia
	Rhodoferax
	Acidovorax
	Curvibacter
	Caenimonas
	Caldimonas
	Hydrogenophaga
	Delftia
	Pelomonas

Class - Family	Genus
Gammaproteobacteria	
Enterobacteriaceae	*Enterobacter*
	Brenneria
	Citrobacter
Moraxellaceae	*Enhydrobacter*
	Acinetobacter
Pseudomonadaceae	*Azotobacter*
	Azomonas
	Azorhizophilus
	Pseudomonas
Pasteurellaceae	*Haemophilus*
Alteromonadaceae	*Alishewanella*
Legionellaceae	*Legionella*
Coxiellaceae	*Aquicella*
Ectothiorhodospiraceae	*Thiohalospira*
Chromatiaceae	*Rheinheimera*
Methylococcaceae	*Methylosarcina*
	Methylosoma
Thiotrichaceae	*Beggiatoa*
Xanthomonadaceae	*Xanthomonas*
	Pseudoxanthomonas
	Dokdonella
	Stenotrophomonas
Deltaproteobacteria	
Bacteriovoracaceae	*Peredibacter*
Bdellovibrionaceae	*Bdellovibrio*
Syntrophobacterales	*Smithella*

Table 19. Classification into genus of pyrosequencing reads of the dominant bacterial families (> 1% abundance) found in calcium carbonate slurries derived from the classification within the various bacterial families was determined at 3% sequence divergence. All analysed samples were treated to be a single library.

Class - Family	Genus	Total Relative abundance (%)	T-RF (bp)[a)]
Deinococci			
Thermaceae	*Thermus*	1	118-123
Actinobacteria			
Propionibacteriaceae	*Propionibacterium*	4	161-165
Nocardiaceae	*Rhodococcus*	2	159-163
Alphaproteobacteria			
Rhodobacteraceae	*Pannonibacter*	4	437
Caulobacteraceae	*Caulobacter*	2	150
	Brevundimonas		403
	Asticcacaulis		437
Sphingomonadaceae	*Sphingomonas*	2	150-152
	Sphingobium		150
	Sphingopyxis		404-405/439
Rhizobiaceae	*Rhizobium*	1	128-130/401-
	Ensifer		403/437
Bradyrhizobiaceae	*Bradyrhizobium*		152
Betaproteobacteria			
Incertae sedis 5	*Tepidimonas*	2	121
	Tepidicella		-
	Aquabacterium		141
	Pelomonas		483
Alcaligenaceae	*Alcaligenes*	6	488-498
	Achromobacter		490
Burkholderiaceae	*Ralstonia*	2	86/429-436
	Limnobacter		151
Oxalobacteraceae	*Massilia*	1	490
Comamonadaceae	*Hydrogenophaga*	10	488
	Acidovorax		488
	Curvibacter		490
	Delftia		488
	Rhodoferax		492
Gammaproteobacteria			
Incertae sedis 7	*Alishewanella*	1	478
Xanthomonadaceae	*Stenotrophomonas*	5	452-454/463-465
	Dokdonella		226
Pseudomonadaceae	*Pseudomonas*	7.5	488-496

a. Values from database MiCA: Virtual Digest (ISPaR) (Shyu *et al.,* 2007)

3.3 Conclusions

Investigation of the microbial diversity based on the identification of culturable bacteria clearly showed an underestimation of the biodiversity of calcium carbonate slurries when using culture-dependent methods. A limited range of bacterial species was identified in biocide-free calcium carbonate slurries and the preservation of slurries with biocides led to a loss of the diversity to a single species of the same genus. Predominantly, bacteria from the genus *Pseudomonas* were isolated from biocide-free or biocide-supplemented calcium carbonate slurries. Schwarzentruber (2003b) demonstrated that *Pseudomonas* is the most frequent genus isolated from calcium carbonate slurries and therefore suspected that the identification of bacterial isolates from calcium carbonate slurries only covered a small portion of the true biodiversity and therefore culture-independent methods are required to elucidate the actual microbial diversity.

By means of culture-independent molecular techniques, the microbial diversity of calcium carbonate slurries was found to be more extensive than ever postulated before. The microbial activity of bacterial populations in calcium carbonate slurry specimens was characterised by means of the CellFacts II® instrument culture-independently and the presence of bacterial cells was observed in all investigated samples. The data obtained by T-RFLP analysis showed that within the corresponding biocide-free or biocide-supplemented samples a perturbance of the microbial communities due to the addition of biocide was not significant in relation to the quantity of predominant OTUs. The addition of biocide caused a rearrangement of the OTU abundance ratios rather than the alteration of OTUs in terms of quantity. However, across all analysed samples the microbial diversity of biocide-free calcium carbonate slurries was greater since in these specimens there was a significant elevation in the number of unique OTUs. Relating the predominance of individual OTUs to the microbial activity determined by means of the CFII device showed that samples with high total cell numbers and high fluorescence values tend toward a narrow diversity and most probably a single species dominated.

Quarterly analysed calcium carbonate slurry samples over a period of one year disclosed that microbial populations inhabiting calcium carbonate slurries are

subjected to seasonal fluctuations. Moreover, microbial community structures were plant specific rather than product specific. Across all analysed calcium carbonate samples the most distinct microbial community was detected in the winter season followed spring, whereas the microbial structure overlapped during the warmer periods of the year (summer and autumn). As a consequence it appears that the transition of the bacterial population into the cold winter months induces more variance compared to the succession in the other three seasons. The seasonal dynamics of microbial populations in calcium carbonate slurries can probably be attributed primarily to the variations in the temperature and cooling performance of the slurry after the wet grinding process. Secondly, the production of calcium carbonate slurries is an "open" large scale industrial process and the raw material used to manufacture calcium carbonate slurries such as the crude marble, the process water usually supplied as tap water from natural watercourses and partially as recirculated water from the process are *per se* subjected to fluctuations in the microbial community. In addition, parameters such as biocide types, biocide dosage intervals, mixing homogeneity of the biocide in the tank, and age of the microbial community driven by the retention time of the product in the tank at the sampling time to some extent influence the outcome of such an analysis. Therefore, interpretation of seasonal and spatial variation in relation to microbial ecology is limited.

Subpopulations of FCM sorted cells, rRNA reverse-transcribed cDNA and PMA treated cells were used in combination with T-RFLP to characterise the fraction of metabolically active species of calcium carbonate microbial communities. All of three methods, with some limitation turned out to be useful to get a picture of the physiological state of bacterial community members. While the acquisition of FCM sorted subpopulations is time-consuming and requires the availability of an expensive FCM sorting instrument, the reverse-transcription of rRNA to cDNA adds an additional step susceptible to molecular analysis bias. Nevertheless, by both methods a clear shift of the bacterial population structure towards viable cells with intact membranes or even metabolically active cells was identified and revealed a significant physiological state heterogeneity within the bacterial populations. The application of PMA to assess viable cells of mixed populations is economic and rapid. The pre-treatment of cells with PMA, a procedure by which cell viability is

assessed based on the integrity of the membrane, is appropriate to monitor the antimicrobial performance of biocides in calcium carbonate slurries. Bacterial communities treated with PMA showed a clear alteration of the microbial OTU composition due to the cut-off of the amplifiable DNA emphasising bacterial OTUs of species with integral membranes. Samples supplemented with biocide and treated with PMA prior to molecular analysis revealed the presence of only a few species (OTUs). Consequently, these species were able to withstand the biocide exposure without loosing the integrity of the bacterial membrane, thus remained viable or became "Active-But-Non-Culturable" (ABNC) since no growth on solid nutrient medium was observed (Kell*, et al.*, 1998).

Elucidation of the phylogenetic relationship by 16S rRNA gene sequencing of bacteria inhabiting calcium carbonate slurries accentuated the microbial diversity detected by T-RFLP analysis and disclosed the presence of members affiliated to numerous bacterial families. Clone libraries showed the dominance of common soil and water bacteria. In addition, whole-community T-RFLP analysis and sequence analysis of clone libraries revealed dominant phylotypes present in calcium carbonate slurries comparably too, and are in line with findings reported in other studies (Ludemann*, et al.*, 2000, Orcutt*, et al.*, 2009). Finally, the microbial diversity of calcium carbonate slurries elucidated by deep amplicon sequencing of 16S rRNA gene fragments corroborated a much greater diversity than previous estimated and provided new insights into the phylogenetic structure. More than 5000 distinct phylotypes at 3% sequence divergence were assigned at the bacterial family level and consequently the phylotype richness of calcium carbonate slurries is greater than ever disclosed by 16S rRNA clone libraries. The seasonal fluctuations of microbial populations of calcium carbonate slurries observed by means of the T-RFLP profiles were confirmed by the data gathered by deep amplicon sequencing. Overall, the most abundant bacterial classes represented across all samples were found to be the *Gamma-*, *Beta-* and *Alphaproteobacteria* as well as the *Actinobacteria*. Whereas phylotypes affiliated to the *Betaproteobacteria* and the *Actinobacteria* peaked in spring, the *Gammaproteobacteria* peaked in autumn at the expenses of the former. In contrast, the *Gammaproteobacteria* were completely underestimated by the preceding analysis of clone libraries and only identified to be dominant when calcium carbonate slurry bacterial isolates were investigated. These proportional

variations of bacterial classes between the seasons reflected the seasonal specific occurrence of bacteria and the ability of the microbial population to adapt to the calcium carbonate slurry environmental niche and the environmental influences (e.g. biocide). The genera found in calcium carbonate slurries in ascending relative abundance were: *Pseudomonas* sp., *Delftia* sp., *Achromobacter* sp., *Propionibacterium* sp., *Hydrogenophaga* sp., and *Pannonibacter* sp..

Summing up, the use of multiple molecular methods such as fingerprint analysis and sequence analysis disclosed a much greater bacterial diversity inhabiting calcium carbonate slurries than previously estimated and these methods applied individually or in combination are a powerful tool to characterise the microbiology of white mineral dispersions. Changes in the bacterial community composition were identified to be seasonal rather than production plant specific. Highly contaminated calcium carbonate slurry showed a narrow microbial diversity due to the predominance of a single bacterial species. Obviously the biocidal treatment of calcium carbonate slurries is not always effective since there are a few bacterial species able to survive and maintain metabolic activity in the presence of the biocide. Finally, the information about the most abundant species inhabiting calcium carbonate slurry is of importance for the standardisation of the current challenge test to investigate the in-use concentration of biocides in the field.

CHAPTER 4

Biocide in WMD - Resistance and Biodegradation

4.1 Introduction

White mineral dispersions have an alkaline pH ranging from 8-10 with a water content of 25-80 % (w/w); the presence of various salts is sufficient to sustain growth of microorganisms in such an environment. Even though WMD seems to be an unusual environmental niche it is a favourable habitat for several bacterial species as evidenced by the bacterial diversity found in the course of this study. Biocides therefore play a central role in the preservation of WMD so as to maintain high quality requirements, such as brightness, rheological parameters, and odour neutrality. Apart from the economic and ecological impact caused by the increased use of biocides, in this context it is essential to highlight the fact that there exist only a limited number of antimicrobials that are suited to preserving WMD. This is due to the more rigorous regulatory situation created by the BPD (Biocidal Products Directive 98/8/EC), as well as the incompatibility of certain antimicrobials with the chemical properties of WMD. These circumstances have led to the shelving of development and registration of new biocidal substances, owing to the laborious and expensive research required.

Bacterial resistance towards biocides is a globally emerging problem and in contrast to antibiotics the mechanism(s) leading to reduced susceptibility of bacteria to biocides are not fully investigated (Tumah, 2009). The problem of resistance to antimicrobial agents used for the preservation of WMD is also increasing (Di Maiuta*, et al.*, 2009a, Schwarzentruber and Gane, 2005). Since the biocide formulations intended for use in WMD are suffering from the unavailability of new compounds and other compounds such as formaldehyde are losing acceptance by the customer, as well as the acquisition of resistance by microorganisms, it will be mandatory for the future to apply the available biocide resources accurately. Despite efforts in optimising biocide application at the WMD production sites, investment for the preservation / disinfection of white mineral dispersions cannot be maintained.

4.1.1 Aims and Objectives

The aims of the following experiments were:

- To evaluate the stability of biocides in WMD.
- To investigate biocide resistance incidence and development in WMD.
- To identify and characterise biocide resistant bacterial species in WMD.
- To assess the cross-resistance of bacteria to various biocides used to preserve WMD.
- To investigate the degradation of biocidal formulation by resistant bacteria in WMD.

The incidence and development of biocide resistant bacteria as well as the stability of biocides in WMD will be of major concern in relation to the optimisation of the preservation strategies which aims to save both environmental as well as economical resources. These findings will provide a better understanding of the biochemical interactions between microbes and biocides and would permit development of new preservation protocols to control bacterial contamination of white mineral dispersions.

4.2 Mechanism of Biocide Action and Activity

Biocides are chemical substances that inactivate viruses, spores or microorganisms such as bacteria and moulds at low concentrations, usually in the range of 1 to 5000 ppm (Kupper*, et al.*, 2005). The antimicrobial performance of biocides is referred to as either bacteriostatic or bactericidal. Bacteriostatic agents inhibit the growth of bacteria and once the antimicrobial agent is depleted the treated cells are often able to recover whereas bactericidal agents damage the target bacteria irreversibly resulting in cell death. Per definition the minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent causing a bacteriostatic effect whereas the lowest concentration leading to the killing of bacteria is named the minimal bactericidal concentration (MBC). However, because several agents exhibit

bacteriostatic or bactericidal effects depending on the applied concentration (Maillard, 2002), in the following sections the term preservation refers to the prevention of bacterial multiplication or even to the killing of bacteria present in the treated product. Additionally, as bacterial cells incur injuries of increasing severity the bacteriostatic effect is followed by the irrevocable bactericidal impact. Biocidal formulations are in general chemically defined substances or blends of preservatives and disinfectants which are widely-used in various industrial applications as well as an integral part of hygiene concepts. The broad range of activity and action based on targeting of multiple cellular entities clearly distinguish biocides from antibiotics (Figure 33).

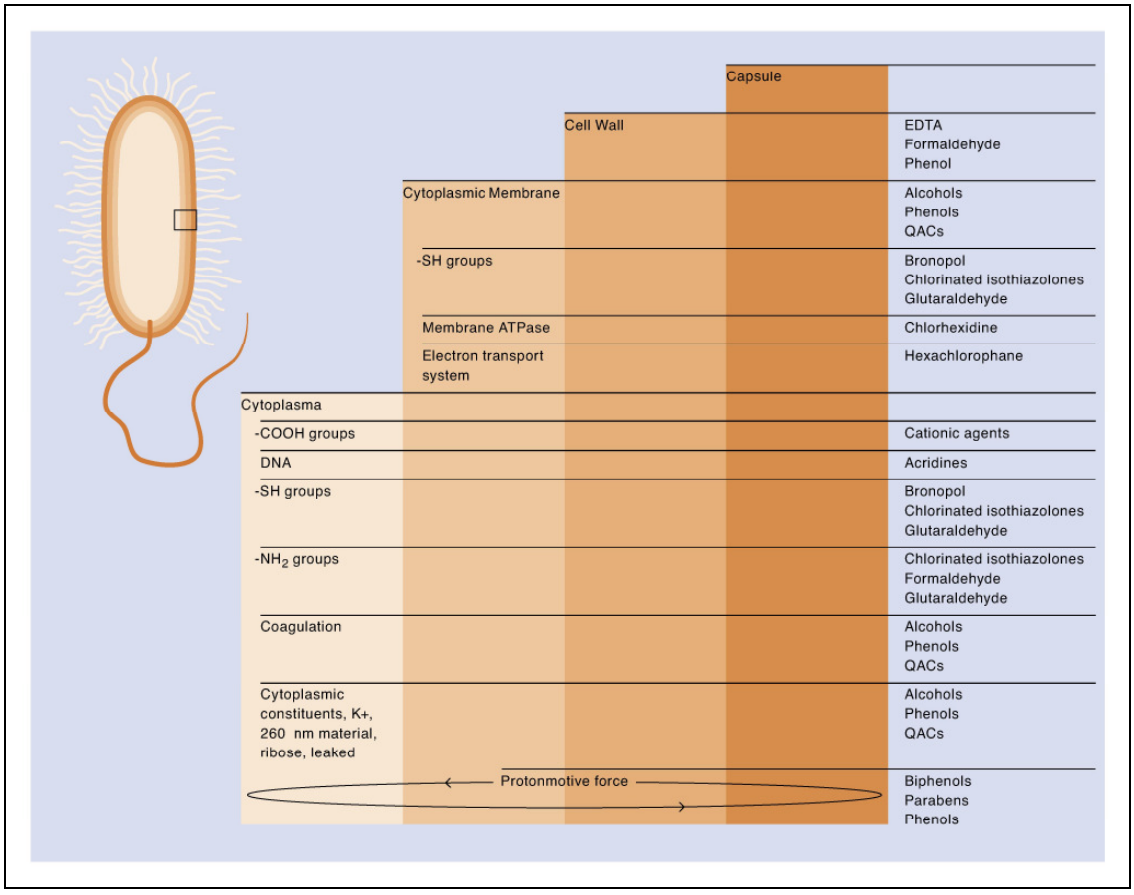


Figure 33. Schematic illustration of the mechanism of action and target sites of biocides (Schwarzentruber, *et al.*, 2007).

The chemical structure of biocides vary among the different biocides and this is reflected in the diversity of the targets and mode of action within a bacterial cell (Maillard, 2002). The activity of biocides implies a sequence of events comprising the association of the biocidal substance with the cell envelope followed by uptake into the cell and the damaging action (McDonnell and Russell, 1999). The cytoplasmic membrane and the cytoplasmic components are the principal targets of antimicrobial agents (Denyer and Maillard, 2002). The bacterial lipidic membrane represents the primary barrier to the entrance of molecules into the cytoplasmic membrane. Whilst hydrophobic biocides having a molecular weight of less than 600 mol g^{-1} pass unhindered across the cell membrane, molecules greater in molecular weight and distinct in hydrophobicity are retained outside (Denyer and Stewart, 1998). Nevertheless, bacterial membranes are not constant within prokaryotes and differences occur between bacterial types and even between a species exposed to environmental stress (Russell, 2003a). Whereas the bacterial cell wall of gram-positive bacteria essentially consists of highly cross-linked peptidoglycan, the cell wall of gram-negative bacteria is a highly complex structure having a periplasm between the inner and outer membrane. The presences of outer membrane proteins (OMP) as well as lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria provide a permeability barrier to chemical compounds. In general, because of the impermeability conferred by the structural differences of the outer envelope, gram-negative bacteria are less susceptible to antimicrobials compared to gram-positive bacteria.

The mode of action of biocides can be categorised into four groups: Electrophiles comprising the group of oxidants (chlorine, peroxides) and electrophilic biocides (aldehydes, isothiazolinones) and the membrane active agent including lytic biocides (phenols, biguanides, QACs) and protonophores (parabens, pyrithiones) (Chapman, 2003a). Membrane-active agents such as phenolic compounds (o-phenylphenol), alcohols (ethanol, isopropanol) and quaternary ammonium compounds (QACs; bezalkonium chloride) interact with the cytoplasmic membrane and lead to the disruption of the cell membrane. Concomitant effects caused by membrane active biocides are the inactivation of lipoproteins whereof many are membrane embedded enzymes as well as the dissipation of the membrane proton motive force which subsequently results in the uncoupling of the oxidative

phosphorylation and inhibition of active transport across the membrane. In the course of the biocidal membrane damage the leakage of intracellular constituents such as potassium, phosphate, nucleotides and nucleosides as well as proteins is the consequence of the loss of membrane integrity ending in the coagulation of the cytoplasma or the lysis of the cell. For electrophilic agents, which include the aldehydes (incl. formaldehyde-releasers), active-halogen compounds, heterocyclic compounds such as isothiazolinone derivates and oxidising agents the antimicrobial effect arise from the strong electron withdrawing ability of the electrophilic group, thus the attack on cellular nucleophilic entities. Biomolecules such as DNA, RNA and proteins containing thiol, amino, amide and imino groups are implicated in the cross-linking reaction with the electrophylically-active biocide. The most significant differences between membrane-active and electrophylically-active agents is that due the irreversible cross-linking reaction of the latter, the antimicrobial activity dissipates due to the exhaustion of freely-available active molecules. In contrast, the antimicrobial activity of membrane-active biocides in general persists given that these agents cause the disintegration of the cells and have continuing activity except they are longer absorbed. For that reason, membrane-active biocides are considered to be of superior effectiveness in terms of long-lasting activity. The choice of biocides to control microbial activity is based on the equilibrium of antibacterial effectiveness and adverse effects.

4.2.1 Factors Affecting the Efficacy of Biocides

The mode of action and the antimicrobial activity of biocides have mostly been studied in broth culture (Hugo and Denyer, 1991) and therefore the MIC of biocides reported in the literature are only in part transferable to white mineral dispersions. There are three main categories of application influencing the activity of biocides: (i) pre-treatment, (ii) in-treatment and (iii) post-treatment factors.

Pre-treatment factors include the growing conditions used for the cultivation of the investigated microorganisms. In practice the WMD bacterial cultures utilised for the determination of the antimicrobial activity of biocides in WMD are batch culture which have been standardised by incubation time. However, in this context it is important to highlight that biocidal agents used to preserve white mineral

dispersions target heterogenic microbial populations rather than on individual species, hence a dynamic system with cells in various physiological states is encountered. Therefore, the biocide performance is to some extent related to the physiological state of the targeted cells since metabolically active cells are more susceptible to biocides whilst starved cells are more resistant to antimicrobials (Russell, 2003; Tumah, 2009). In this context it is essential to highlight that because of the microbial diversity and the heterogeneous physiological structure of bacterial populations inhabiting white mineral dispersions an inconsistent response to the biocide can be presumed. Furthermore, pre-treatment involves the conditioning of bacterial cells with chemical agents in relation to the impermeability of bacterial cell membranes to biocides. Pre-exposure with non-ionic surfactants (e.g. polysorbate 80), cationic surface-active agents such as cetyltrimethylammonium bromide (CTAB) and permeabilisers like ethylenediaminetetraacetic acid (EDTA) are common.

In-treatment factors refer to abiotic factors arising from the external physical environment such as pH, temperature and the presence of organic and inorganic constituents which can affect the antimicrobial activity of biocides. The activity of antimicrobial agents is usually increased at elevated temperatures (e.g. phenols), but some biocides at high temperatures are less stable and so result in reduced activity (e.g. isothiazolinones at temperatures > 50 ºC). The activity of biocides is also influenced by the environmental pH, positively or negatively, since changes in pH can ionise the biocidal substance as well as the surface of bacteria causing a change in the affinity and hence intensity of the biocidal effect. The recommended pH range for some biocidal compounds used for the preservation of calcium carbonate slurry is summarised in Figure 34.

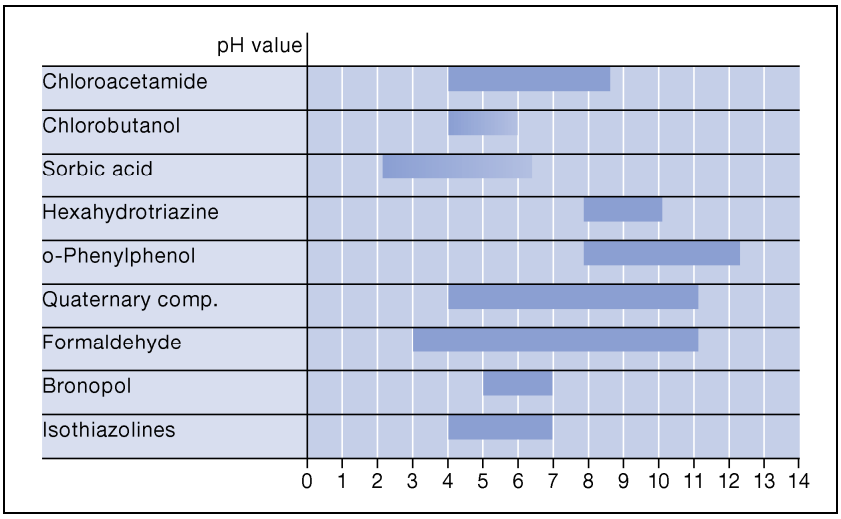


Figure 34. Optimal pH range of application of various biocides.

For example glutardialdehyde is less stable but more active at alkaline pH because the electrophilic interaction with the targeted cell entities is enhanced (Russell, 2003d). Additionally, as mentioned above modification of the concentration of an antimicrobial agent can have a direct influence on its effectiveness. An important characteristic of biocides is the concentration exponent (dilution coefficient) η which measures the effect of concentration changes of antimicrobials in relation to their lethal activity rate. Substances with high η-values such as phenolics and alcohols result in an expanded time to achieve a comparable killing effect when the concentration is reduced, whilst compounds with a low η-value are influenced to a lesser extent (e.g. QACs or formaldehydes). Organic and inorganic matter present in the biocide action ambient may interfere with the microbiocidal activity. Examples of the mechanisms by which these impurities influence the biocidal activity are the competition with the biocide for the targeted cellular entities or even the sequestration of the biocide molecules generating a reduction in biocide molecule availability.

Factors influencing the biocide post-treatment are related to the recovery of microorganisms which have been exposed to antimicrobial compounds. The recovery mechanisms of biocide injured, but still-viable, microorganism on solid media and their colony forming ability are not completely understood (Russell, 2003d), this may be crucial for the determination of biocidal activity i.e. MIC. These include factors influencing the determination of viable count such as the diluent used to carry out the

serial dilution, the composition of the recovery medium and the temperature and incubation period.

When using biocides for industrial applications it is of importance to determine and quantify the principal factors influencing the activity of biocides. Apart from the biocides activity, the large-scale applicability of biocides in industrial processes such as the preservation of calcium carbonate slurries has also to take into account the economic efficiency and the compatibility of the applied biocide system with the material to be protected. Further key points are the regulatory framework, the risk to humans and the environment as well as the acceptance on the part of the customer.

4.3 Biocides Used for the Preservation of White Mineral Dispersions

The requirements of preservation and the usage of suitable biocide systems in white mineral dispersions is not only limited to the antimicrobial activity. Some of the most important criteria which a biocide system has to meet in order to be used in white mineral dispersion have been summarised as follows (Schwarzentruber and Gane, 2005):

- Heat-stability of the biocidal substances up to 60 ºC
- Alkaline rather than acidic properties of the biocidal formulations
- Compliance with the regulatory requirements (e.g. BPD, FDA, BfR)
- Optimal short-term impact (disinfection) as well as long-term activity (preservation)
- No negative influence on the physiochemical properties of WMD
- May not migrate into finished goods (e.g. papers, surfaces) used for materials with food contact
- Biodegradability of >80%, preferably 100% (OECD guideline 301D)
- Do not impair the nitrification of the biological wastewater treatment (inhibition < 30%)
- Positive redox potential required

The adoption of the BPD (Biocidal Products Directive 98/8/EC) has caused a significant reduction in the number of biocidal actives, whereby of a total of approximately 1000 notifiable actives only about 360 have been notified (Kähkönen and Nordström, 2008), and only a few of these are applicable in WMD (Table 20). In addition, some biocidal compounds used for the preservation of calcium carbonate slurries, such as formaldehyde-releasers, are under pressure due to their possible classification as carcinogenics, while some EU member states have reduced their occupational exposure limits. End users too are refusing to take delivery of products that use compounds like o-phenylphenol (personal communication P. Schwarzentruber). In addition to these reasons, certain compromises are being accepted as a consequence of shortened research activities in the field of preservation systems.

Table 20. Biocidal substances used currently for the preservation of white mineral dispersions.

Categories			
Declining acceptance	**Customer rejection**	**Available active compounds**	
Formaldehyde releasing Compounds	**Phenolics**	**Heterocyclic N, S Compounds**	**Aldehydes**
- EDDM (O-formals) - TMAD (N-formals) - DAZOMET (Thione) - Hexahydrotriazine - Adamantanechloride - THPS - DMO	- o-Phenylphenol (OPP)	- Methylisothiazolin (MIT) - Chloro-methyl-isothiazolin (CIT) - Benzisothiazolin (BIT) **Compounds with activ. halogen atoms** - Dibromo-nitrilo-propion-amide (DBNPA) - BNPD (Bronopol)	- Glutaraldehyde **Oxidizing Agents** - Hydrogenperoxid

The fact that not all biocides have been considered and accurately investigated for their compatibility with white mineral dispersion indicate the opportunity of discovering new antimicrobial actives potentially applicable in this field. In the following sections the most important antimicrobial compounds used for the preservation of white mineral dispersions are discussed, however, to exhaustively

review the area of antimicrobials and biocide systems is beyond the scope of this thesis.

4.3.1 Aldehyde Based Compounds

As a consequence of the broad spectrum of activity, the most widely used aldehydes among the aldehyde-based biocides are formaldehyde, glutardialdehyde and o-phthalaldehyde. Other aldehydes with some antimicrobial and sporicidal activity are acetaldehyde, glyoxal (ethanedial), succinaldehyde, 2-propenal and adipaldehyde (hexanedial). Aldehydes are highly reactive molecules and strong electrophilic agents which exert the antimicrobial activity when reacting with nucleophilic cell entities. The following section deals mainly with formaldehyde-releasing agents and glutardialdehyde.

4.3.1.1 Formaldehyde-Releasing Agents

As a consequence of its toxicity to many microorganisms, formaldehyde (FA) is a widely used biocide in various industrial applications such as the production of paper, resins, glue, and in wood processing (Glancer-Soljan*, et al.*, 2001). Formaldehyde is a water-soluble gas and is used as a disinfectant in the vapour state or as an aqueous solution of up to 38% (w/w). Formaldehyde is slower than glutardialdehyde in the action and also has a bactericidal, mycobactericidal, fungicidal, sporicidal and virucidal activity. The toxic effect of formaldehyde on many microorganisms has been well studied and can be traced back to its reaction with nucleic acids and different amino acids in proteins. Formaldehyde is an extreme reactive molecule which interacts with the primary amide and amino groups of proteins as well as with the amino groups of nucleic acid bases thus inhibit DNA and RNA activities by forming cross-links. Indisputably, the cross-linking efficiency of formaldehyde accounts for the principal activity. Furthermore, based on the reaction with carboxyl, sulfhydryl and hydroxyl groups it has been proposed that formaldehyde acts as a mutagenic agent (McDonnell and Russell, 1999). Among other criteria such as toxicity and irritation source, this was the reason why formaldehyde, recently recommended by the IARC (International Agency for Research on Cancer) to be classified as carcinogenic, is under pressure even though

it is one of the most extensively investigated biocides. However, the European Chemicals Bureau did not follow the IARC recommendation yet (Schwarzentruber and Hoppler, 2004).

Besides undesirable side-effects such as increased viscosity and an insufficiently balanced range of activity, formaldehyde is too volatile and reactive to be used directly in WMD (Schwarzentruber, 2003b). Instead, formaldehyde-releasing compounds offer an alternative source without these disadvantages. (Ethylenedioxy)dimethanol (EDDM) and tetramethylolacetylene diurea (TMAD) are two formaldehyde releasers widely used as preservatives in industrial aqueous systems (Paulus, 2005, Pfuhler and Wolf, 2002, Selvaraju*, et al.*, 2005). Other formaldehyde-releasing agents are derivates of imidazole (imidazolines), amine-formaldehyde–reaction–products such as derivates of hexamine (hexamethylene tetramine or 1,3,5,7-triaza-1-azonia-adamandate) and tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione (Dazomet), triazines and oxazolo-oxazoles (4,4-Dimethyloxazolidine). However, the applicability of formaldehyde-releaser in white mineral dispersion is determined by the compatibility, stability and the amount of released formaldehyde under these environmental circumstances. In the preservation of white mineral dispersion EDDM in combination with isothiazolinones has been successfully used. The protection of white mineral dispersions requires a concentration of 200 to 250 ppm (a/d; 75% solids) of EDDM (Schwarzentruber, 2003b). It is known that the activity of formaldehyde decreases significantly in the presence of contaminating matter (organics) or microbial clumping (biofilms) (McDonnell and Gerald, 2007).

4.3.1.2 Glutardialdehyde

Glutardialdehyde (GDA) is a highly reactive molecule and like formaldehyde exhibits antibacterial, sporicidal, fungicidal and virucidal activity. GDA is used in a variety of industrial applications (e.g. cooling water systems, oilfield operation, leather tanning industry, cosmetics) as a low-temperature liquid disinfectant of temperature-sensitive devices (e.g. endoscopes) and as a fixative for example in electron microscopy. Moreover, GDA is non-corrosive to stainless-steel and has no deleterious effects on sensitive materials such as plastics and rubbers. In contrast to

formaldehyde, glutardialdehyde is more active at alkaline pH, despite the fact that with increasing pH the shelf live and hence the availability of reacting aldehyde groups is decreased primarily due to self cross-linkage. GDA reacts with various biomolecules, particularly proteins and inhibits the synthesis of DNA and RNA. The mechanism of action of GDA has been described as resulting from the strong association to the outer layers of the bacterial cells and due to the cross-linking reaction with amino and thiol groups. The free exposed amino groups of lysine and arginine are direct targets of the cross-linking reaction and have been demonstrated to interact strongly with glutardialdehyde (McDonnell and Russell, 1999). Overall, the cross-linking of unprotonated amines of proteins on the cell surface lead to rapid inhibition of vital cell functions. A controversial issue is that organic matter has been stated to have no effect on the activity of GDA, although the interaction with amino groups of proteins would suggest this characteristic (Moore and Payne, 2003). In recent years, combinations of 100 ppm (a/d; 75% solids) glutardialdehyde with isothiazolinones and/or 2-bromo-2-nitropropane-1,3-diol (BNPD) have taken a central role in the preservation of white mineral dispersions (personal communication P. Schwarzentruber).

4.3.2 Isothiazolinones

Benzisothiazolinone (BIT), 5-Chlor-2-methyl-4-isothiazolin-3-one (CMIT), 2-Methyl-4-isothiazolin-3-one (MIT) and 2-n-Octyl-4-isothiazolin-3-one (OIT) are isothiazolinone derivates containing a nitrogen and sulphur atom in the ring system. Isothiazolinones are biocides which exhibit an antimicrobial activity due to electrophilic attack on cellular thiol groups, given that their activity is quenched by thiol-containing compounds. CMIT has also been reported to act against non-thiol amino acids, it may therefore react with amines. Isothiazolinones are widely used as preservative for cutting oils and as in-can preservative in the paint, pigment and paper industry as well as in cosmetics.

The most prominent mixture of isothiazolinone is common under the trade name Kathon® and is a mixture of three parts CMIT and one part MIT. This mixture has been established to have antimicrobial activity against a wide range of bacteria and is used in a concentration range of 2.25 to 9 ppm without showing a

Pseudomonas gap. As a consequence of skin-irritant properties, the concentration of CMIT in cosmetic products is restricted to 15 ppm and 7.5 ppm for rinse-off products and leave-on products, respectively. Schwarzentruber (2003b) has shown that 3 to 5 ppm (a/d; 75% solids) Kathon® achieves a good preservation of calcium carbonate slurries. BIT is primarily a bacteriostatic agent but in contrast to the other two isothiazolinones it is a hypersensitising agent. CMIT acts as a bactericidal agent at lower concentrations than BIT. In addition, CMIT has been reported to associate rapidly with the bacterial cell membrane, thus resulting in the traverse of the cell membrane followed by a rapid growth inhibition of the treated cells (Diehl and Chapman, 1999). Isothiazolinone compounds are not heat-stable, while MIT is the most stable over a wide range of pH and temperatures. As from August 2001 any product containing more than 15 ppm CMIT/MIT in the ration 3:1 must be labelled with an appropriate R phrase warning in the European Community (Directive 2001/59/EC). As a result of the high pH of white mineral dispersion and thus a lack of mould occurrence, the fungicidal and algaecidal OIT is not considered to be necessary for the preservation of WMD.

4.3.3 Compounds with Activated Halogen Atoms

The most prominent compounds of this kind used for the preservation of white mineral dispersions are 2,2-Dibromo-3-nitrilopropionamide (DBNPA) and 2-Bromo-2-nitropropane-1,3-diol (BNPD). The antimicrobial activity of these substances arise form the nucleophilic reaction of cellular entities with the electrophilic carbon atom of such substances. DBNPA is a potent but not persistent antimicrobial agent which has been demonstrated to be an effective slimicide for use in paper making systems and cooling towers. The use of DBNPA at pH ranges 7 to 9.5 is limited due to the breakdown of this compound in alkaline conditions and therefore fails to inhibit bacteria. The application of DNBNA in white mineral dispersion can be referred to as short-term rather than as long-lasting protection. An amount of 270 ppm (a/d; 75% solids) are required to achieve significant preservation (Schwarzentruber, 2003b).

BNPD is an aliphatic halogenonitro compound with potent antimicrobial efficiency. BNPD react to a great extent with thiol groups, and free radical mediated toxicity has been suggested to be an accompanying effect of the action mechanism

(Chapman, 2003a). BNPD is most stable under acidic conditions and the release of formaldehyde under alkaline conditions contributes insignificantly to the antimicrobial performance. A previous report demonstrated that BNPD was even affective against formaldehyde-resistant bacteria demonstrating that the antimicrobial activity is not only based on the action of the potentially released formaldehyde (Sondossi, *et al.*, 1986). BNPD has been used as a preservative in cosmetics and pharmaceutical products but the limited stability in alkaline products constrains its use in white mineral dispersions. The half-life of BNPD at pH 8 and 25 ºC is of about 4 days. Finally, BNPD should not be used in combination with secondary amines since the reaction produces carcinogenic nitrosamine as a by-product (Legin, 1996). A combination with OPP also leads to an increased risk of corrosion for stainless steel tanks and pipings (personal communication P. Schwarzentruber). For pigment slurries 35 ppm BNPD (a/d; 75% solids) are recommended (Schwarzentruber, 2003b).

4.3.4 Phenolics

Phenol derivates are membrane-active biocides and are used as disinfectants and as preservatives in a variety of products. The antimicrobial activity of phenols results from the hydroxyl group which is very reactive and preferentially forms hydrogen bonds with proteins. Phenols adsorb onto the microbial cell and then progressively damage the cell membrane by inducing the leakage of intracellular material including potassium ions (Maillard, 2002), and leads to the disruption of transport, respiratory and energy coupling activity (Denyer and Stewart, 1998). Once they penetrate the cell, reactions within the cytoplasm include the inhibition of enzymes. At higher concentrations phenols rapidly cause cell lysis of both gram-positive and gram-negative bacteria.

The compound o-phenylphenol (OPP) belongs to the “non-coal-tar” phenols which are made in large-scale synthetic processes. OPP is a white crystalline powder which is not particularly soluble in water. Whilst for practical use OPP is used as the sodium or potassium salt which is readily soluble in water, the antimicrobial activity is retained in the phenolate anion form as well. As a consequence of its favourable toxicity data (Paulus, 2005), OPP has been used for the preservation of citrus fruits

and in recent years due to its alkaline character OPP gained importance in the preservation of calcium carbonate slurries. However, OPP in calcium carbonate slurry is more effective as a preservative rather than as a disinfectant and because of the relatively low solubility level, 200 – 300 ppm (a/d; 75% solids) are required to achieve optimum preservation performance (Schwarzentruber, 2003b). Falkinham (2009) have shown that OPP did not affect the growth of *Pseudomonas pseudoalcaligenes* and *Mycobacterium immunogenum* cells therefore did not perform as a disinfectant. To conclude, OPP has been shown to be ineffective against bacteria protected by slime layers such as sessile cells in biofilms. As from the 1st January 2011 OPP will be classified as a pesticide by the European Union (commission directive 2009/160/EC amending Council Directive 91/414/EEC) the relevance of this substance for the preservation of white mineral dispersions has all but ceased.

4.3.5 Combinations of Biocides

For the optimal protection of white mineral dispersions often a single compound biocide is not sufficient (Schwarzentruber, 2003b). In addition, the chemical properties of biocidal compounds in term of speed of action and long-lasting activity are relevant properties to achieve a stable and protection of WMD. Since the production of WMD is not an aseptic process and is performed in an "open" system using raw materials of natural source (minerals, tap water) the optimal biocide comprise a disinfectant to kill the present contamination and a protective agent to guarantee cleanness along the storage and supply chain. The combinations of preservatives with disinfectants in biocide formulations have proven to be practical for the protection of WMD. Combinations typically comprise fast but short-term acting compounds such as isothiazolinone, DBNPA or BNPD with rather low but long-term efficient compounds such as formaldehyde-releasers, glutardialdehyde or o-phenylphenol. Figure 35 visualise the target profile of biocide combinations for the protection of white mineral dispersions. Whilst formaldehyde and glutardialdehyde relating to the stability and speed of action are interjacent, o-phenylphenol has a poor disinfection efficacy (Schwarzentruber, 2003b). Therefore, combinations of antimicrobials lately gained in importance to preserve white mineral dispersions.

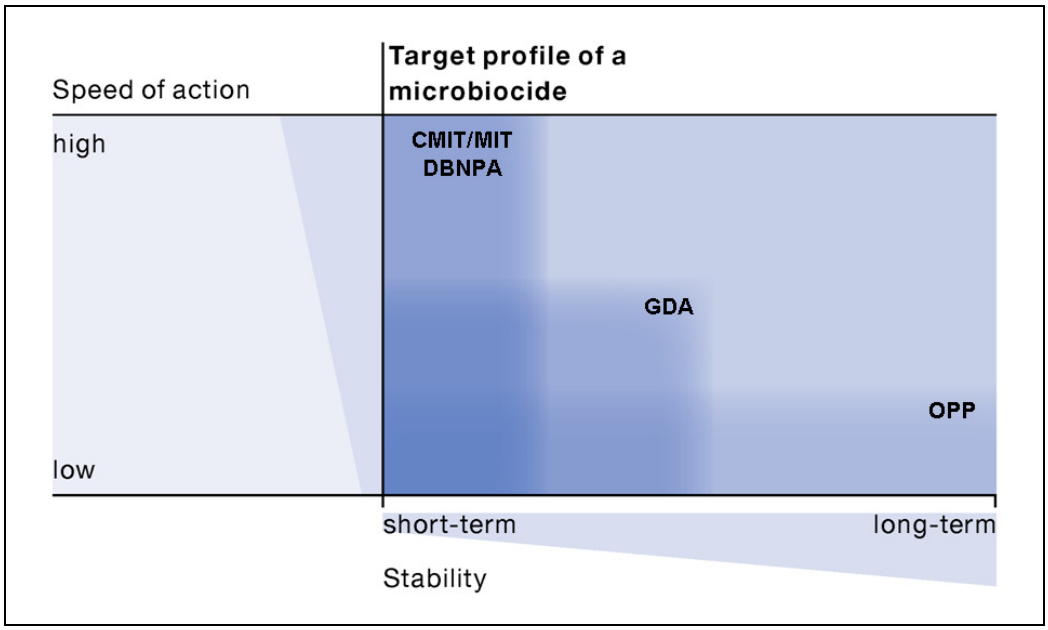


Figure 35. Activity profile of biocide combinations in white mineral dispersions.

4.4 Bacterial Biocide Resistance

Bacterial resistance to disinfectants and preservatives is an emerging but not new problem causing serious concern in relation to economic as well as health issues (Chapman, 2003a). Several studies have reported reduced susceptibility of bacteria to various biocidal compounds and basic principles of the resistance mechanism have been exhaustively reviewed (Chapman, 2003a, McDonnell and Russell, 1999, Russell, 1995, Tumah, 2009). The term "biocide resistant" is relative. Overall, the terminology pertaining to "biocide resistance" is addicted to definition and to apply designations analogously to the antibiotics is still subject of controversy in the scientific community (Russell, 2003c). The term "reduced susceptibility" has been suggested to be more appropriate in this context (Beumer*, et al.*, 2000). However, the differences in MIC between biocide susceptible and resistant bacteria are much smaller compared to antibiotics since biocides act on several entities of the cells whereas antibiotics target specific sites (Chapman, 1998). In general bacteria are considered to be resistant to antibiotics if they persist above a MIC breakpoint defined by the National Committee for Clinical Laboratory Standard (NCCLS) (Chapman, 1998). However, in the use of preservative and disinfectants the susceptibility breakpoint for a biocide is not recommended by any regulatory or

industrial organisation and varies as the case arises. Therefore, bacteria are considered to be resistant once the in-use concentration of a biocide fails to achieve the expected protection of the treated product or process. The term biocide tolerance has been introduced to describe reduced susceptibility to biocides, however, biocide tolerance might arise from temporarily low susceptibility phenotypes because of environmental changes (Chapman, 2003a) and maybe lost in the absence of selective pressure.

Bacterial resistance to biocides can be categorised into two groups: either intrinsic resistance as a natural property of an organisms or acquired resistance by the mutation or acquisition of a plasmid or transposon (McDonnell and Russell, 1999). The principal mechanisms of bacterial resistance toward biocides are summarised in (Table 21). Intrinsic resistance, the most described resistance form, includes membrane impermeability due to the structure of the outer membrane to prevent the uptake or penetration of biocides (e.g. gram-negative bacteria), efflux systems which actively extrude biocides from the cytoplasm (e.g. *mex* pump in *P. aeruginosa*) or the production of enzymes which bring about biodegradation of the biocidal compound (e.g. inactivation of aldehydes and phenols by *Pseudomonas*). The mutation and the acquisition of resistant determinants can confer resistance to antimicrobial actives and the acquisition of resistance genes is particularly important with regard to cross-resistance and co-resistance (Chapman, 2003b). Examples of acquired resistance are similar to those described above and comprise the membrane impermeability, extrusion and breakdown of biocides due to the acquisition of genetic material conferring these properties. Moreover, decreased affinity of the biocide to the target due to mutation has been reported for example for 5-chlor-2-(2,4-dichlorphenoxy)-phenol (triclosan) which showed reduced effectiveness in mutants lacking the enoyl reductase enzyme (Tumah, 2009).

Table 21. Resistance mechanisms of bacteria to biocides. Adapted from (Pagès, *et al.*, 2009).

Mechanism	Category	Susceptibility to other biocides[a)]	Cross-resistance
Permeability	intrinsic / (acquired)	no	yes
Efflux	intrinsic / acquired	reduced	yes
Degradation	intrinsic / acquired	reduced	no
Target site Mutation	acquired	reduced	no
Phenotypic change	following exposure	reduced	yes
Induction - stress response	following exposure	variable	yes

a) susceptibility according to the concentration of biocides

Both cross-resistance and co-resistance result in the insusceptibility of bacteria to more than one biocidal agent (Chapman, 2003b). Per definition cross-resistance describes the bacterial resistance towards a set of biocides with analogue performance routes, e.g. which attack the identical target, use common uptake routes or affect similar metabolic pathways within bacterial cells. Instead, the arrangement of genes or gene cassettes on mobile genetic elements (e.g. plasmids) conferring resistance to a set of biocidal agent is termed co-resistance.

A considerable contribution to the resistance of bacteria against antimicrobial agents is made by the occurrence of biofilms in their natural environment. Biofilms represent a sessile form of bacterial communities and are the result of an interaction of bacteria with surfaces. Unlike planktonic cells, biofilms consist of multispecies microbial communities embedded in a slime-like matrix of extracellular polymeric substances (EPS) (Stoodley, *et al.*, 2002). The main components of the EPS are polysaccharides, proteins and nucleic acids. Stoodley et al. (2002) in his review described the development of a biofilm as a five-stage process which includes the initial attachment of cells onto a surface, the production of the EPS matrix, the development and maturation of the biofilm architecture and finally the dispersion of single cells or biofilm fragments with the potential of restarting the biofilm development process. Additionally, a complex physiological interaction between the bacterial cells in terms of nutrient and oxygen availability, cell-to-cell signalling (quorum sensing) and the overexpression of genes belonging to the stress response

exist in the biofilm microenvironment. It is widely accepted that bacterial cells of biofilms are less susceptible to biocide, thus withstand to greater extent the biocidal activity of agents (Tumah, 2009). Several factors contribute to the reduced susceptibility and originates from the limited access of the biocide molecule to the cell due to the protective action of the EPS, the metabolic activity and stress response of individual cells (e.g. presence of persistent cells), the close interaction between the cells (e.g. genetic exchange) and the increased production of degradative enzymes. In connection with white mineral dispersions the development of biofilms not only represent a problem in term of resistance development but also contribute to the formation of sediments in the product storage tanks and pipelines acting as a foci for the contamination of fresh product. Finally, the formation of biofilm in stainless steel tanks is associated with biological corrosion and as a consequence the manifestation of pitting corrosion.

4.5 Results and Discussion

4.5.1 Formaldehyde-Resistant Bacteria in WMD

One problem with formaldehyde-releasing preservatives reported in the field is the ability of various bacterial strains to metabolise formaldehyde. In several cases, formaldehyde degradation has been related to the growth of pink-pigmented facultative methylotrophic bacteria belonging to the family *Methylobacteriaceae* (Anesti, *et al.*, 2005, Schwarzentruber, 2003b, Van Dien, *et al.*, 2003). Methylotrophic bacteria belong to a group of microorganisms with an efficient metabolic pathway for using single carbon compounds (C_1 compounds) as their energy source. Formic acid has been suggested to be the central metabolite in the conversion of C_1 compounds into biomass (Crowther, *et al.*, 2008). Thus, in the mentioned cases, destabilisation of the mineral dispersion observed was due to the pH drop caused by an accumulation of formic acid. The aim of the following experiments was to investigate the biodegradation of formaldehyde in broth and in WMD by two formaldehyde-resistant bacterial WMD isolates. In addition, the minimal inhibition concentration of EDDM, the stability of formaldehyde released by EDDM and the amount of formic acid produced in WMD were determined. Finally, the phylogenetic affiliation of the facultative methylotrophic bacteria was assessed by using culture-independent and cultivation approaches. The following data provide insight about the degradation progression of formaldehyde by a formaldehyde resistant mixed bacterial population.

4.5.1.1 Release and Stability of Formaldehyde in WMD

The release rate of formaldehyde-releasing biocides is fundamental to estimating the amount of free formaldehyde in preserved calcium carbonate slurries, since formaldehyde-releasing biocides are differentiated by fast and slow formaldehyde-releasing types. Furthermore, the stability of the formaldehyde in the slurry is indicative of the preservation efficiency time period. To determine the formaldehyde release rate of EDDM biocide as well as formaldehyde stability in calcium carbonate dispersion, sterile Hydrocarb 90-GU 75% dispersion was supplemented with 2700 ppm (a/l) EDDM biocide, incubated at 30 ºC and both formaldehyde and formic acid concentrations were monitored daily during 7 days. Formaldehyde concentrations

remained steady over the entire evaluation period (Figure 36). The recorded average level of formic acid during the entire incubation period was 186 ppm (a/l).

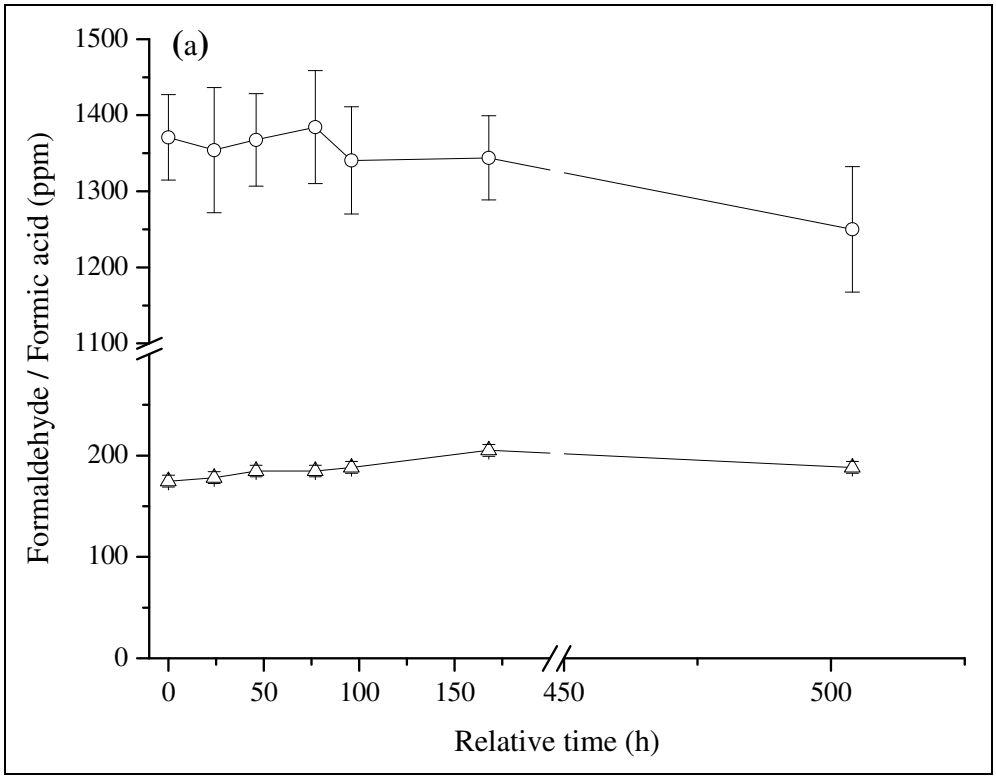


Figure 36. Stability of formaldehyde released by EDDM. Concentration of (○) formaldehyde and (△) formic acid in H90-GU 75% dispersion supplemented with 2700 ppm (a/l) EDDM.

However, the average rate of formaldehyde and formic acid release into calcium carbonate slurry was estimated to be 50% and 7% respectively, based on the initial dosed amount of EDDM biocide (Figure 37).

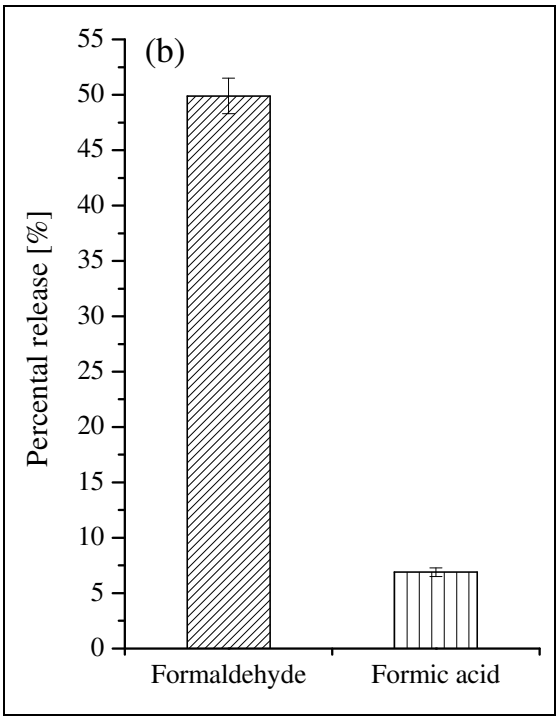


Figure 37. Formaldehyde and formic acid release rate of EDDM calculated in respect to the measured formaldehyde concentrations in Figure 36. The average percent release was calculated in relation to the initial EDDM concentration from the means of each point. Vertical bars represent standard deviation of three independent experiments.

The data suggested that the concentration of EDDM released formaldehyde by hydrolysis and the formic acid in sterile WMD remained stable for up to one week. The elevated amount of retrieved formic acid at time 0 of the analysis indicates the spontaneous oxidation of the formaldehyde taking place in the container of the biocide formulation. By virtue of this, the biocide specifications usually cover a range in terms of percental content of the single active compounds. In this specific case the content of EDDM in the applied biocidal formulation is specified to range from 85-95%, hence both the formaldehyde and formic acid releasing rate are affected by variations in the EDDM concentration. Therefore, it can be assumed that the concentration of the active compounds in the biocide blends vary from batch to batch and in some cases might deviate from the dosed concentration. Thus, the concentration of formic acid would appear to be driven by the chemical equilibrium of spontaneous formaldehyde oxidation.

4.5.1.2 Isolation and Identification of Formaldehyde-Resistant Bacteria

A total of sixteen beige and four pink-pigmented formaldehyde-resistant colonies isolated by means of EDDM-supplemented MH-agar were identified by sequencing approximately 700 bp of the 16S rRNA gene. Sequence analysis of the isolated strains revealed two main groups of bacteria capable of growing in the presence of formaldehyde-releasing biocide in calcium carbonate slurries (Table 22). Phylogenetic classification revealed that the beige colonies all belonged to the genus *Pseudomonas*. The pink-pigmented colony types were affiliated to the genus *Methylobacterium*. Beige and pink-pigmented colonies did not always coexist on the same plate. Every time combined growth was detected, the *Pseudomonas* colonies were typically visible 24 h into incubation, whereas the *Methylobacterium* colonies were only visible after an incubation period of 5 to 7 days.

Table 22. Calcium carbonate dispersion isolated species identified on the basis of 16S rRNA gene sequence alignment, with use of blastn and RDPII databases.

Appearance on MH-A[a]	Taxonomic affiliation	Closest relative[b]	Accession no.[c]	Maximum identity[d]	Length (bp)[e]
	Gammaproteobacteria				
beige	*Pseudomonas*	*Pseudomonas mendocina*	DQ178226	99%	705
beige		*Pseudomonas putida*	DQ182328	99%	768
beige		*Pseudomonas pseudoalcaligenes*	DQ837704	99%	768
	Alphaproteobacteria				
pink	*Methylobacterium*	*Methylobacterium extorquens*	AF531770	99%	698
pink		*Methylobacterium radiotolerans*	AB175641	100%	742

a. Mueller-Hinton agar supplemented with 1000 ppm EDDM (MH-A)
b. Closest relative from comparison with blastn and RDPII databases
c. Accession no. of the closest relative entry
d. Maximal identity for the covered sequence length
e. Sequenced lenght of 16S rRNA gene

The pink-pigmented bacteria *Methylobacterium extorquens* and *Methylobacterium radiotolerans* have often been suspected as being the sole species responsible for biodegradation of formaldehyde in calcium carbonate slurries (Schwarzentruber, 2003b) and previous experiences presumed that in white mineral dispersions, the pH drop is caused by the destabilising effect of an overproduction of formic acid by

formaldehyde-degrading microorganisms. In most cases where formaldehyde-resistant bacteria were detected in shelved samples, the WMD production plants seldom identified bacterial growth immediately after sample collection using Easicult® dipslides (Bode Chemie, Hamburg). Beige colonies, probably all belonging to the genus *Pseudomonas*, were occasionally accompanied by pink-pigmented methylotrophic bacteria, which appeared at the earliest five days into incubation of calcium carbonate slurries plated on EDDM-supplemented MH-A. This is not least because the facultative methylotrophic *Methylobacterium* spp. propagates very slowly compared to *Pseudomonas* spp. (Garrity*, et al.*, 2005, Lee*, et al.*, 2004). *M. extorquens* belongs to the facultative methylotrophs, a group of microorganisms with an efficient metabolic pathway for using single carbon compounds (C_1 compounds) as their energy source (Chistoserdova *et al.* 2004). Moreover, it has been shown that formic acid is the main branch point in the methylotrophic metabolism of *M. extorquens* (Crowther*, et al.*, 2008). A closer examination of the rare sequence tags gathered in the microbial diversity analysis of WMD by means of deep amplicon sequencing in the previous section 3.2.4.2 revealed that bacterial species affiliated to the family *Methylobacteriaceae* occurred in a minority in calcium carbonate slurry. Of the analysed sequence tag of sample H60-AV (winter 2008) and H90-GU (winter 2008), 0.6% and 0.1% of the sequence tags respectively were affiliated to the family of *Methylobacteriaceae*. In the sample H60-GU (spring 2008) a single tag sequence corresponding to a relative abundance of 0.01% was identified. These data suggest that bacteria of the family of *Methylobacteriaceae* sporadically occur in calcium carbonate slurry contaminated with mixed bacterial populations. These bacteria are inferior in terms of propagation because of the slow growth kinetics, however, once the residual biocide is restricted to formaldehyde these bacteria are able to dominate.

4.5.1.3 MIC of EDDM against Formaldehyde-Resistant Bacteria

Previous lab-scale experiences with formaldehyde-sensitive *Pseudomonas* strains have shown that 375 ppm (a/l) of EDDM is a sufficient amount to inhibit growth and thus colony formation on agar. On-site studies suggested that in the field (Omya plants) a concentration of 600–750 ppm (a/l) EDDM has to be recommended in order to avoid sub-optimal preservation performance of WMD, thus preventing the

development of resistance (Schwarzentruber, 2003b). In this study the MIC of EDDM in calcium carbonate slurry for both investigated microorganisms was assessed after biocide exposure times of 24 and 48 h, respectively (Table 23). Accumulation of biomass by the formaldehyde-resistant *P. putida* was invariably evident at an EDDM concentration of 600 ppm (a/l). The minimum inhibition concentration for *P. putida* 24 h after EDDM addition was assessed above 1200 ppm (a/l). In contrast, 48 h after the addition of biocide the MIC was assessed at 900 ppm (a/l) EDDM. The underestimation of the MIC after 24 h of exposure can be assumed to indicate the time of action required by the released formaldehyde to accumulate and develop its antimicrobial activity in WMD. In contrast, *M. extorquens* in WMD ceased propagation at an EDDM concentration greater than 2400 ppm (a/l) independently of the time of exposure. In this context it is important to emphasise that ideally 50% of the dosed EDDM is converted to free formaldehyde, therefore the MIC of formaldehyde is half the amount in respect to EDDM. However, other authors have reported formaldehyde MIC of 30 ppm (a/l) for *M. extorquens* and 400 to 1000 ppm (a/l) for *Pseudomonas* spp. determined in other growth media (Yanase *et al.* 1995; Yamazaki *et al.* 2001; Marx *et al.* 2003). Concomitantly to the MIC experiments, both organisms were inoculated in biocide-free WMD to confirm that inhibition was solely attributable to free formaldehyde in solution.

Table 23. Minimum inhibition concentration of the formaldehyde releaser EDDM for both organisms isolated from calcium carbonate dispersion.

Organism	Minimal Inhibitory Concentration (MIC) of EDDM (ppm a/l)	
	Time of incubation with EDDM (h)	
	24	48
Pseudomonas putida	1200	900
Methylobacterium extorquens	2400	2400

4.5.1.4 Degradation of Formaldehyde in Culture Broth

The biodegradation of formaldehyde by both *Pseudomonas putida* and *Methylobacterium extorquens* was investigated in TSB supplemented with either 1000 ppm EDDM or 1000 ppm TMAD biocide. Unlike the faster growth of *P. putida* in formaldehyde-free TSB, the recorded growth kinetics in EDDM-supplemented TSB shows moderate biomass accumulation and notable formaldehyde detoxification during the first 25 h of incubation, in tandem with production of formic acid at concentrations up to 380 ppm (Figure 38a). The maximum formaldehyde consumption rate for *P. putida* was determined at 11.7 ppm h^{-1}. Biomass increased measurably thereafter, and the accumulated formic acid was subsequently metabolised. Towards 50 h of incubation, the formic acid concentration settled at a constant level whereas the formaldehyde was completely exhausted. In comparison, the facultative methylotrophic *M. extorquens* showed a different propagation pattern in the presence of EDDM biocide. 24 h of cultivation were necessary to produce a measurable increase in biomass (Figure 38b). The maximal formaldehyde consumption rate recognised for *M. extorquens* was 3.9 ppm h^{-1}; a concomitant degradation of formaldehyde and accumulation of biomass were seen towards 30 h of incubation. In addition, after 80 h of cultivation a pulse of 1000 ppm EDDM was added to the culture; this induced an additional concentration of 356 ppm formic acid in the media, while the formaldehyde concentration increased by just 58 ppm.

Comparison experiments using TMAD as the formaldehyde supplier gave different findings. TMAD was observed to release less formaldehyde than EDDM and an initial high concentration of formic acid was measured (Figure 39a and b). In TMAD-supplemented TSB, biomass accumulation by *P. putida* as well as by *M. extorquens* became measurable sooner than was the case with EDDM-supplemented TSB. Both strains exhausted the formaldehyde within 50 h. Formic acid concentrations ranged from 214 ppm to 156 ppm for *P. putida*, and 310 to 206 ppm for *M. extorquens*.

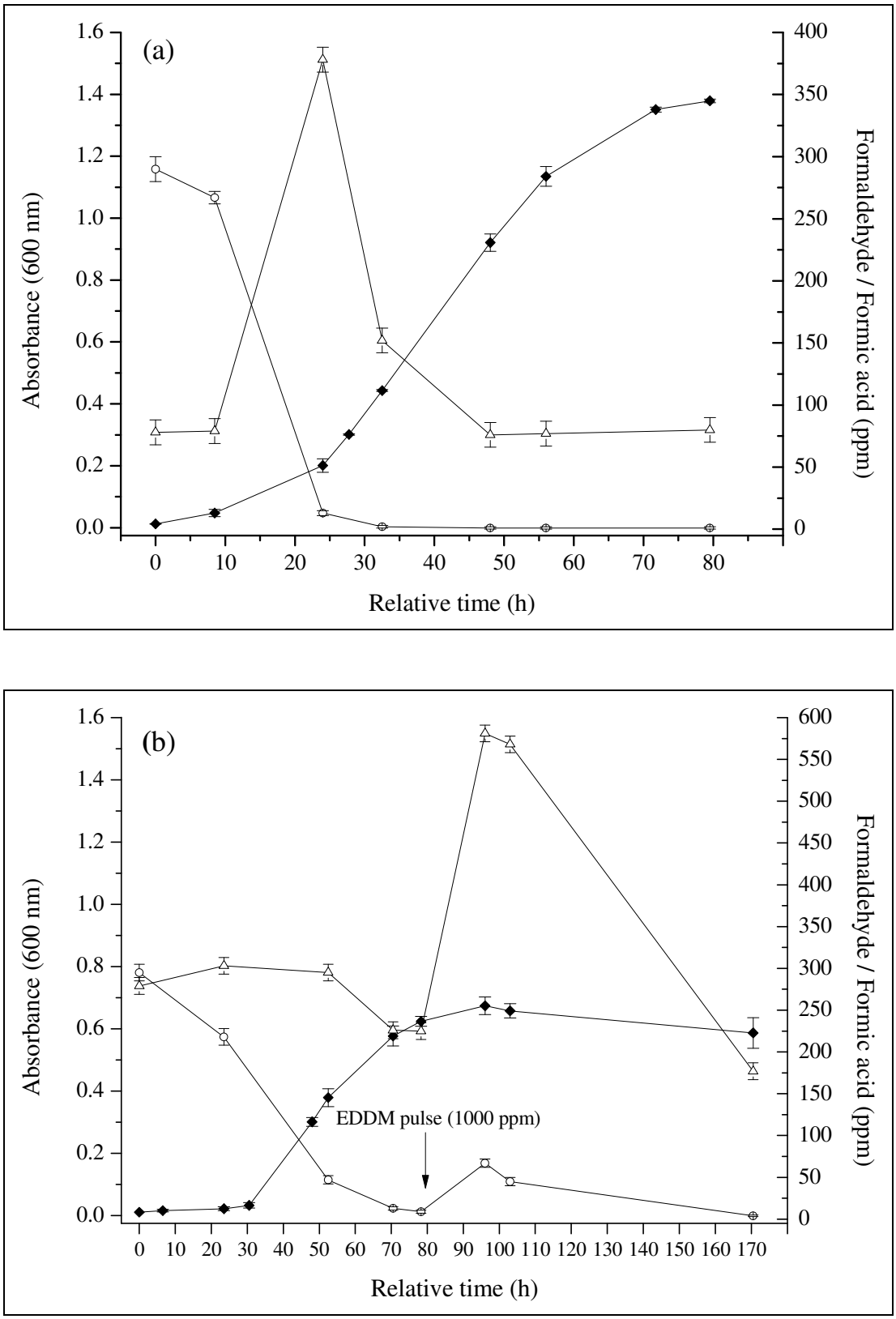


Figure 38. Oxidation of formaldehyde released by the biocide (ethylenedioxy)dimethanol (EDDM, 1000 ppm) in Tryptic Soy Broth. (a) *Pseudomonas putida*. (b) *Methylobacterium extorquens*. (♦) Absorbance (600 nm); (○) Formaldehyde; (△) Formic acid.

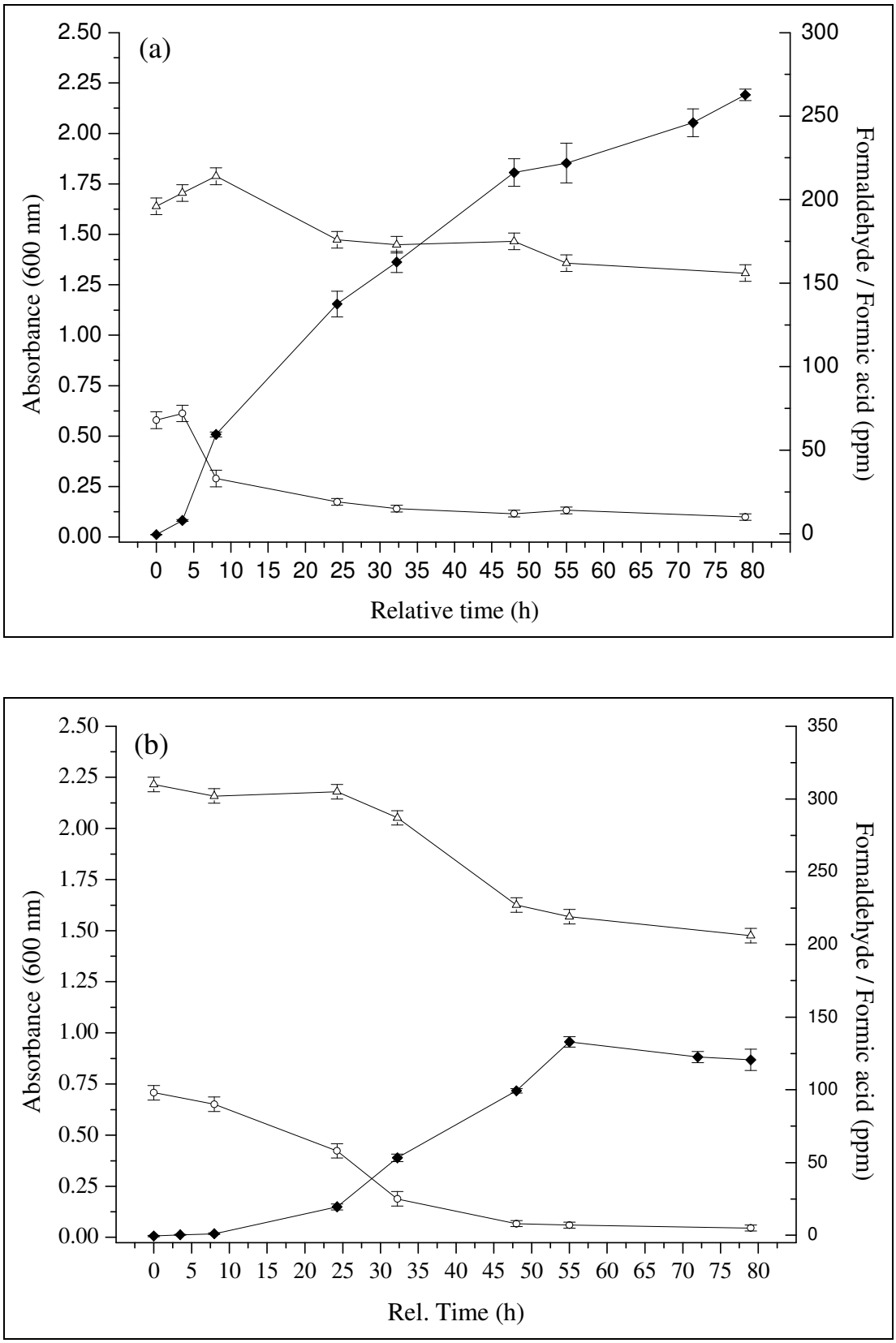


Figure 39. Oxidation of formaldehyde released by the biocide tetramethylolacetylene diurea (TMAD, 1000 ppm) in Tryptic Soy Broth. (a) *Pseudomonas putida*. (b) *Methylobacterium extorquens*. (♦) Absorbance (600 nm); (○) Formaldehyde; (△) Formic acid.

However, due to probable limitation of the substrate, degradation rates of formaldehyde in TMAD-supplemented media were lower than in media containing EDDM (Adroer*, et al.*, 1990). These findings support the assumption that, regardless of the formaldehyde-releasing compound used, consequent degradation and growth dynamics depend on the free, available formaldehyde in solution. The formic acid either produced by the degradation of formaldehyde or carried over from the biocide container (e.g. spontaneous oxidation) is metabolised too. In metalworking fluid Selvaraju et al. (2005) investigated the activity of formaldehyde-releasing biocides (hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine and 4,4-dimethyloxazolidine) and found a high tolerance of *Mycobacterium immunogenum* and *Pseudomonas fluorescens* to formaldehyde.

4.5.1.5 Degradation of Formaldehyde in WMD

Total cell count and membrane potential of the cells in calcium carbonate slurries were measured with a CellFacts II® instrument. The growth behaviour of the investigated organisms was examined individually in EDDM-supplemented calcium carbonate slurry. In Hydrocarb 90-GU 75% dispersion supplemented with 1500 ppm (a/l) EDDM and inoculated with *P. putida* slurry culture a steady cell count was observed. An increase in relative fluorescence measured by CellFacts II® provided evidence of physiological activity by the cells (Figure 40). The formaldehyde concentration remained stable over a period of 7 days, after which there was measurable consumption of the initial formaldehyde amount with concomitant production of formic acid. At the end of the experiment, after 16 days of incubation, the formaldehyde amount was 30% below its initial concentration and the formic acid produced had reached 100 ppm (a/l).

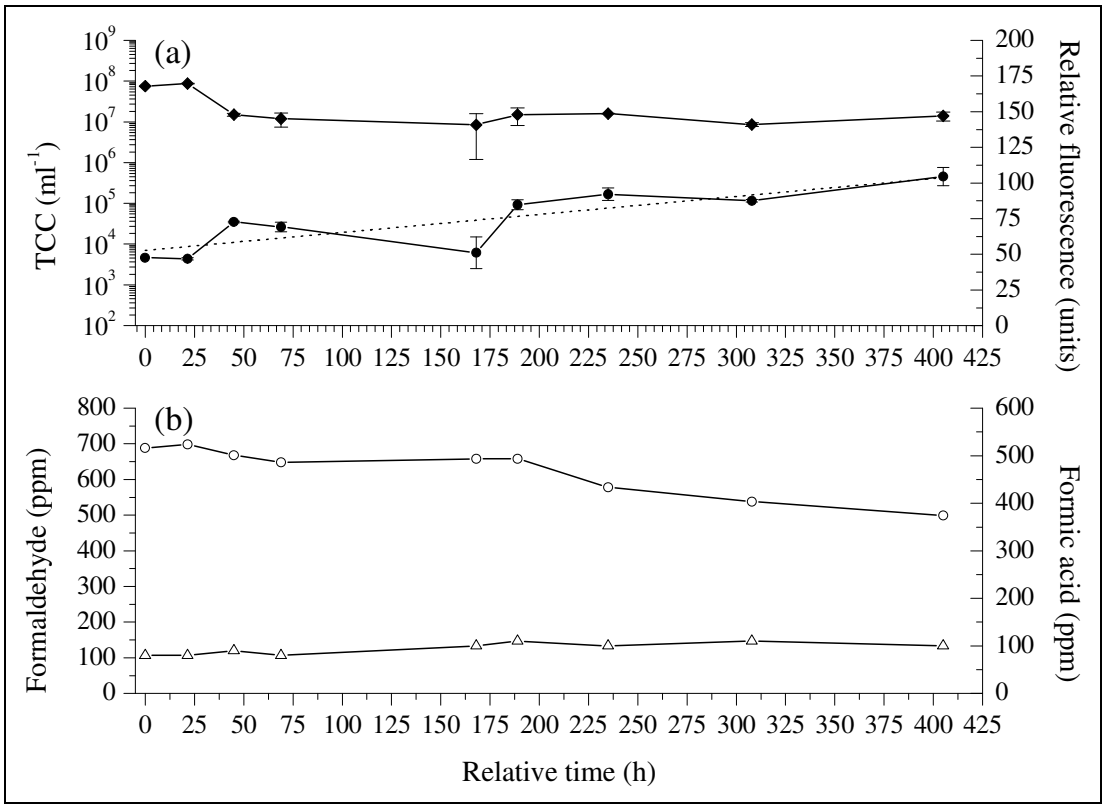


Figure 40. Growth kinetics of *P. putida* in H90-GU 75% dispersion supplemented with 1500 ppm a/l EDDM. (a) (♦) Total cell count and (●) Relative fluorescence measured by CellFacts II®. (b) (○) Formaldehyde and (△) Formic acid concentrations. Dotted line shows linear trend of the fluorescence.

Degradation of formaldehyde in WMD by *M. extorquens* was recorded after 50 h incubation (Figure 41). After 16 days of incubation, residual formaldehyde was measured at 20 ppm (a/l) (<2% of initial concentration), whereas the concentration of formic acid peaked at 558 ppm (a/l) on day 12. Assuming a zero-order reaction ($F_t = -k \cdot t + F_0$, were F_t is the concentration at a particular time, F_0 is the initial concentration and t is the time) returned a formaldehyde consumption rate, $-k$, of 40.5 ppm (a/l) d^{-1}. The relative fluorescence and hence the physiological activity of the cells increased during the experiment while there was no significant increase in viable counts of *M. extorquens*.

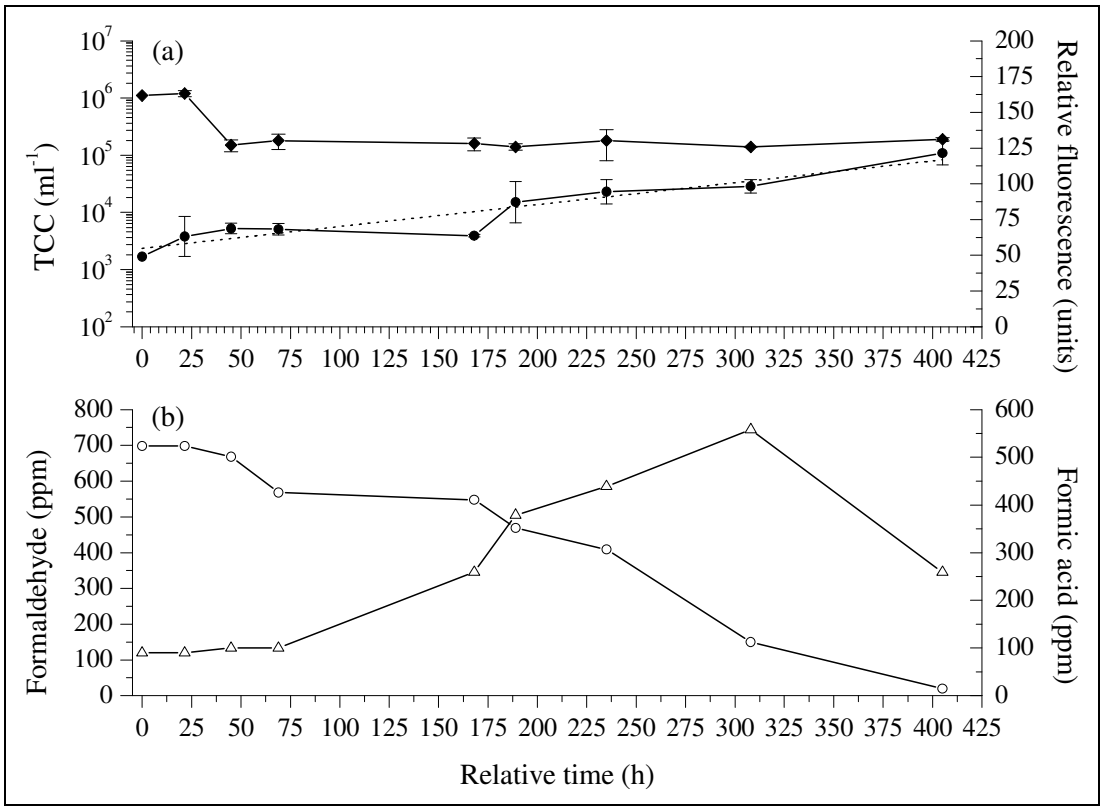


Figure 41. Growth kinetic of *M. extorquens* in H90-GU 75% dispersion supplemented with 1500 ppm (a/l) EDDM. (a) (♦) Total cell count and (●) Relative fluorescence measured by CellFacts II®. (b) (○) Formaldehyde and (□) Formic acid concentrations. Dotted line shows linear trend of the fluorescence.

The steady cell count observed in slurry for both bacteria species indicates that maximal cell density was reached after the initial inoculation. *P. putida* cultured in EDDM preserved WMD showed reduced formaldehyde metabolic activity, hence a reduced formaldehyde tolerance, compared to cultures in broth. Previous studies have reported the presence of both a formaldehyde dehydrogenase (*fdh*, EC 1.2.1.46) and a formaldehyde dismutase (*fdm*, EC 1.2.99.4) in various microorganisms (Fujii*, et al.*, 2004, Glancer-Soljan*, et al.*, 2001, Ito*, et al.*, 1994, Yamazaki*, et al.*, 2001, Yanase*, et al.*, 2002, Yanase*, et al.*, 1995, Yasuhara*, et al.*, 2002). *Pseudomonas*-specific *fdm* oxidises formaldehyde in the absence of an electron acceptor, whereas glutathione-independent *fdh* requires the presence of NAD^+ (Yanase *et al.* 1995; Fujii *et al.* 2004). As a result it can be presumed that the lower tolerance to formaldehyde by *Pseudomonas* spp. is related to the characteristically high pH of the slurry. The high slurry pH leads to an alkalinisation of the cytoplasm, thus to a

reduction of activity by the related enzymes, compared to the enzyme activity in neutral media (Saito and Kobayashi, 2003). On the other hand, *M. extorquens* in slurry showed high tolerance to formaldehyde. This might also be attributed to the fact that the biodegradation of formaldehyde in *P. putida* is related to a detoxification process either via formaldehyde dehydrogenase or different assimilation pathways (Roca*, et al.*, 2008, Roca*, et al.*, 2009), whereas in *M. extorquens* the formaldehyde is used as an energy and carbon source. On the basis of this study, the isolated microorganisms are able to degrade formaldehyde and release a considerable amount of formic acid into calcium carbonate slurry.

4.5.1.6 Phylogenetic Analysis of Methylotrophic Bacteria in WMD

Methylotrophic bacteria belong to a group of microorganisms with an efficient metabolic pathway for using single carbon compounds (C_1 compounds) as their energy source. Methane, methanol, methylated amines, methylated glycines, halomethanes and methylated sulphur are just some in a wide range of C_1 compounds consumed by methylotrophs. Methylotrophic bacteria have been recovered in the environment by targeting different genes involved in the C_1-metabolic pathway (Chistoserdova*, et al.*, 2009). Although *Methylobacterium extorquens* AM1 is one of the best-studied facultative methylotrophic microorganisms, methylothrophy is in fact widespread across bacterial groups. It is found in *Alpha-*, *Beta-* and *Gammaproteobacteria* as well as in Gram-positive bacteria (Acharya*, et al.*, 2005, Chistoserdova*, et al.*, 2004). Depending on the available C_1 compound, the substrate is assimilated in the periplasm or in the cytoplasm. This complex metabolic pathway consists of a wide set of enzymes that are involved in the catalytic degradation of methane to carbon dioxide. Those enzymes with specific catalytic function are in turn encoded by a set of genes forming a module. Different specific modules oxidise C_1 compounds. With a few exceptions, these reactions produce formaldehyde. Theoretically, a methylotrophic microorganism requires at least one functional module to survive and thus to detoxify formaldehyde. Figure 42 illustrates one of the most widely distributed formaldehyde oxidation modules in most gram-negative methylotrophs and is the one involving tetrahydromethanopterin (H_4MPT) as a cofactor (Marx *et al.* 2003; Chistoserdova *et al.* 2004). After entering the cell,

formaldehyde condenses with one of the two pterin cofactors to form the respective methylene derivates.

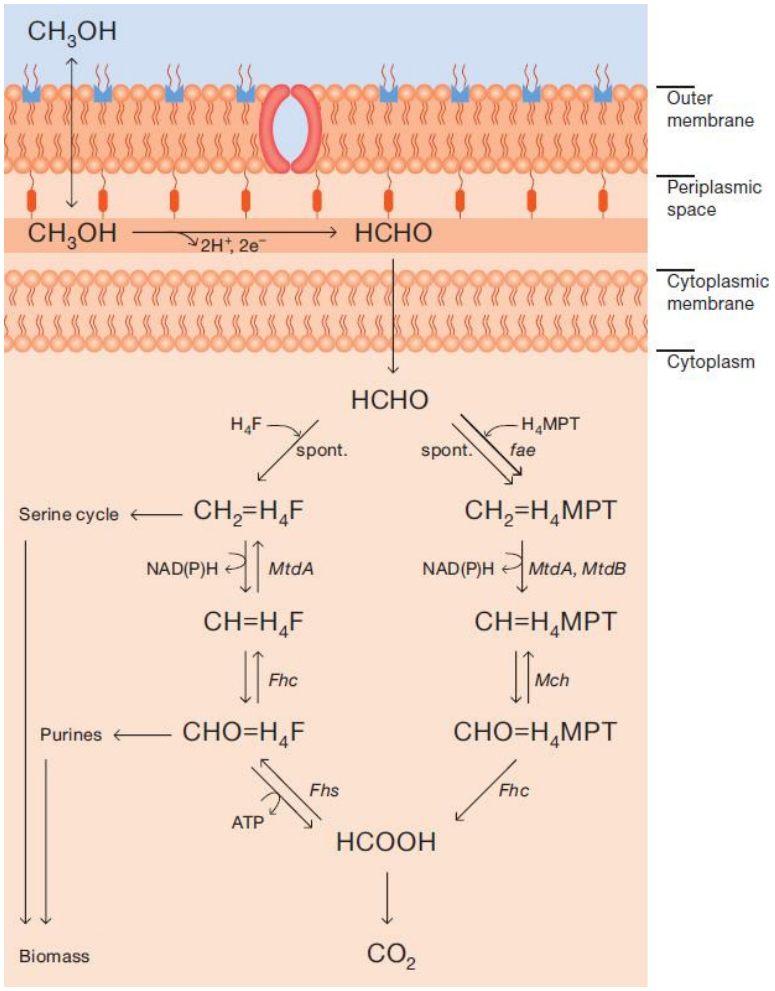


Figure 42. Comparison of the two metabolic pathways in *Methylobacterium extorquens* AM1. Reactions occurring spontaneously or catalysed by an enzyme are indicated by arrows labelled spont., or with the corresponding enzyme name. Reversible enzymatic reactions are indicated by bilateral arrows. *Fae* H_4MPT-dependent formaldehyde activating enzyme, *MtdA* NADP-dependent methylene H_4MPT dehydrogenase, *MtdB* NAD(P)-dependent methylene-H_4MPT cyclohydrolase, *Fch* ethenyl-H_4F cyclohydrolase, *Fhs* formyl-H_4F synthetase, *Fhc* Formyltransferase/hydrolase complex. Adapted from (Marx*, et al.*, 2003).

The reaction of formaldehyde with H_4F seems to occur spontaneously and methylene-H_4F either can be assimilated through the serine cycle or may ultimately be oxidised to formate. Formate can then be oxidised to CO_2 through the action of formate dehydrogenase (Laukel*, et al.*, 2003). Alternatively, formaldehyde can be oxidised through a similar pathway that is linked to the folate analogue H_4MPT. It

has been shown that this condensation is catalysed by the specific enzyme *fae* (formaldehyde activating enzyme), which is required for methylotrophic growth (Marx*, et al.*, 2003). Finally, formic acid is released from the complex and can be oxidised to CO_2 in order to generate energy or used for the production of biomass. Crowther *et al.* (2008) have suggested that formic acid is the central metabolite in the assimilation of C_1 compounds into biomass from the domination of methylene-H_4F synthesis through formic acid. Thus, the spontaneous direct condensation reaction of H_4F serves as an overflow valve and more formaldehyde can be metabolised ATP-independently once the formaldehyde concentration reaches toxicity levels (Marx*, et al.*, 2005).

Detection of methylotrophs in calcium carbonate slurries by the standard plate count method is a laborious and time-consuming process that requires 5 to 10 days of incubation to obtain initial results. Furthermore, the detection limit of 100 cfu ml^{-1} for the plate count method is insufficiently sensitive to detect the seldom large community of methylotrophic microbes populating WMD. Therefore, insights about the diversity of methylotrophic bacteria in calcium carbonate slurry were elucidated by using the gene encoding the formaldehyde activating enzyme *fae*. The broadest range of divergent gene sequences has been detected using either the *fae* (formaldehyde activating enzyme) or *fhcD* (formyltransferase/hydrolase complex subunit D) genes which are part of the H_4MPT-linked formaldehyde oxidation pathway common to most methylotrophs (Chistoserdova*, et al.*, 2009, Nercessian*, et al.*, 2005a, Nercessian*, et al.*, 2005b). Comparison were made from the *fae* sequence data acquired either from DNA extracted directly from calcium carbonate slurry, or extracted from pink-pigmented colonies formerly cultivated on MH-A containing 1000 ppm (a/l) EDDM. Sequence analyses revealed the phylogenetic affiliation of the detected *fae* genes (Figure 41). Representatives of two different clusters were recognised: sequences revealed the presence of members of both *Alpha-* and *Gammaproteobacteria*. Considering the speciality of the WMD environment, phylogenetic analysis revealed a surprising diversity of methylotrophic bacteria in WMD. Similarly, *Methylosinus* and *Methylobacter* as well as other methanotrophs, i.e. bacteria able of oxidising methanol to formaldehyde have been found in alkaline environments such as soil from coal mines (Han*, et al.*, 2009). A number of previous studies have shown that PCR recovery of *fae* gene sequences is a powerful tool for

characterising methylotrophic bacteria in environmental samples (Kalyuzhnaya, *et al.*, 2004, Nercessian, *et al.*, 2005a, Vorholt, *et al.*, 2000). Using the functional gene *fae* sequence affiliated with methanotrophs and methylotrophs, as well as uncultivated organisms of the classes *Alpha-*, *Beta-*, and *Gammaproteobacteria* were identified (Nercessian *et al.* 2005b).

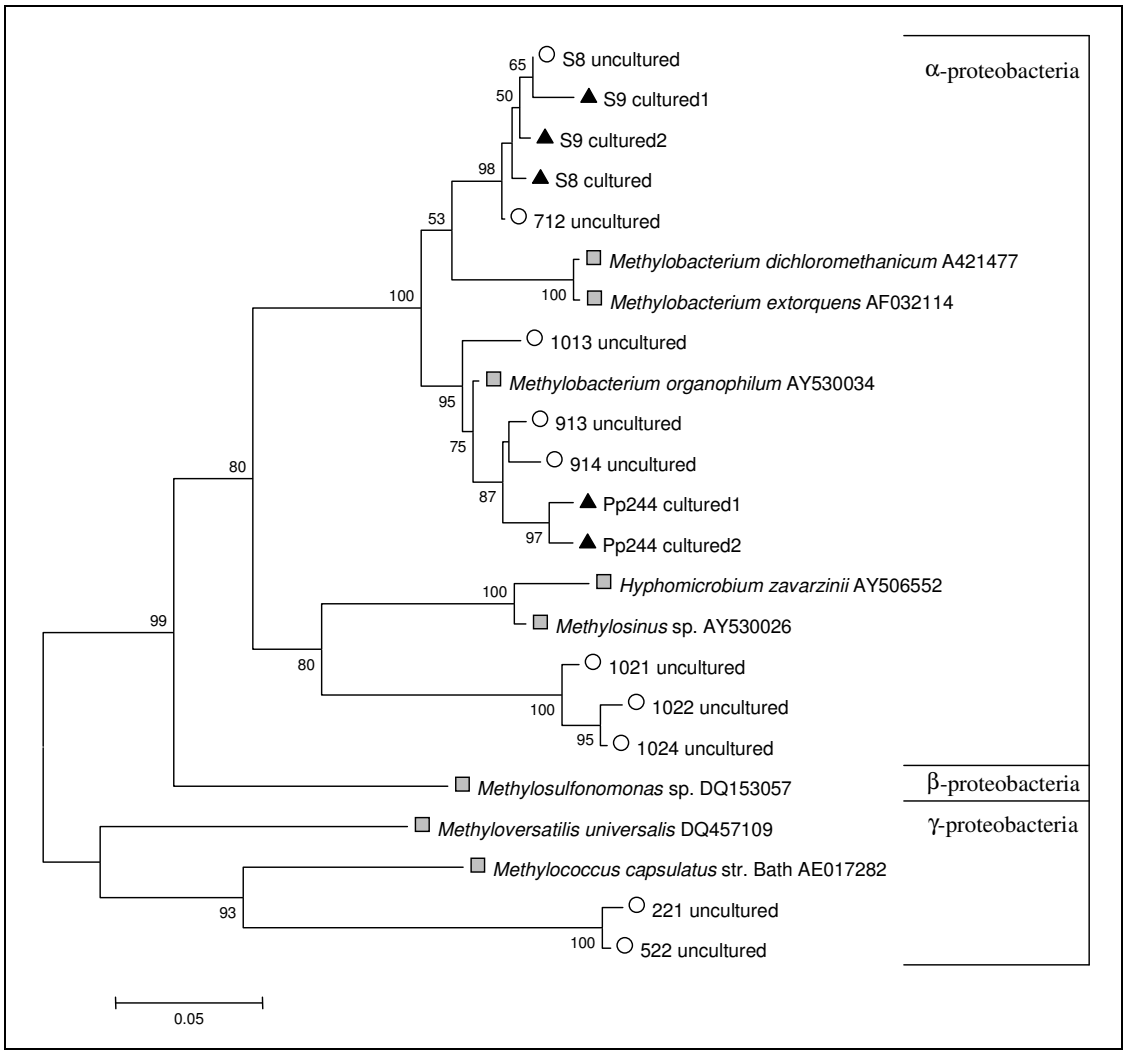


Figure 43. Phylogenetic tree constructed from partial *fae* gene sequences for cultured colonies and DNA extracted *in situ* from uncultured WMD. Samples were collected at the Omya plant in Norway. Method: neighbour-joining; Model: Kimura 2-parameter; Phylogeny test: bootstrap (1000 replicates), values > 50 are shown. The scale bar represents 0.05 substitutions per nucleotide position.

4.5.1.7 Mixed Microbial Populations in Formaldehyde-Preserved WMD

Frequently, the two isolated formaldehyde-degrading microorganisms *P. putida* and *M. extorquens* were detected simultaneously in WMD samples originally taken in the field. However, several limitations were encountered during attempts to artificially engineer a mixed culture of both organisms in the laboratory by simplifying and simulating the real biocide and microorganism regimes in WMD tanks (Figure 44).

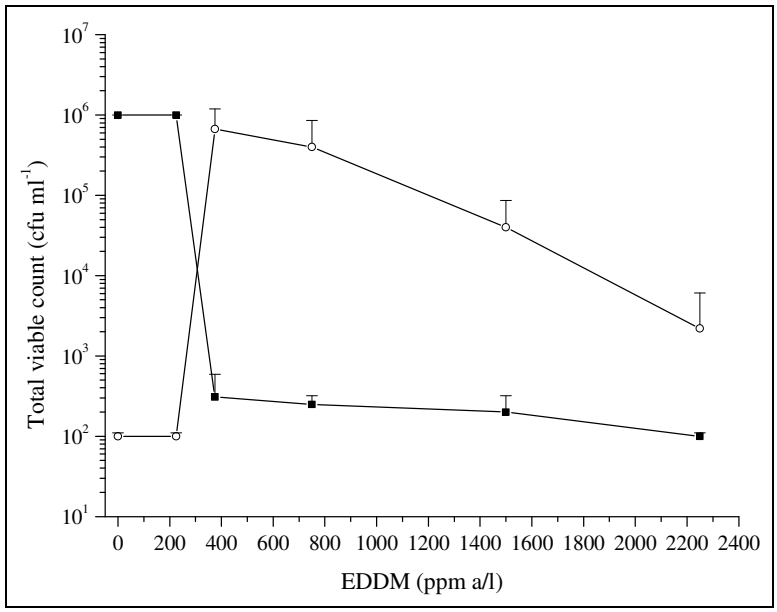


Figure 44. Occurrence of (■) *P. putida* and (○) *M. extorquens* in H90-GU 75% dispersion supplemented with various amounts of EDDM and inoculated with both microorganisms.

Artificially engineered calcium carbonate dispersion cultures containing both organisms were only achieved when preserved slurry containing detectable *M. extorquens* cells was inoculated multiple times with WMD containing *P. putida*. Addition of 225 ppm (a/l) EDDM to WMD inoculated with both bacteria species favoured domination by *P. putida*, whereas *M. extorquens* could not be recognised even after seven days of incubation. In contrast, in dispersions containing 375 ppm (a/l) up to 2250 ppm (a/l) EDDM, *M. extorquens* dominates the slurry environment whilst *P. putida* was recognised at levels below 10^3 cfu ml^{-1}. Furthermore, in four-week-old cultures that originally harboured both organisms, only *P. putida* could be retrieved. When this four-week-old culture was supplemented with EDDM at 1500 ppm (a/l), *M. extorquens* resumed domination and *P. putida* was suppressed by the high formaldehyde concentration in the WMD.

4.5.1.8 Activity of FDH and FDM in Formaldehyde-Degrading *P. putida*

Taking a closer look at the enzymes involved in the degradation of formaldehyde revealed that the formaldehyde-resistant *Pseudomonas putida* strain isolated from calcium carbonate slurry preserved with EDDM harbours the gene for both a glutathione-independent formaldehyde dehydrogenase *fdh* and a formaldehyde dismutase *fdm*. Whereas the formaldehyde dehydrogenase oxidises one molecule of formaldehyde into formate which is further oxidised by the enzyme formate dehydrogenase to CO_2 (Ito, *et al.*, 1994, Roca, *et al.*, 2009), the formaldehyde dismutase catalyses the production of one molecule of both formic acid and methanol from two molecules of formaldehyde (Yanase, *et al.*, 2002).

In order to identify the gene involved in the degradation of formaldehyde released from EDDM, the relative gene expression of both genes was quantified (Figure 45).

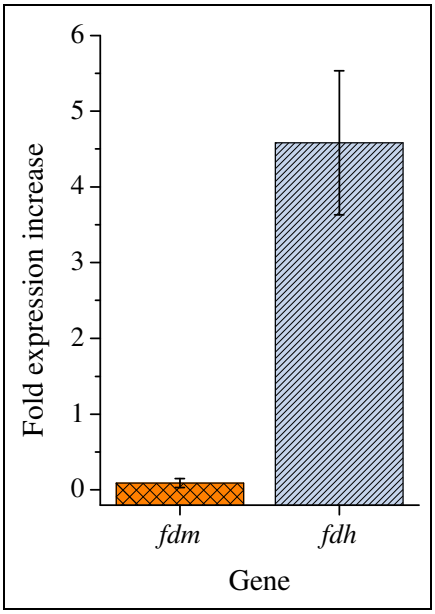


Figure 45. Relative fold increase in expression of the genes *fdm* and *fdh* in *P. putida* after 30 minutes exposure to 750 ppm (a/l) EDDM in TSB measured by quantitative PCR (qPCR). The 16S rRNA gene was used to normalise the data and the relative expression was calculated according to the $2^{-\triangle\triangle C}{}_t$ method. Data are presented as mean ± standard error of mean (biological replicates n = 2, technical replicates n = 4).

An increase of 4.5 fold in the relative expression of the formaldehyde dehydrogenase gene *fdh* was observed after exposing formaldehyde-free precultured *P. putida* to formaldehyde for 30 minutes. The data suggested that the presence of formaldehyde in the growth medium of the formaldehyde-resistant *P. putida* strongly induced the expression of mRNA from the *fdh* gene in order to make more efficient the detoxification process. In contrast, the relative expression of the formaldehyde dismutase gene *fdm* was not increased, hence excluding the involvement of this enzyme in the detoxification of the formaldehyde. Finally, Roca *et al.* (2009) analysed the formaldehyde detoxifying gene clusters of *Pseudomonas putida* and reported an increased expression of the noncanonical promoter regions of the genes encoding the formaldehyde dehydrogenase and formate dehydrogenase in the stationary phase independently of the presence of formaldehyde or formate in the culture medium.

4.5.2 Resistance to other Biocidal Formulations common used in WMD

As a consequence of using one single active agent for many years, formaldehyde, on its own has over time become of limited applicability for the preservation of calcium carbonate slurry (Schwarzentruber, 2003b). Therefore, shortly after the occurrence of resistant bacteria against the in-use formaldehyde-based biocides, multiple uses of two or three different biocidal compounds covering a broader range of action sites were introduced for the preservation of WMD. Of particular significance for the preservation of calcium carbonate slurry has been the combination of either formaldehyde-releasing agents or glutardialdehyde with isothiazolinones. In addition, where acceptance from the customer is given, the biocidal compound o-phenylphenol is a potent single biocidal compound and offers the advantages of being an excellent preservation agent with favourable toxicological properties (non-sensitising, non-formaldehyde-releasing). Furthermore, its compatibility with calcium carbonate slurry because of its alkaline character and heat stability make this antimicrobial agent very interesting.

In the course of this thesis biocide-resistant bacteria from calcium carbonate slurries were isolated and characterised. In the following sections the impact of biocide resistance of calcium carbonate slurry bacteria with regard to the fold

increase of the MIC of the in-use biocide has been discussed. The resistance and the susceptibility level of the bacterial species which were able to endure the in-use concentration of the various biocides were characterised and compared to the MIC of the calcium carbonate slurry bacterial population H90-i (mainly *Pseudomonas* sp.) found in slurry produced under laboratory conditions in small scale as well as to a slurry culture of a *Pseudomonas putida* type strain H90-Pp adapted to growth in calcium carbonate. Resistance was analysed by means of the challenge test and the MIC of the biocides against the corresponding resistant bacteria were determined as the concentration preventing bacterial growth detection over three cycles of inoculation.

All biocide-resistant calcium carbonate slurry cultures, GDA/CMIT/MIT-resistant (rGCM), EDDM/CMIT/MIT-resistant (rECM) and OPP-resistant (rOPP), were obtained from product storage tanks located at WMD manufacturing plants that used the related biocide to preserve the products. Biocide-resistant colonies isolated from biocide-supplemented calcium carbonate slurry were identified by sequencing approximately 800 bp of the 16S rRNA gene. Sequence analysis of the isolated strains revealed that all resistant cultures, rGCM, rECM and rOPP, contained by bacteria belonging to the genus *Pseudomonas*. The closest relatives based on the similarity of the 16S rRNA gene sequence were the species *Pseudomonas pseudoalcaligenes*, *Pseudomonas mendocina* and *Pseudomonas alcaliphila*.

Table 24. Calcium carbonate slurry isolated species from the biocide-resistant culture identified on the basis of 16S rRNA gene sequence alignment, with use of blastn and RDPII databases.

Taxonomic affiliation	Closest relative[a]	Accession No.[b]	Max. Identity[c]	Length (bp)	T-RFs (bp)
Pseudomonas	*Pseudomonas pseudoalcaligenes*	AB109888	99	756	489
	Pseudomonas mendocina	AF232713	99	778	489
	Pseudomonas alcaliphila	AB030583	99	925	489

a. Closest relative from comparison with blastn and RDPII databases
b. Accession number of the closest relative entry
c. Maximum identity for the covered sequence length

4.5.2.1 Evaluation and Significance of Biocide Resistant Bacteria in WMD

All resistant calcium carbonate bacterial cultures (rGCM, rECM and rOPP) were subject to challenge tests using increasing concentrations of the corresponding biocide. The challenge test is in general used to assess the efficacy of biocides in calcium carbonate slurry and is of major importance to evaluate the dose response of the bacteria to the biocide and to predict the robustness of the preservation (Schwarzentruber, 2003b). Moreover, the multiple cycle challenge test involves the addition of bacteria to the test product and provides an indication whether the product is adequately preserved (Russell, 2003b). The determined biocidal MIC is considered to yield stable product preservation from the production plant to the consumer over a sustained time interval, and the frequently long storage period of the product in a microbiologically acceptable manner. However, in specific cases post-treatment with biocide prior to shipping or even at the customer site cannot be excluded. In the following Figures (Figure 46 to Figure 48) the biocide dose response of the resistant calcium carbonate bacterial cultures rGCM, rECM and rOPP to the corresponding biocide were investigated in calcium carbonate slurry by means of a three cycle challenge test. Additionally, the preservation performance of all three biocides (GDA/CMIT/MIT, EDDM/CMIT/MIT and OPP) against the calcium carbonate slurry cultures H90-i and H90-Pp were determined.

The rGCM resistant bacteria were obtained from slurry from a calcium carbonate producing plant using the biocidal formulation GDA/CMIT/MIT as preservative were the in-use concentration of 1350 ppm (c/l) no longer prevented bacterial contamination. Figure 46a illustrates the dose response of the rGCM culture to the biocide GDA/CMIT/MIT. The preservation performance of the biocide formulation GDA/CMIT/MIT against the H90-i and H90-Pp slurry culture is shown in Figure 46b and c.

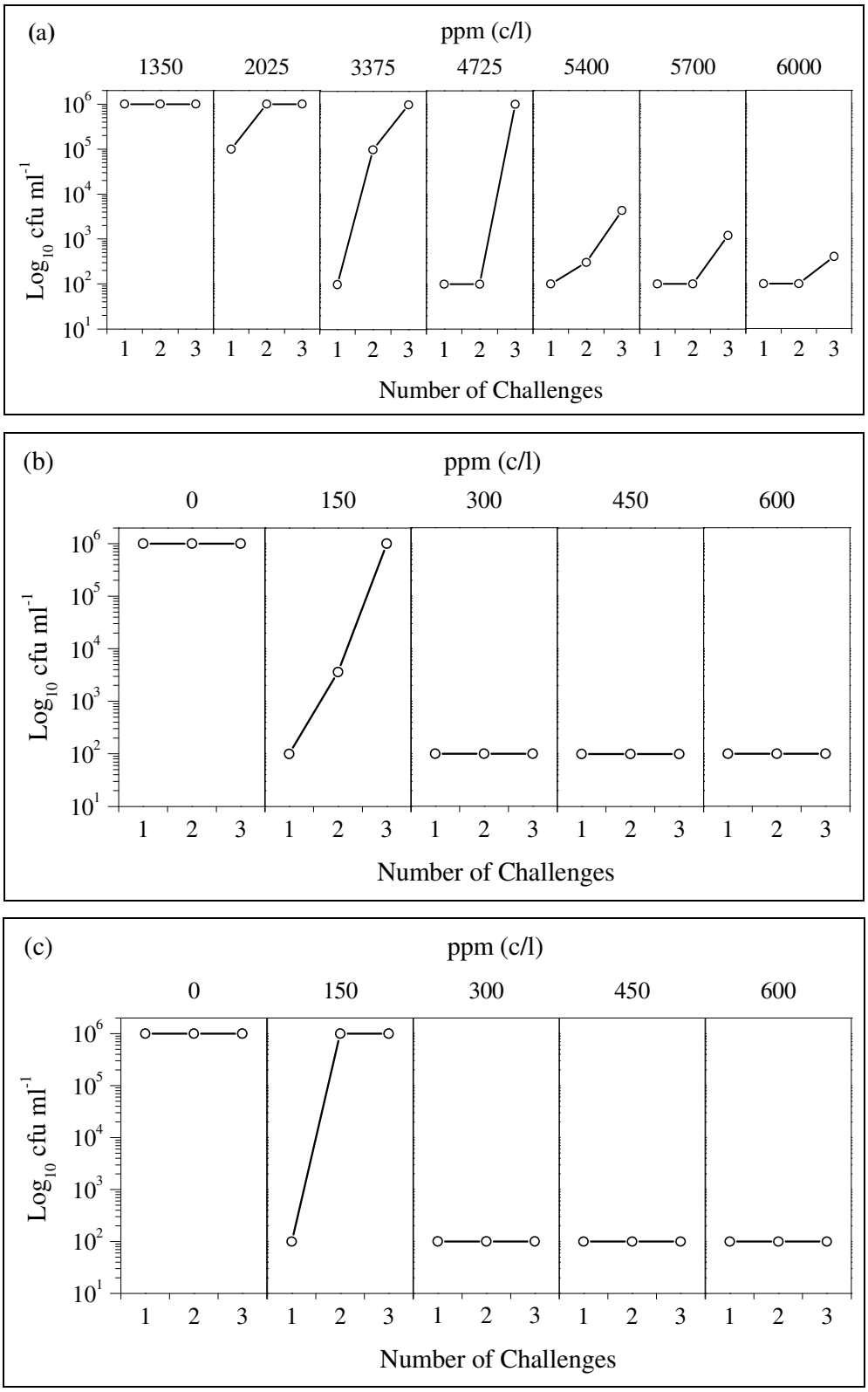


Figure 46. Antimicrobial performance of GDA/CMIT/MIT against (a) rGCM, (b) H90-i and (c) H90-Pp slurry culture determined by means of the challenge test. There were no more than three inoculations performed. TVC detection limit was 10^2 cells ml^{-1}.

Against the resistant slurry culture rGCM the biocide GDA/CMIT/MIT at a concentration of 3375 ppm (c/l) demonstrated preservation of the first bacterial challenge cycle only. This corresponds to a GDA concentration of 725 ppm (a/l) and is not surprising because reduced susceptibility to glutardialdehyde, even at concentrations up to 1000 ppm, has been reported (Azachi*, et al.*, 1996, Manzoor*, et al.*, 1999). However, even a concentration of 6000 ppm (a/l) did not completely inhibit bacterial growth below the detection limit over three cycles of bacterial challenge. Using the susceptible slurry cultures H90-i and H90-Pp to perform the challenge tests demonstrated a preservation performance of the biocidal formulation GDA/CMIT/MIT over three challenge cycles at 300 ppm (c/l) for both cultures.

The bacterial slurry culture resistant to the biocidal formulation EDDM/CMIT/MIT was originally isolated from the storage tank of a calcium carbonate slurry plant using either EDDM/CMIT/MIT or GDA/CMIT/MIT formulations to preserve the products depending on customer acceptance of formaldehyde. The in-use concentration of the biocidal formulation EDDM/CMIT/MIT in the product storage tank was 750 ppm (c/l). Figure 47a shows the dose response of the rECM culture to the biocide EDDM/CMIT/MIT. The preservation performance of the biocide formulation EDDM/CMIT/MIT against the H90-i and H90-Pp slurry culture are shown in Figure 47b and c.

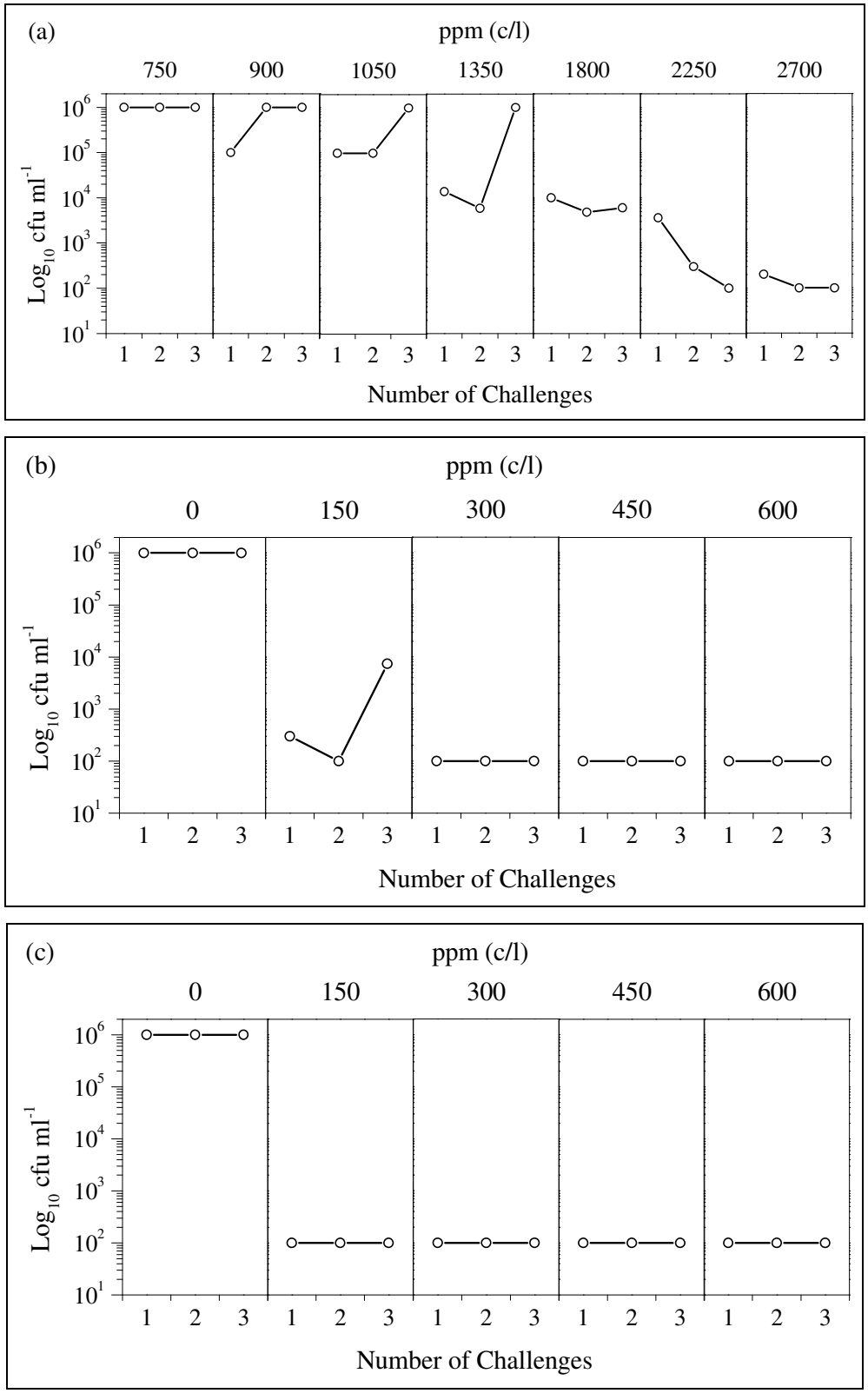


Figure 47. Antimicrobial performance of EDDM/CMIT/MIT against (a) rECM, (b) H90-i and (c) H90-Pp slurry culture determined by means of the challenge test. There were no more than three inoculations performed. TVC detection limit was 10^2 cells ml^{-1}.

Using the biocidal formulation EDDM/CMIT/MIT against the resistant culture rECM, preservation over three bacterial challenge cycles was achieved at a concentration of 2700 ppm (c/l). Similar to the results for the biocidal formulations GDA/CMIT/MIT, the biocidal formulation EDDM/CMIT/MIT achieved preservation over three bacterial challenge cycles at 300 ppm (c/l) and 150 (ppm (c/l) against the susceptible culture H90-i and H90-Pp respectively.

Comparing the dose responses of the rGCM and rECM bacteria slurry cultures revealed significant differences between the bacterial challenge cycles. With increasing concentrations of the GDA/CMIT/MIT biocidal formulation, preservation after the first and second bacterial challenge cycle was evident and at the same time a decrease of the total viable count after the third cycle was observed. The enhanced performance of the biocide after the first and second bacterial challenge cycle may result from the fast reactivity and action of GDA with the bacterial cells introduced into the calcium carbonate slurry. However, the resulting drop in performance after the second challenge cycle was probably due to the instability of actively available GDA. Further increment of the biocide concentration leads to an accumulation and prolonged action of GDA, hence inhibition of the resistant bacteria over three bacterial challenge cycles. On the contrary, with increasing concentrations of the EDDM/CMIT/MIT biocidal formulation there was an overall reduction of the total viable count across the three bacterial challenges and a decreased total viable count on the third challenge cycle was observed. These observations lead to the conclusions that formaldehyde acts slower compared to glutardialdehyde, but formaldehyde is significantly more stable in calcium carbonate slurry than GDA and exhibits a more efficient long lasting preservation performance against the resistant bacteria at lower concentrations.

Several limitations should be pointed out by comparing the dose response of the bacteria slurry culture resistant to GDA/CMIT/MIT and EDDM/CMIT/MIT since this also depends on the mechanism of resistance and on the genetic and phenotypic adaptation of the respective cultures. Moreover, both biocide formulations contain MIT and CMIT in the range of 0.25% to 0.38% and 0.75% to 1.15% respectively, to support the bactericidal performance rather than the preservation performance of the biocidal formulation. However, the antimicrobial activity of CMIT and MIT among the bacterial challenge cycles is restricted. MIT is actually used to chemically

stabilise CMIT and does not significantly contribute to the antimicrobial activity (Schwarzentruber, 2003b) because when used as a single biocide compound it is generally applied at concentrations between 50 and 150 ppm (Diehl, 2002) which are far-off from the in-use concentrations used in calcium carbonate slurries (1.8 to 5.1 ppm a/l). CMIT acts at lower concentrations (0.6 to 6.8 ppm) (Moore and Payne, 2003, Paulus, 2005) which are in the range, or even below the concentrations used in calcium carbonate slurries (5 to 15 ppm a/l), but CMIT is heat-unstable as described by Schwarzentruber (2003b) and degraded as shown in the following investigations. In summary, the results above clearly showed that the slurry culture rGCM is more resistant to the relative biocidal formulations than the rECM culture.

The bacterial slurry culture resistant to OPP was originally isolated from the storage tank of a calcium carbonate slurry plant using OPP. The in-use concentration of OPP in the product storage tank was 660 ppm (a/l). The dose response of the rOPP culture to increasing OPP concentrations is shown in Figure 48a. The preservation performance of OPP against the H90-i and H90-Pp slurry culture are shown in Figure 48b and c. At any assessed concentration the biocide OPP did not show any preservation performance over three challenge cycles with OPP-resistant bacteria. Because of the reduced solubility of OPP and the formation of OPP-crystals above a concentration of 3000 ppm (a/l) in calcium carbonate slurry (P. Schwarzentruber personal communication) the assessed concentrations of OPP did not exceed 3000 ppm (a/l).

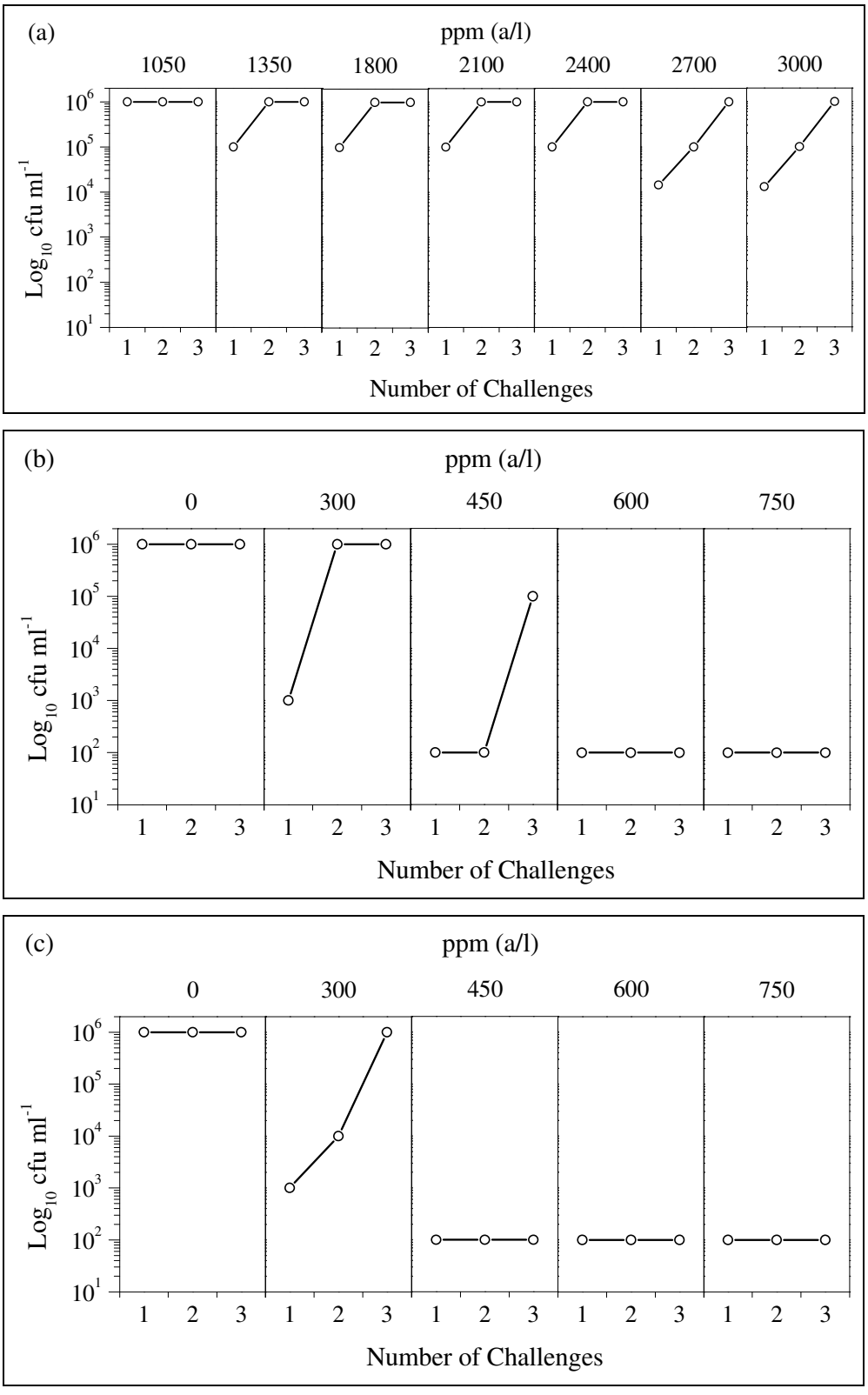


Figure 48. Antimicrobial performance of OPP against (a) rOPP, (b) H90-i and (c) H90-Pp slurry culture determined by means of the challenge test. There were no more than three inoculations performed. TVC detection limit was 10^2 cells ml^{-1}.

However, even after the first bacterial challenge a concentration of 3000 ppm (a/l) was not able to inhibit the resistant bacteria below the 10^4 cfu ml^{-1}. In comparison, a minimum concentration of 600 ppm (a/l) and 450 ppm (a/l) are required to achieve a preservation over three challenge cycles with the susceptible slurry cultures H90-i and H90-Pp. These data are in line with those reported by Schwarzentruber (2003b) where an optimum preservation of calcium carbonate slurry was achieved at 600 to 900 ppm (a/l) OPP. Even though OPP is an excellent preservative in an alkaline environment such as calcium carbonate slurry, OPP has a poor bactericidal performance and therefore the disinfection activity in the bacterial challenge cycles can be disregarded (Schwarzentruber, 2003b).

The "new" MIC values of biocides against resistant and susceptible bacteria slurry cultures determined by means of the challenge test are summarised in Table 25. The MIC of GDA/CMIT/MIT biocidal formulation against culture rGCM was 4.4 times higher compared to the in-use concentration of this biocidal formulation. Similarly, 3.6 times more biocide was required to achieve growth inhibition of the rECM culture by the biocidal formulation EDDM/CMIT/MIT. As a consequence of the solubility limitation of OPP at 3000 ppm (a/l), 4 time higher concentration of OPP compared to the in-use concentrations did not preserve calcium carbonate slurry challenged with the OPP-resistant bacterial culture. Chapman (1998) collected bacterial strains from systems where a failure of preservation occurred and reported that the majority of the isolates, mainly *Pseudomonas* sp. and *Burkholderia* sp., showed MIC values 2 to 5 times higher that equivalent sensitive strains of various biocides (GDA, CMIT/MIT, BIT). In contrast to these data, the MIC of both biocides against the H90-i and H90-Pp were significantly lower and biocide concentrations of 2.5 to 9 times less were required to inhibit bacterial growth. By comparing the new MIC to the MIC determined for the susceptible bacterial culture H90-i the increase of the required biocide concentrations in order to inhibit growth of the biocide-resistant bacteria in calcium carbonate slurry was even higher. The increase in biocide concentrations used to preserve calcium carbonate slurry over three bacteria challenges was 20, 9 and 5-fold higher for the biocidal formulations GDA/CMIT/MIT, EDDM/CMIT/MIT and OPP respectively. These MIC values clearly showed that the WMD samples obtained from the biocide-using production plants were contaminated with bacteria that were adapted or even resistant to the

applied biocide formulation. On the other hand, bacterial slurry cultures that had been prepared in the laboratory were susceptible to all the tested biocidal formulations. A breakdown of the biocidal formulations to the single active compounds evidence that the increase in the application of biocide against biocide-resistant bacteria is not applicable, not at least because of the environmental and economical impact but also due to the regulatory framework and maximum permissible concentration for the use of antimicrobial substances (e.g. CMIT) in the calcium carbonate slurry downstream products.

Table 25. Minimum inhibitory concentration of biocide formulations against both resistant and susceptible WMD bacterial cultures.

Slurry culture	Biocide formulation	In-use biocide concentration (ppm)	MIC[a] (ppm c/l)	Fold increase[b]	Overall increase[c]
rGCM	GDA/CMIT/MIT	1350	6000	4.4	20
rECM	EDDM/CMIT/MIT	750	2700	3.6	9
rOPP	OPP	750	3000	4	5
H90-i	GDA/CMIT/MIT	1350	300	lower	-
	EDDM/CMIT/MIT	750	300	lower	-
	OPP	750	600	lower	-
H90-Pp	GDA/CMIT/MIT	1350	300	lower	-
	EDDM/CMIT/MIT	750	150	lower	-
	OPP	750	525	lower	-

a) OPP (active/liquid)
b) Relative to the in-use biocide concentration
c) Relative to the susceptible HC90-i culture

4.5.2.2 Stability of Resistance in WMD

The susceptibility and response of bacteria to biocidal compounds can vary in relation to the growth environment, physiological adaptations as well as phenotypic changes leading to reduced antimicrobial susceptibility and have been previously documented (Beumer*, et al.*, 2000). Such a transient change in susceptibility has been defined as pseudo-resistance (Heinzel, 1998) or phenotypic tolerance (Chapman, 2003a) and both are also considered to be intrinsic resistance. Examples of this phenomena are the altered susceptibility to antimicrobials of bacteria with reduced grow rates or the induction of resistance by nutrient depletion (Russell, 1995). Additionally, increase of the MIC to different biocides has been induced by serial subculture of bacteria in increasing biocide concentrations (Walsh*, et al.*, 2003). The induced resistance for isothiazolinones and QACs (Quaternary Ammonium Compounds) has lead to a 2-fold and 4-fold increase respectively of the MIC and to the alteration of the outer membrane proteins (OMP) between the biocide-free and biocide-exposed bacteria (Winder *et al.* 2000; Walsh *et al.* 2003).

For the purpose of investigating the resistance stability of the biocide-resistant slurry cultures described above, each slurry culture was subcultured for 10 passages in biocide-free calcium carbonate slurry. After 0, 5 and 10 passages the culture was inoculated into biocide-free or biocide-supplemented slurry and the TVC was determined after 24 and 48 hours (Table 26).

Table 26. Resistance stability of biocide-resistant bacteria in calcium carbonate slurry.

Passage	Biocide[a)]	rGCM	rECM	rOPP
0	+	1.0E+06	1.0E+06	1.0E+06
	-	1.0E+06	1.0E+06	1.0E+06
5	+	1.0E+06	1.0E+06	1.0E+06
	-	1.0E+06	1.0E+06	1.0E+06
10	+	1.0E+06	1.0E+06	1.0E+06
	-	1.0E+06	1.0E+06	1.0E+06

a) supplemented with the relevant biocide

GDA/CMIT/MIT 1350 ppm (c/l)
EDDM/CMIT/MIT 750 ppm (c/l)
OPP 750 (a/l)

The level of resistance against the in-use concentration of GDA/CMIT/MIT, EDDM/CMIT/MIT and OPP biocide was not influenced by subculturing the resistant cultures in biocide-free calcium carbonate. Independently of the number of passages in biocide-free calcium carbonate slurry the resistance against the biocides was always expressed once the cultures were transferred to biocide-supplemented calcium carbonate slurry. These findings support the conclusion that resistance against biocides in calcium carbonate slurries is not a transient change of susceptibility as a result of physiological adaptation of the bacteria to the in-use concentration of biocides but expression of true resistance.

Distinct from applications in industrial processes, pure bacterial cultures can be stepwise adapted under laboratory conditions to develop antimicrobial resistance, however in practice such adaptations are unlikely (Russell, 2003c). In fact, subculturing biocide-resistant bacteria from calcium carbonate slurry into nutrient-reach medium such as Tryptic Soy Broth (TSB) caused a decrease of the resistance level. However, after 2 to 3 passages of acclimatisation in the presence of suboptimal biocide concentration the resistance was regained to the in-use concentration of the relative biocide in TSB. Nevertheless, in consideration of the fact that the biocidal formulation available for the preservation of WMD are exhaustive and the resistant cultures showed stability of resistance in calcium carbonate slurry this fact is of concern for the applicability of biocides in calcium carbonate slurry.

4.5.2.3 Biocide Cross-Resistance in WMD

Cross-resistance of the WMD bacterial cultures rECM, rGCM, rOPP as well as the formaldehyde-resistant bacterial strains *P. putida* and *M. extorquens* were determined by exposing the cultures to either half, double or the in-use concentration of various common biocide formulations employed to preserve calcium carbonate slurries (Table 27). A single bacteria challenge was performed. In addition to the biocidal formulations relevant to the resistant strains, the biocide formulations CMIT/MIT, GDA/BIT, BNPD/CMIT/MIT, EDDM/BNPD/CMIT/MIT, DMO and GDA/MIT/BP were included in this investigation.

Table 27. Biocide cross-resistance patterns of WMD bacteria cultures.

Biocidal formulation (% w/w)	Biocide concentration (ppm c/l)	rECM	rGCM	rOPP	*P. p**	*M. e**
no biocide control	0	■	■	■	■	■
GDA/CMIT/MIT (21.5 / 1.15 / 0.38)	675	□	■	□	□	□
	1350	□	■	□	□	□
	2025	□	■	□	□	□
EDDM/CMIT/MIT (85 / 0.75 / 0.25)	375	■	■	▒	□	□
	750	■	□	□	□	□
	1125	■	□	□	□	□
CMIT/MIT (1.15 / 0.38)	600	■	■	■	□	□
	1200	■	■	■	□	□
	1800	■	■	■	□	□
GDA/BIT (16.7 / 6.7)	675	■	■	□	□	■
	1350	□	■	□	□	▒
	2025	□	■	□	□	▒
GDA/MIT/BNPD (21.5 / 1.53 / 10)	900	□	■	□	□	□
	1800	□	■	□	□	□
	2700	□	□	□	□	□
OPP (100)	735	□	■	■	□	□
	1470	□	■	■	□	□
	2205	□	■	■	□	□
BNPD/CMIT/MIT (14 / 1.05 / 0.35)	375	□	■	□	□	□
	750	□	■	□	□	□
	1125	□	□	□	□	□
EDDM/BNPD/CMIT/MIT (67.5 / 10 / 0.75 / 0.25)	375	□	▒	□	□	□
	750	□	□	□	□	□
	1125	□	□	□	□	□
DMO (78)	375	■	■	■	■	■
	750	■	■	□	▒	■
	1125	■	□	□	□	■

□ no growth (< 10^2 cfu ml^{-1})

▒ moderate growth (< 10^5 cfu ml^{-1})

■ heavy growth (> 10^6 cfu ml^{-1})

*) EDDM (formaldehyde) resistant strains

As was to be expected, bacterial growth in each resistant WMD culture was not affected by the corresponding biocide. The calcium carbonate slurry cultures H90-i and H90-Pp were also exposed to all concentrations of biocidal formulations and growth of these susceptible cultures was not detected at any concentration. The rOPP bacteria slurry culture was resistant to CMIT/MIT only, showing that multiple biocidal compound formulations are efficient against this culture. However, none of the resistant cultures rECM, rGCM or rOPP were susceptible to the CMIT/MIT biocidal formulation which at the highest investigated concentration of 1800 ppm (c/l) equals 21 ppm (a/l) CMIT and 7 ppm (a/l) MIT. Resistance to CMIT has been reported by several authors and has been suggested to be associated with the alteration of the outer membrane proteins (OMP) (Chapman, 2003a). The ineffective CMIT concentration determined for the resistant cultures in calcium carbonate slurry is higher than reported previously. Winder *et al.* (2000) found an MIC of 2.5 ppm (a/l) for CMIT and 49 ppm (a/l) for MIT against the biocide-resistant *Pseudomonas aeruginosa* PAO1 species.

The WMD bacterial culture rECM revealed cross-resistance against formaldehyde-releasing biocides such as 4,4-dimethyl oxazolidine (DMO). The growth of this bacterial culture in WMD ceased when exposed to glutardialdehyde-based biocides, o-phenylphenol (OPP) and combinations of formaldehyde-releaser with BNPD indicating specific resistance to formaldehyde and to isothiazolinones or combinations thereof. In contrast, the WMD bacterial culture rGCM was resistant to almost all concentrations of the tested biocide formulations. Exceptions were formaldehyde-containing biocides combined with either CMIT and MIT or 2-bromo-2-nitropropane-1,3-diol (BNPD) although the lowest concentration had no effect on bacterial growth in WMD. These data suggested that this bacterial culture is to some extent susceptible to formaldehyde-releasers, especially to O-formals such as EDDM rather than oxazolidine which releases less formaldehyde (Giuliani, 2008). These findings differ from those reported by Chapman (1998) where the cross-resistance between glutardialdehyde and formaldehyde was described. Finally, the formaldehyde degrading *P. putida* was inhibited by all tested biocides at every concentration, whereas *M. extorquens* proliferated in the presence of GDA/BIT and DMO. Therefore, it can be assumed that *M. extorquens* is susceptible to isothiazolinones (CIT/MIT) or BNPD in combination with formaldehyde or

glutardialdehyde, respectively, as well as to OPP. These results are in line with those reported by Schwarzentruber (2003b) who reported that microorganisms resistant to formaldehyde can be eradicated by using active agents other than formaldehyde. Overall the biocidal formulation EDDM/BNPD/CMIT/MIT showed an antimicrobial performance against all tested slurry bacterial cultures.

Summing up, these results clearly show that a range of biocide resistant strains obtained from calcium carbonate slurries harbour multiple resistance to various biocidal formulations. This investigation showed that the multiple resistance of bacterial WMD isolates must be taken seriously as an inherent factor affecting future preservation strategies for WMD. For this reason, WMD preservation research activities have to be focused on novel and innovative preservation approaches to maintain the high hygiene and quality requirements of WMD.

4.5.2.4 Degradation of GDA/CMIT/MIT Biocide in WMD

Glutardialdehyde (GDA) has an enhanced antimicrobial activity at alkaline pH, however, this active compound is susceptible to both high temperature and alkaline pH values. Although successful in use for the preservation of calcium carbonate slurries, GDA encounters several difficulties because of the native alkaline pH of calcium carbonate slurries and the occasionally high temperature of the slurries at the point of biocide dosage. The thermostability of GDA in different media was investigated at two incubation temperatures over an exposure period of three days (Figure 49). The stability of GDA is both pH and temperature dependent. Temperature seems to be a critical parameter which promotes the degradation of GDA in alkaline media. In order to distinguish whether the aqueous or solid phase of calcium carbonate slurry is involved in GDA degradation both pressure filtrate of slurry and whole slurry were analysed. In respect to the temperature impact on GDA the data were similar for both incubation temperatures, 30 ºC and 60 ºC respectively.

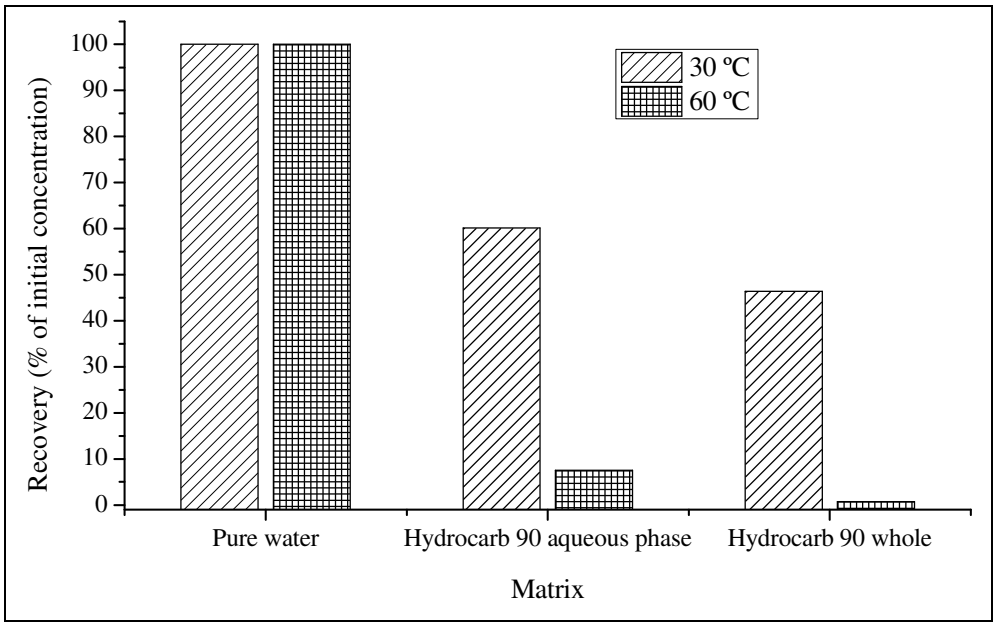


Figure 49. Recovery rate of glutardialdehyde in various media incubated at 30 ºC and 60 ºC over a period of 72 hours. A concentration of 300 ppm (a/l) GDA was dosed prior to incubation. pH: pure water 6.9, Hydrocarb 90 aqueous phase 8.5 and Hydrocarb 90 whole 9.3.

These data demonstrated that with rising temperature the detectability of GDA in the medium decreased significantly. However, in pure water a 100% retrieval of the initial GDA concentration was recorded for both tested temperature regimes. After 3 days of incubation at 30 ºC the percentage retrievability of GDA in slurry filtrate was 60%, as compared with 45% in the whole slurry. Due to higher thermolability of GDA in the whole slurry compared to the slurry filtrate this finding suggested that the solids in the slurry potentially catalyse the deterioration of GDA.

Nevertheless, GDA is a potent antimicrobial compound which offers high antimicrobial efficiency in respect to the preservation of calcium carbonate slurries. Therefore, in Figure 50 the residual antimicrobial efficiency of GDA supplemented calcium carbonate slurry after the naturally occurring GDA recovery rate drop off is shown. Hydrocarb 90-GU calcium carbonate slurry was supplemented with 250 ppm (a/l) GDA and incubated at 30 ºC and the GDA concentration measured periodically. In defined intervals (1, 2, 3, 7 and 14 days) portions of the sample were removed and inoculated with contaminated calcium carbonate slurry (biocide susceptible native bacteria Covercarb 60-GU). After 24 hours of additional incubation the total viable count was measured on PCA plates.

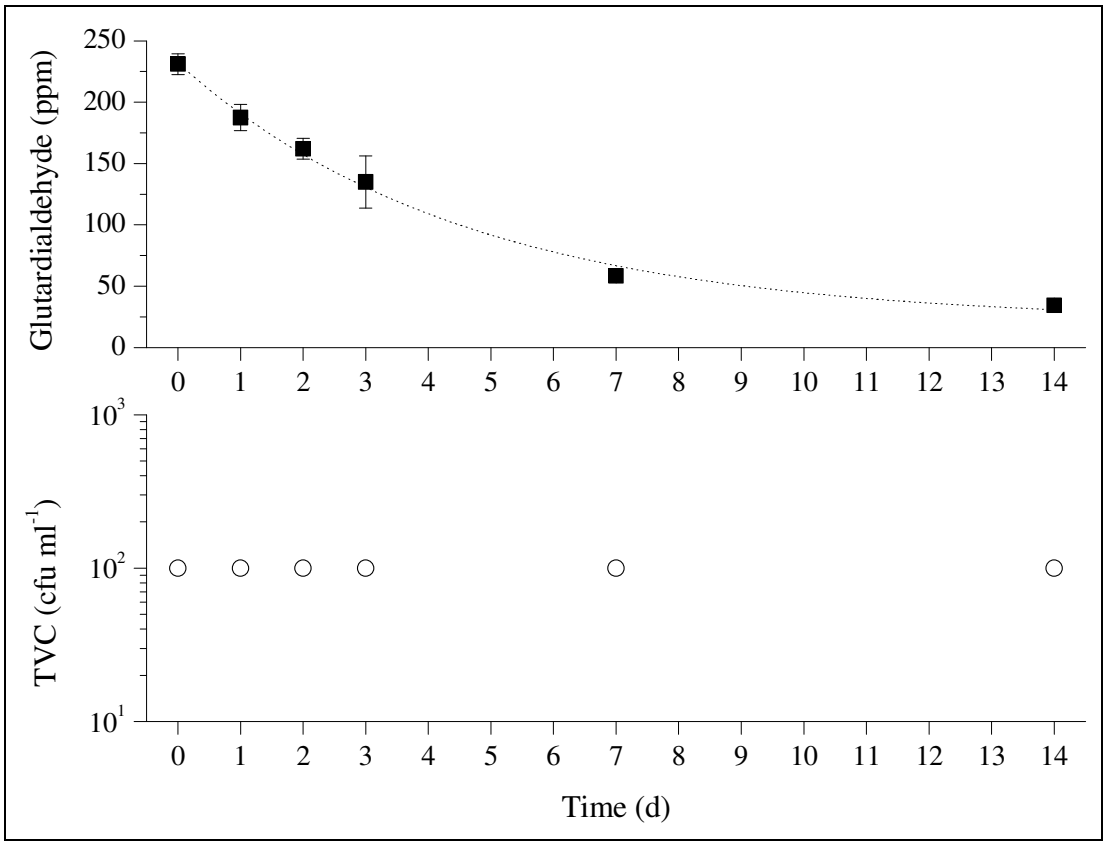


Figure 50. Stability and antimicrobial efficacy of GDA (■) in H90 calcium carbonate slurry (pH 9.3) incubated at 30ºC over a period of 14 days. The GDA dosed concentration was 250 ppm (a/l). (○) TVC was determined 24 h after the inoculation of portions of the sample at defined intervals (1, 2, 3, 7 and 14 days) with biocide susceptible calcium carbonate bacteria. TVC detection limit 10^2 cfu ml^{-1}. Fitted curve represent exponential decay according to the formula: $y = y_0 + a \cdot e^{-(x/t)}$; ($R^2 = 0.993$) of two independent replicates.

Even though the concentration of GDA decreases significantly after 3 days incubation, a preservation performance over the investigation time of 14 days was observed. This fact supports the conclusion that without losing the antimicrobial properties, the chemical structure of GDA in calcium carbonate slurry is modified and no longer detectable by HPLC. It has been described that at high pH equilibrium between the open-chain molecule and the hydrated ring structure of GDA exists (Moore and Payne, 2003). Additionally, polymerisation of GDA is promoted at high pH values and above pH 9 a significant loss of aldehyde groups can occur. Finally, these data demonstrated that GDA still retains antimicrobial activity even though it is no longer detectable by HPLC.

Little is known about the bacterial mechanism of resistance against GDA, however *Mycobacterium chelonae* GDA-resistant cells isolated from GDA-treated

endoscopes shows a reduction of the cell wall polysaccharide fractions arabinogalactan and arabinomannan and this has been proposed to be responsible for the reduced GDA permeability of the cell wall (Manzoor, *et al.*, 1999). Analogously, in *Mycobacterium* the reduced susceptibility to GDA has been reported to be associated with increased hydrophobicity of the cell wall and in *Burkholderia cepacia* the enzyme aldehyde dehydrogenase (EC-number 1.2.1.3) has been detected (Chapman, 2003b). Moreover, the enzyme aldehyde oxidase (EC-number 1.2.3.1) belonging to the xanthine oxidase family has been described as being involved in the oxidation of aldehydes to the corresponding carboxylic acid using molecular oxygen or other electron acceptors (Uchida, *et al.*, 2003, Yasuhara, *et al.*, 2002), whereupon glutardialdehyde has shown a higher substrate specificity than formaldehyde (Kataoka, 2008, Uchida, *et al.*, 2005). In the course of this investigation, DNA primers were designed covering the gene regions of the enzyme aldehyde dehydrogenase (EC-number 1.2.1.3) and aldehyde oxidase (EC-number 1.2.3.1) and PCR analysis of the GDA-resistant strain disclosed the presence or both genes in the genome of the GDA-resistant bacterium *P. mendocina* obtained from the calcium carbonate slurry culture rGCM. Therefore, degradation of GDA by both enzymes is a potential source of glutaric acid which will act on the pH and hence on the stability of the calcium carbonate dispersions.

In order to identify whether GDA is actively metabolised, the biodegradation of GDA in calcium carbonate slurry was investigated over a period of 7 days. At the same time the concentration of glutaric acid was monitored. Figure 51 illustrates the degradation of GDA and the production of glutaric acid in Hydrocarb 90-GU by GDA-resistant bacteria. Over the period of analysis the TVC of the sample inoculated with GDA-resistant bacteria was greater than 10^6 cfu ml^{-1} whereas the sterile control samples remained below the detection limit of 10^2 cfu ml^{-1}. Glutardialdehyde (calculated initial concentration 250 ppm a/l) was supplied into the calcium carbonate slurry using a GDA/CMIT/MIT biocidal formulation, however, the CMIT/MIT (calculated initial concentration 15.5 and 5 ppm a/l respectively) concentration was not determined.

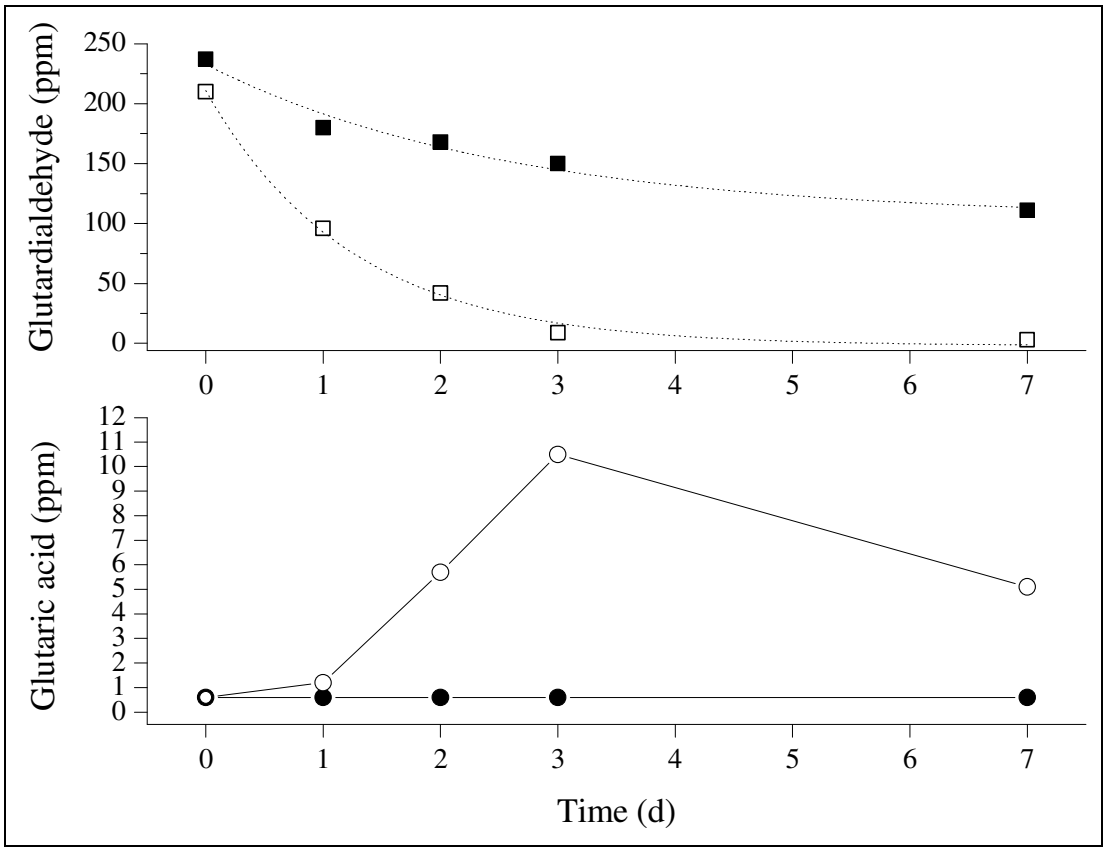


Figure 51. Degradation of glutardialdehyde and the production of glutaric acid in H90 calcium carbonate slurry (pH 9.1) incubated at 30ºC. Closed symbols: no inoculum control. Open symbols: inoculated with rGCM slurry culture. (square) GDA, (circle) glutaric acid. GDA was dosed via GDA/CMIT/MIT biocide and the initial GDA concentration was 250 ppm (a/l). Detection limits: glutardialdehyde 0.3 ppm (a/l) and glutaric acid 0.6 ppm (a/l). Fitted curves represent exponential decay according to the formula: $y = y_0 + a \cdot e^{-(x/t)}$. No inoculum control $R^2 = 0.952$; Inoculated sample $R^2 = 0.994$.

On the one hand, as shown in Table 27, none of the assayed CMIT/MIT concentrations either alone or in combination with GDA prevented the growth of the rGCM slurry culture whilst other authors have reported the MIC of the isothiazolinones-base biocide Kathon™ WT (CMIT/MIT 3:1; Rohm&Haas) to be 1.5 ppm (a/l) against planktonic cells from rotating biological contactors (Laopaiboon*, et al.*, 2001). On the other hand CMIT/MIT has been combined with GDA to achieve a short-term disinfection rather than preservation. CMIT is chemically unstable and rapidly decomposes in the alkaline environment of calcium carbonate slurry whereas MIT remains stable but contributes very little to the antimicrobial activity of the formulation (Schwarzentruber, 2003b). The stability of CMIT and MIT in calcium carbonate slurry is outlined in the following section 4.5.2.6.

GDA in calcium carbonate slurry is obviously completely biodegraded within 3 days by GDA-resistant bacteria. The glutaric acid concentration peaked at day 3 at 10.5 ppm (a/l) and was metabolised afterwards. The low retrieval of glutaric acid led to the assumption that GDA degradation generates glutaric acid as an intermediate metabolite and subsequently glutaric acid is probably further metabolised to carbon dioxide. In comparison, in H90 without bacteria 47% of the dosed glutardialdehyde was recovered after 7 days incubation and glutaric acid was not detectable. Comparing the decay curves of GDA in sterile calcium carbonate slurry (closed symbols) of Figure 50 and Figure 51 is limited by the utilisation of different GDA biocide formulations nonetheless revealed a similar drop of GDA within the first 3 days of analysis but a significant deviation of the retrieved GDA concentration at day 7. This may result from the different formulated biocide commodity solutions used to perform the investigations (GDA vs. GDA/CMIT/MIT) or from the presence of CMIT/MIT in the sample inoculated with rGCM. The degradation of GDA has also been reported in other studies and in rotating biological contactors fed with waste water containing 180 ppm GDA as sole carbon source, an average GDA removal of 71% was achieved after 28 days (Laopaiboon*, et al.*, 2003). Finally, the data presented above clearly showed that an accumulation of glutaric acid in calcium carbonate slurries as a consequence of GDA metabolism by resistant bacteria and hence a negative impact of the acid on the dispersion relative to pH and viscosity bacteria can be ruled out.

4.5.2.5 Degradation of OPP in WMD

Bacterial species, mainly *Pseudomonas* sp., able to metabolise hydroxylated biphenols have been described (Higson and Focht, 1989). In bacterial cells the biodegradation of aromatic compound such as OPP occurs by hydroxylation to catechols and subsequent ring cleavage. In addition, pyrocatechol as described by Schwarzentruber (2003b) can be further oxidised either to succinate and acetate or to acetaldehyde and pyruvate. In an other study the degradation pathway of OPP by a *Pseudomonas* sp. has been proposed to include the formation of 2,3-dihydroxybiphenyl by means of NADH-dependent monooxygenase and benzoic acid (Kohler*, et al.*, 1988).

The concentration of OPP and pyrocatechol are shown in Figure 52 and were measured over a period of 14 days both in sterile H90 calcium carbonate slurry and in H90 inoculated with the OPP-resistant slurry culture. Unfortunately, it was not possible to determine the concentration of acetate because of a large unknown peak masking the acetate peak in the ion chromatogram.

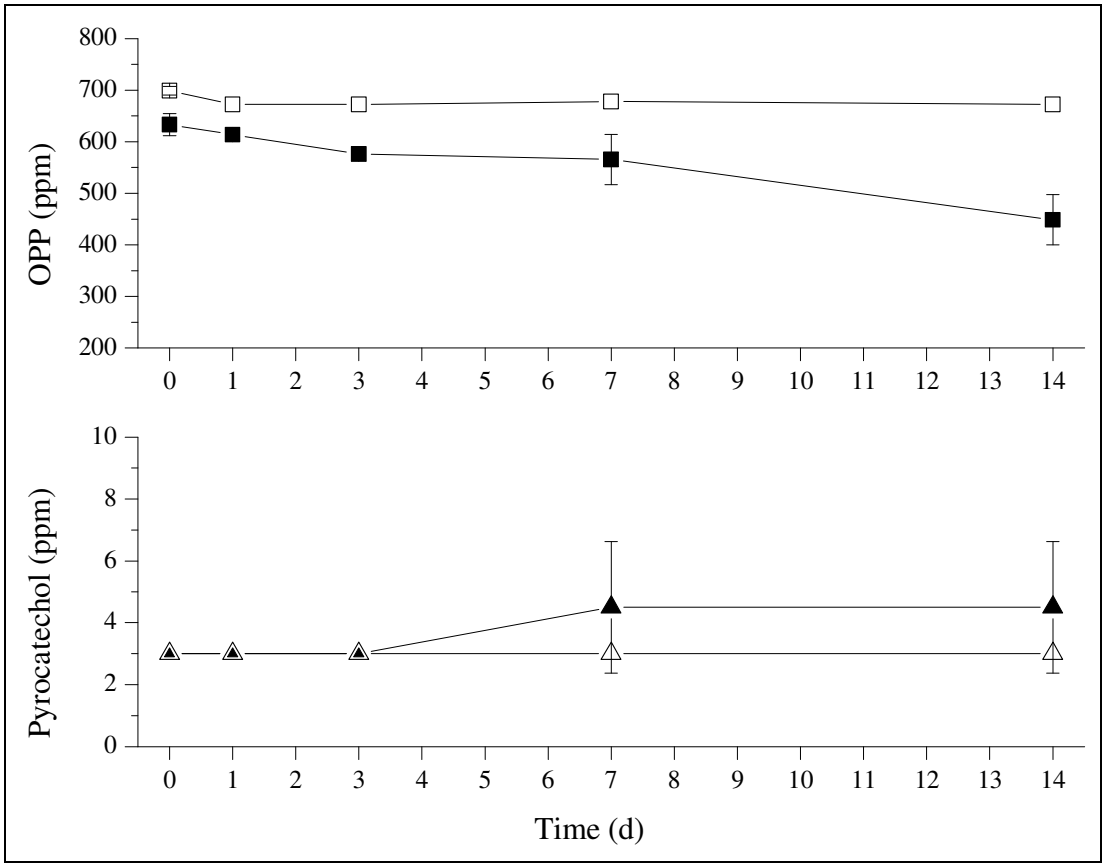


Figure 52. Degradation of o-phenylphenol and production pyrocatechol in H90 calcium carbonate slurry incubated at 30ºC. The initial dosed OPP concentration was 660 ppm (a/l). Open symbols: no inoculum control. Closed symbols: inoculated with rOPP slurry culture. (square) OPP, (triangle) pyrocatechol. Detection limit pyrocatechol 3 ppm (a/l). Values are means and vertical bars represent standard deviation of two independent experiments.

The results clearly showed that in sterile H90 slurry samples the OPP concentration remains steady over a period of 14 days and pyrocatechol was not detected. In contrast, the Hydrocarb 90 inoculated with OPP-resistant bacteria showed a decline of the OPP concentration of 30% over the 14 days of analysis. However, the concentration of pyrocatechol remained close to the detection limit (3 ppm a/l). The data suggests that the mechanism of resistance adopted by the OPP-resistant bacteria

is not only based on the degradation of the active OPP. After a period of acclimatisation, at day 7 only 10% of the OPP was degraded whilst an additional 20% was degraded from day 7 to 14. The degradation of OPP did not result in significant accumulation of the intermediate metabolites pyrocatechol, therefore it can be assumed that the degradation ends up in the complete exhaustion of the OPP derived metabolite by the primary metabolic pathways. Kohler *et al.* (1988) suggested on the basis of the induction of the oxygen uptake rates that pyrocatechol and benzoate are intermediates formed when OPP-degrading *Pseudomonas* sp. cells are grown on OPP. Finally, in view of the fact that OPP is a membrane active biocide it can not be excluded that the irretrievable OPP is bound by the biomass without causing any adverse effect on the cells which might possess altered outer membrane structures.

4.5.2.6 Degradation of EDDM/CMIT/MIT Biocide in WMD

Due to the evidence, as shown in the chapters above, that biological degradation of formaldehyde in calcium carbonate slurry takes place, it seems obvious that the degradation of the biocidal formulation EDDM/CMIT/MIT by the relevant EDDM/CMIT/MIT-resistant slurry occurs. Formaldehyde, CMIT and MIT concentration was determined over a period of 14 days in Hydrocarb 90 calcium carbonate slurry supplemented with EDDM/CMIT/MIT biocide and either inoculated or not with EDDM/CMIT/MIT-resistant slurry culture (Figure 53).

The data shows that the EDDM/CMIT/MIT-resistant bacterial culture was able to degrade all biocidal compounds of this formulation. In sterile H90 calcium carbonate slurry the EDDM-released formaldehyde and MIT remained steady over the period of analysis and after 14 days incubation, 81% and 94% respectively of the initial concentration was still recoverable. CMIT, known to be chemically unstable in the alkaline slurry environment, was reduced by about 85%. In contrast, in the H90 slurry inoculated with the resistant slurry culture a total viable count greater $>10^6$ cfu ml^{-1} was detected over the period of analysis and the formaldehyde was almost completely exhausted after 14 days of incubation (1.7% residual concentration). These data clearly show that the formaldehyde released by EDDM is metabolised in a similar way to its degradation by *P. putida* and *M. extorquens* as shown in section

4.5.1. The formaldehyde was rapidly exhausted by the resistant bacteria and assuming a zero-order reaction ($F_t = -k \cdot t + F_0$, were F_t is the concentration at a particular time, F_0 is the initial concentration and t is the time) returned a consumption rate, $-k$, of 19.8 ppm (a/l) d^{-1}. In comparison, the formaldehyde consumption rate of *M. extorquens* in calcium carbonate slurry described in the section above was 40.5 ppm (a/l) d^{-1}.

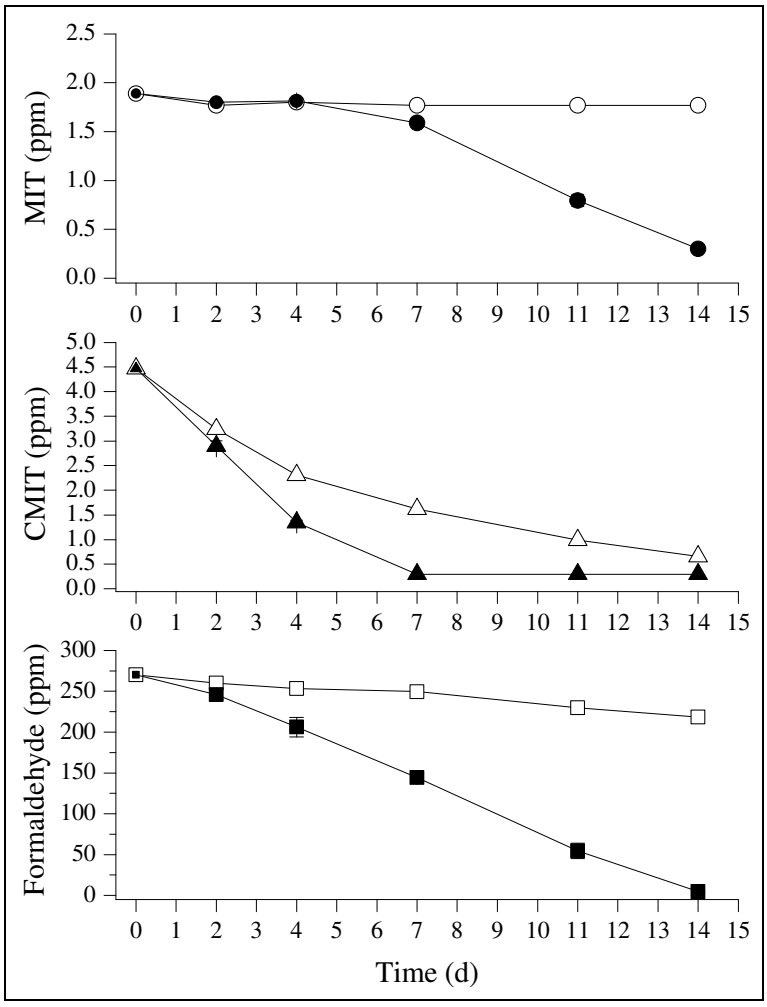


Figure 53. Degradation of EDDM/CMIT/MIT biocide in H90 calcium carbonate slurry incubated at 30ºC. Open symbols: no inoculum control. Closed symbols: inoculated with rECM slurry culture. Values are means and vertical bars represent standard deviation of two replicates. Detection limits: 0.3 ppm (a/l).

The isothiazolinone compounds showed different degradation and the compounds CMIT and MIT were exhausted below the detection limit after 7 and 14 days, respectively. However, CMIT is unstable in the alkaline environment of calcium

carbonate slurry and it appears that in the presence of bacteria this compound is exhausted even more rapidly. MIT is rather than CMIT stable in the alkaline slurry environment, but after 7 days of acclimatisation the resistant bacteria started to degrade this compound. Other studies have reported that CMIT rapidly associates with bacterial cells (Diehl and Chapman, 1999), therefore it can not be excluded that the CMIT is depleted in the calcium carbonate matrix because of this effect. Nevertheless, isothiazolinones are degraded via a nucleophilic attack cleaving the ring structure and the formation of the intermediate malonic acid which finally results in formaldehyde and formic acid (Schwarzentruber, 2003b). These data clearly demonstrate that resistance of bacteria to biocide is often linked to the metabolisation and exhaustion of the biocidal compounds.

4.6 Conclusions

Two different formaldehyde-degrading microorganisms, *Pseudomonas putida* and *Methylobacterium extorquens*, were isolated from calcium carbonate slurry containing the formaldehyde-releasing biocide (ethylenedioxy)dimethanol. The results indicated that in slurry, *M. extorquens* is more tolerant of formaldehyde than *P. putida*. In slurry, microbial-induced oxidation of formaldehyde caused a temporary accumulation of formic acid, which is presumed to be responsible for a pH drop and destabilisation of the calcium carbonate slurry suspension systems. In addition, the residual formaldehyde concentration was observed to drive dominance and recovery of individual formaldehyde-resistant microorganisms in the slurry. Summing up, these findings support the hypothesis that degradation of formaldehyde in calcium carbonate slurries stems from the interplay of formaldehyde-resistant *Pseudomonas* spp. and methylotrophic microorganisms. *M. extorquens* seems to be responsible for the major degradation of formaldehyde in preserved calcium carbonate slurry. Thereafter, when the formaldehyde concentration is sufficiently low, *P. putida* takes over and suppresses the growth of *M. extorquens*. This leads to the conclusion that when formaldehyde-resistant *P. putida* is recognised in formaldehyde-treated slurries showing a pH drop and destabilisation, some formaldehyde-degrading species were or are still present in the slurry as an unculturable fraction. The cooperation of formaldehyde-degrading bacteria is

assumed to be a complex process that involves alternation of species dominance depending on the residual formaldehyde concentration. Overall, this investigation indicated that biodegradation of formaldehyde in calcium carbonate slurry is brought about by alternating dominance of bacterial genera of mixed formaldehyde-resistant microbial populations. Chemically inhibiting the degradation ability of the highly formaldehyde-tolerant bacterial fraction in mixed bacterial populations – i.e. methylotrophic bacteria species – can be regarded as a potential tool for overcoming shortfalls in the preservation performance of formaldehyde in calcium carbonate dispersions.

Synergistic combinations of biocidal compounds such as aldehyde-based or aldehyde-releasing compounds with isothiazolinones, BNPD or DBNPA have been used for many years to preserve WMD (Schwarzentruber, 2003b, Schwarzentruber and Gane, 2005). Nevertheless, WMD-contaminating bacteria resistant to several combinations of biocidal actives have been discovered. In calcium carbonate slurries bacteria resistant to several combinations of biocidal actives have been characterised and these species were at least resistant to the in-use biocide blend used in the manufacturing plant from where they had been recovered. The resistance was found to be maintained in calcium carbonate slurry and not lost once the physiological conditions changed such as the lack of biocide. However, identification of the resistant species by means of molecular techniques revealed that all resistant species were affiliated to the genus *Pseudomonas*. *Pseudomonas* spp. exhibit a broad metabolic adaptability and are ubiquitously involved in the deterioration of various matrices consequently assisting or compromising many industrial processes (McDonnell and Gerald, 2007). The bacterial species resistant to the formaldehyde-releasing biocide EDDM blended with CMIT/MIT (rECM) was specifically resistant to formaldehyde and isothiazolinones whereas the OPP-resistant culture showed isothiazolinones and phenol-specific resistance only. Surprisingly, the bacterial culture resistant to the combination of glutardialdehyde/isothiazolinones (rGCM) harboured multiple resistance to various biocidal formulations. Furthermore, time-related experiments showed that the biocidal compounds such as glutardialdehyde, formaldehyde and isothiazolinone in WMD were metabolised by the resistant bacteria and not detectable within 7 to 14 days.

In order to achieve preservation of calcium carbonate slurry contaminated

with resistant bacteria and treated with the relevant biocidal formulation, MIC values up to 4-fold higher compared to the in-use biocide concentrations were required. Even more important was the increase of the MIC when comparing the resistant strains to susceptible calcium carbonate slurry bacteria which was determined to be between 5 to 20-fold greater. Russel (2003b) proposed that the MIC alone is not a suitable value to evaluate biocide resistance and suggested that susceptibility is restored once the biocide is no longer used. As mentioned above this was not the case for the resistant calcium carbonate slurry bacteria. In addition, Russel (2003b) mentioned several causes and mistakes in the usage of biocides leading to a reduced susceptibility against biocides were specified: (i) biocide residues in the environment to which bacteria can adapt, (ii) the employment of insufficient biocide concentrations ("topping-up" practice), (iii) incorrect use of biocides (systems affected by high biomass, no house-keeping) and (iv) the use of ineffective biocide against the target bacteria. Nevertheless, in the application of biocides as a preservative in calcium carbonate slurry the MIC is a primary and crucial parameter. On the one hand the more rigorous regulatory situation created by the BPD (Biocidal Products Directive 98/8/EC) have led to the situation wherein the development and registration of new biocidal substances has been radically reduced or even abandoned and a handful of biocides suitable to preserve calcium carbonate slurries remain. On the other hand the limits on the biocide concentrations that can be used, as specified by the regulatory frameworks (e.g. isothiazolinone), disallow a continuous rise of the in-use biocide concentrations in calcium carbonate slurries. As a consequence of the biocide formulations used being acidic, an increase of the in-use concentration typically influences the dispersion stability of the alkaline calcium carbonate slurry and tends to result in a pH drop and viscosity increase. Finally, apart from the ecological impact an adjustment of the in-use biocide concentrations always affects the economic efficiency of a preservation system.

The development of biocide resistance and the incidence of multiple biocide-resistant bacteria in calcium carbonate slurries must be taken seriously as a primary factor affecting future preservation strategies for WMD. In order to avoid bacterial resistance developing in WMD two approaches have to be considered rather than increasing biocide dosage: (i) the evaluation of technical process measures to achieve eradication of resistant microorganisms from the WMD production and storage

process and (ii) the enhancement of the biocide agent by non-antimicrobial compounds (Poole, 2003). A number of compounds are known to be able to destabilise bacterial cell membranes and thus increase permeability (Alakomi*, et al.*, 2006, Vaara, 1992). This approach may solve the emerging problems, either resistance or adaptation, by ensuring that an accumulation of the biocidal agent at the action site is achieved due to the enhancing effect of biocide and enhancer compound (Denyer and Maillard, 2002). The breakthrough of a biocidal enhancer offers a potential tool to revolutionise the use of biocidal agents in the WMD producing industry. The investigation of new preservation technologies therefore must be taken seriously in order to maintain the high hygiene and quality requirements of calcium carbonate slurries.

CHAPTER 5

Enhancement and Synergy of Biocides in WMD

5.1 Introduction

Nowadays it is inevitable that biocide resistance will develop in calcium carbonate slurries (Schwarzentruber, 2005). A lack of biocidal activity having been recognised, the only available options are to increase the biocide dosage, or to substitute the antimicrobial compound currently in use with another one (Schwarzentruber, 2003b, Schwarzentruber and Gane, 2005). Apart from the economic and ecological impact caused by increased application of biocide, in this context it is essential to highlight the fact that there exist only a limited number of antimicrobials that are suited to preserving WMD. This is due to the more rigorous regulatory situation created by the BPD (Biocidal Products Directive 98/8/EC), as well as the incompatibility of certain antimicrobials with the chemical properties of WMD. These circumstances have led to the shelving of development and registration of new biocidal substances, owing to the laborious and expensive research required.

The development of biocide resistance and the incidence of multiple biocide-resistant bacteria in calcium carbonate slurries must be taken seriously as a primary factor affecting future preservation strategies for WMD. For this reason, WMD preservation research activities have to focus on novel and innovative preservation approaches to maintain high standards of hygiene and quality. Adoption of the BPD has caused a significant reduction in the number of available biocides, whereby of a total of approximately 1000 notifiable biocides only 360 have been notified (Kähkönen and Nordström, 2008), and only a few of those are applicable in WMD. Moreover, some biocidal compounds used for the preservation of calcium carbonate slurries, such as formaldehyde-releasers, are under pressure due to their possible classification as carcinogenics, while some EU member states have reduced their occupational exposure limits. In addition end customers are refusing to take delivery of products that use compounds like o-phenylphenol (personal communication P. Schwarzentruber). The few remaining actives such as glutardialdehyde, isothiazolinones (CMIT/MIT/BIT), 2-bromo-2-nitropropane-1,3-diol (BNPD) or 2,2-dibromo-3-nitrilopropionamide (DBNPA) are less stable, while the acidic character of some compounds constitutes a technical hurdle for optimally preserving calcium carbonate slurries which are rather alkaline.

Of the strategies for overcoming biocide resistance and to keep within

technical limitations on the application of biocides, one approach is to use combinations of antimicrobial compounds. Combinations of biocides have been described to exhibit a synergistic effect in that the effectiveness of the combined antimicrobials is greater than the sum of the individual compounds (Botelho, 2000, Denyer, *et al.*, 1985, Denyer, *et al.*, 1986, Lambert, *et al.*, 2003). The synergy that is apparent is an effect brought about by combining reduced concentrations of different antimicrobials, rather than combining the in-use biocides with non-antimicrobial compounds. The term "enhancement" is therefore a more appropriate term to describe an increase of the antimicrobial activity of a biocide by means of a compound which does not exhibit antimicrobial activity by itself (Hodges and Hanlon, 1991). Recent studies have reported the enhancement of biocides by non-antimicrobial compounds such as EDTA (Alakomi, *et al.*, 2006, Ayres, *et al.*, 1999, Vaara, 1992) and polyethylenimine (Alakomi, *et al.*, 2006, Helander, *et al.*, 1997, Khalil, *et al.*, 2008). Generally the enhancement activity has been suggested as originating from destabilisation of the bacterial cell membrane of gram-negative bacteria, thus increasing its permeability to the antimicrobials. This effect has been reported in connection with a vast assortment of other compounds such as polycations, lactoferrin, transferrin and citric acid (Hancock and Wong, 1984). Additionally, interference with the quorum sensing system (Vestby, *et al.*, 2009) or the substrate as a promoter of the uptake of antimicrobials has already been described (Denyer and Maillard, 2002, Maillard and Russell, 2001). Metals such as silver (Silvestry-Rodriguez, *et al.*, 2007), mercury (Morrier, *et al.*, 1998), copper (Sondossi, 1990) and caesium (Avery, 1995) have also been noted to exhibit biocide potential or even toxicity towards bacteria. In general, heavy metal ions are more toxic than the other classes of metal ions (Harrison, *et al.*, 2005).

5.1.1 Definition "Biocide Enhancer" in WMD

Rather than increasing biocide dosage another strategy employed to overcome the lack of new antimicrobial substances applicable to WMD and the occurrence of resistance mechanisms is to use combinations of biocides with non-biocidal compounds. These non-biocidal compounds, referred to as biocide "enhancers", do not exhibit inherent antimicrobial activity - yet they support and/or enhance the

activity of antimicrobials. The estimation of synergy between combinations of antimicrobials has been described in detail as the sum of the fractional inhibitory concentration assessed by means of the checkerboard titration principle (Berenbaum, 1978). However, using fractional inhibitory concentrations to investigate synergistic effects of antimicrobials assumes a linear dose response and it has been suggested that the synergism occasionally arises from the combination of antimicrobials with different dose-responses, rather than from their synergistic performance (Lambert*, et al.*, 2004, Lambert*, et al.*, 2003, Lambert and Lambert, 2003).

In the case of WMD, a modified definition of the terms biocide enhancement and synergy have been introduced: (i) a biocide enhancer is a substance that is not noted to be a biocide and which at the applied concentration does not exhibit antimicrobial activity in WMD. A biocide enhancer is thought to be able to reconstitute the antimicrobial efficacy of in-use biocide concentrations against non-susceptible and biocide-resistant bacteria contaminating WMD; (ii) A synergistic biocidal formulation is considered to be a blend of two or more antimicrobial actives to preserve WMD and by definition the effect of the combination is greater than the effect of either compound on its own. Finally, of particular importance are the specifications of enhancer/synergistic compounds due to essential chemical compatibility with WMD and the economic and ecological sustainability. The application of a biocide enhancer is a promising tool to improve measures to combat biocide-resistant bacteria in the WMD producing industry.

5.1.2 Aim and Objectives

The aims of the followings experiments were:

- To evaluate the biocide enhancement performance of various compounds against biocide-resistant bacteria in WMD.
- To investigate alternative preservation systems and protocols.

The application of these findings should make a more effective treatment of white mineral dispersions possible. Furthermore, it helps to avoid biocide overdosing, and thus would save both environmental as well as financial resources. This thesis not only investigates the new biocide systems on monocultures of bacteria on the

laboratory scale, but also the effect on mixed populations present at the production sites. On this basis, previous biocide strategies will be revisited and new preservation strategies have been designed.

5.2 Results and Discussion

5.2.1 Lithium – an Universal Biocide Enhancer

Lithium has been determined as exhibiting antiviral activity (Amsterdam*, et al.*, 1990), as well as antimicrobial activity against gram-negative bacteria (Eisenberg*, et al.*, 1991), gram-positive bacteria and synergistic activity when combined with antibiotics (Lieb, 2002, Lieb, 2004). In addition, even though the molecular mechanism of electropermeabilisation is not fully understood, lithium is routinely used to perform lithium-cation induced electrotransformation of bacteria. This effect is believed to be related to a transient increase in permeability of the cell membrane (Papagianni*, et al.*, 2007, Ramon and Fonzi, 2009).

Since lithium is easy to introduce into calcium carbonate slurry via dispersant neutralisation, the main objective of this study was to evaluate the enhancement performance of lithium to the antimicrobial activity of the biocidal formulations in use in calcium carbonate slurries against inherent biocide-resistant bacteria. The effects of concentration and the mode of introduction of lithium into calcium carbonate slurry were determined. In addition, the inherent activity and the mechanism of enhancement of lithium were investigated.

5.2.1.1 Manufacture of WMD containing Lithium

Lithium was introduced into the calcium carbonate slurries used in the following experiments via the dispersant (fully or partially neutralised with lithium) or during the wet grinding of marble in the form of lithium carbonate as well as added to the final slurry product by means of a lithium carbonate solution. The nomenclature and details of the lithium-supplemented calcium carbonate slurries investigated in this study are summarised in Table 28.

Table 28. Details of the lithium supplemented calcium carbonate slurry products.

Product ID	Dispersant Neutralisation (%)	Lithium content[a)] (ppm a/l)	Lithium measured[b)] (ppm a/l)	Remarks
H90-Li	100 Li	1580	407	Lithium via disperant
H90-MgNa5050	50:50 Mg:Na	0	0	Standard H90 slurry
H90-MgLi5050	50:50 Mg:Li	920	N.D.	Lithium via disperant
H90-MgLi7525	75:25 Mg:Li	660	N.D.	Lithium via disperant
H90-gLi	50:50 Mg:Na	1500	711	ground with Li_2CO_3

a. theoretical value
b. measured by ion chromatography
N.D. not determined

The lithium concentrations summarised in Table 28 show that the determined lithium concentration of slurry, dispersed with fully lithium-neutralised dispersant (H90-Li) as well as ground in the presence of lithium carbonate (H90-gLi), deviated significantly from the theoretical calculated value based on the dosed dispersant or lithium carbonate amount, respectively. The lithium concentration was initially determined by means of ion chromatography in the aqueous phase of the calcium carbonate slurry after removing the solids by pressure filtration. These data suggested that the lithium introduced into the calcium carbonate slurry via dispersant was not completely dissolved in the water phase and not completely dissociated from the polyacrylate dispersant. Similarly, lithium introduced into the calcium carbonate slurry in the form of lithium carbonate reacted with the polyacrylates and was partially sequestered to neutralise free carboxyl groups of the polyacrylate polymer. These observations were corroborated by the fact that using inductively coupled plasma optical emission spectrometry (ICP-OES) to determine the total lithium content revealed a concentration of 1499 ppm (a/l) lithium for the H90-Li slurry manufactured with fully lithium-neutralised dispersant.

5.2.1.2 Addition of Lithium in WMD via Neutralised Dispersant

The enhancement efficacy of lithium to EDDM biocide was investigated in various calcium carbonate slurries where lithium was introduced into the product via lithium-neutralised dispersant (Table 29).

Table 29. Enhancement of the biocidal activity of EDDM against formaldehyde-resistant bacteria (*Pseudomonas* sp. isolated from Hydrocarb V40 T-ME 60% slurry) by various concentrations of lithium introduced into WMD via dispersant.

Product ID	**Total Viable Count (cfu ml^{-1})**			
	M. extorquens		*Pseudomonas sp.*	
EDDM (ppm a/l)	0	1500	0	1500
HC90-MgNa5050	$>10^6$	3.2E+04	$>10^6$	1.6E+04
HC90-Li	$>10^6$	< 100	$>10^6$	< 100
HC90-MgLi5050	$>10^6$	4.4E+04	$>10^6$	8.0E+04
HC90-MgLi7525	$>10^6$	2.4E+04	$>10^6$	1.6E+04

After a single challenge with the formaldehyde-resistant WMD culture of *P. putida* (isolated from V40 MCC slurry) or facultative methylotrophic *M. extorquens* significant growth of both bacterial species was observed in all calcium carbonate slurry products without biocide. However, product H90-Li dispersed with lithium-neutralised dispersant showed inhibition of both formaldehyde-resistant microorganisms when 1500 ppm (a/l) EDDM biocide had been added to the slurry. On the other hand, the H90-MgLi slurries manufactured by means of dispersant neutralised with only 25% or 50% lithium all showed considerable bacterial contamination.

The biocide enhanced performance to EDDM of a H90-Li dilution series in conventional lithium-free H90-MgNa5050 was also examined (Table 30).

Table 30. Enhanced performance of lithium in serial dilutions of H90-Li to 1500 ppm (a/l) EDDM biocide against formaldehyde-resistant WMD bacteria.

Product	**Lithium (ppm)**	**Total Viable Count (cfu ml^{-1})**	
		M. extorquens	*Pseudomonas sp.*
HC90-MgNa5050	0	$>10^6$	$>10^6$
HC90-Li	1580	< 100	< 100
Dilutions[a)]			
1:2	790	$>10^6$	3.2E+04
1:4	395	$>10^6$	5.0E+04
1:8	198	$>10^6$	5.0E+04

a) ratio HC90-Li : HC90-MgNa5050

These results indicated that a 1:2 dilution of H90-Li with conventional H90 supplemented with 1500 ppm (a/l) EDDM showed a lack of lithium-derived biocide enhanced efficacy against both *M. extorquens* and *Pseudomonas* sp. formaldehyde-resistant bacteria.

5.2.1.3 Addition of Lithium into WMD via Lithium Carbonate

The biocide enhancing performance of lithium introduced into WMD in the form of lithium carbonate was determined by comparing the addition of lithium carbonate during the mineral grinding step with the dosing of lithium carbonate directly into the final product. The calcium carbonate slurry H90-gLi ground in the presence of 1500 ppm (a/l) lithium in the form of lithium carbonate showed excellent enhancing properties for both in-use concentrations of the biocide formulations EDDM/CMIT/MIT and GDA/CMIT/MIT against the related resistant WMD culture (Figure 54). Independent of which biocide formulation was used, a concentration of 1500 ppm (a/l) lithium was sufficient to preserve calcium carbonate slurry over three bacterial challenges with the related biocide-resistant culture.

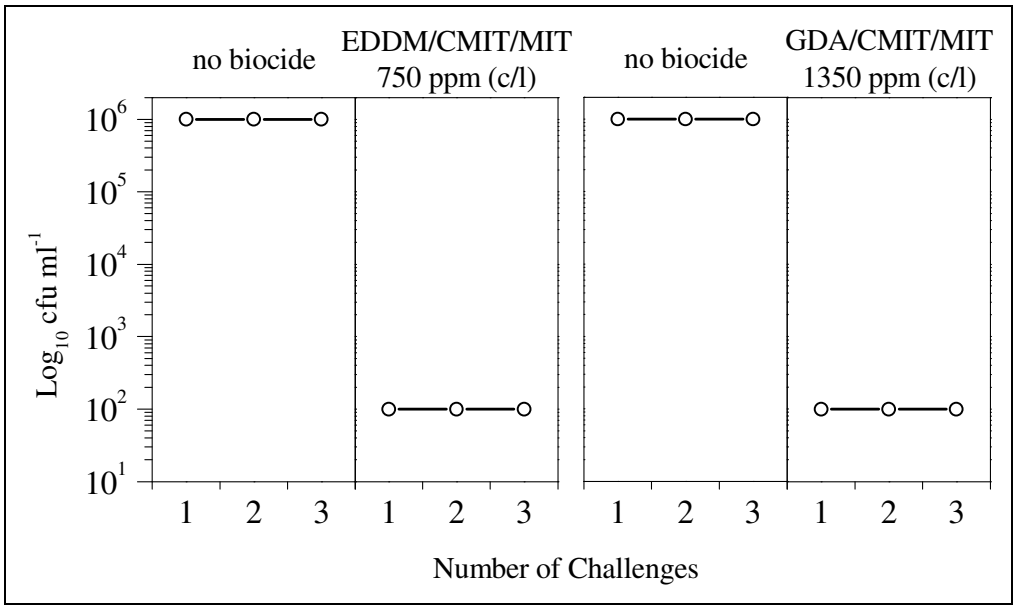


Figure 54. Enhancement of EDDM/CMIT/MIT and GDA/CMIT/MIT biocide formulations in H90-gLi calcium carbonate slurry ground in the presence of lithium carbonate (1500 ppm a/l lithium). Calcium carbonate samples were inoculated with the resistant bacteria rECM and rGCM, respectively.

The minimal enhancement concentration (MEC) of lithium was elucidated for both biocidal formulations against their respective biocide-resistant microorganisms by dosing various concentrations of lithium carbonate into regular H90-MgNa5050 calcium carbonate slurry (Figure 55 and Figure 56). The MEC of lithium for the biocide formulation EDDM/CMIT/MIT and GDA/CMIT/MIT over three challenges with biocide-resistant bacteria were determined to be 1050 ppm (a/l) and 1500 ppm (a/l), respectively. No significant differences in total viable count were either observed in biocide-free/lithium-containing calcium carbonate slurry samples or *vice versa* inoculated with the biocide-resistant bacteria culture.

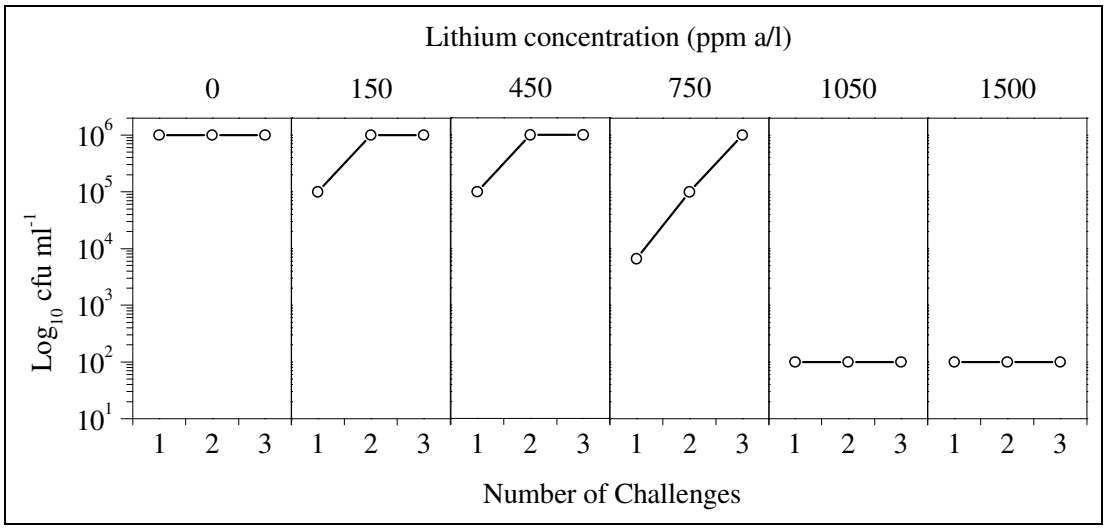


Figure 55. Biocide enhancement performance of lithium against rECM slurry culture in the presence of 750 ppm (c/l) EDDM/CMIT/MIT determined by means of the challenge test. There were no more than three inoculations performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}.

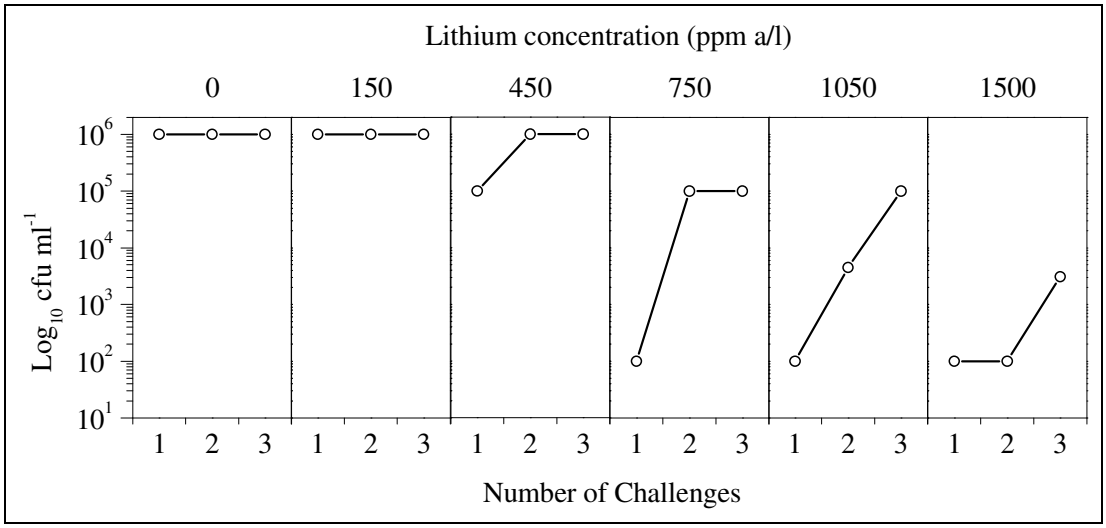


Figure 56. Biocide enhancement performance of lithium against rGCM slurry culture in the presence of 1350 ppm (c/l) GDA/CMIT/MIT determined by means of the challenge test. There were no more than three inoculations performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}.

The challenge testing results clearly show that the biocide enhancing property of lithium is dose-dependent, and that the slurry culture rECM was more susceptible to the enhancing effect of lithium. A closer investigation of the enhancing concentration of lithium for a single bacterial challenge revealed that complete eradication of the

resistant bacteria was achieved at a dose of between 750 ppm (a/l) and 1050 ppm (a/l). Furthermore, in the presence or absence of biocide no long-term influence on the viscosity stability of either H90-gLi or H90 supplemented with lithium carbonate was observed over a period of 30 days, indicating that lithium carbonate is fully compatible with the biocide systems used (Figure 57).

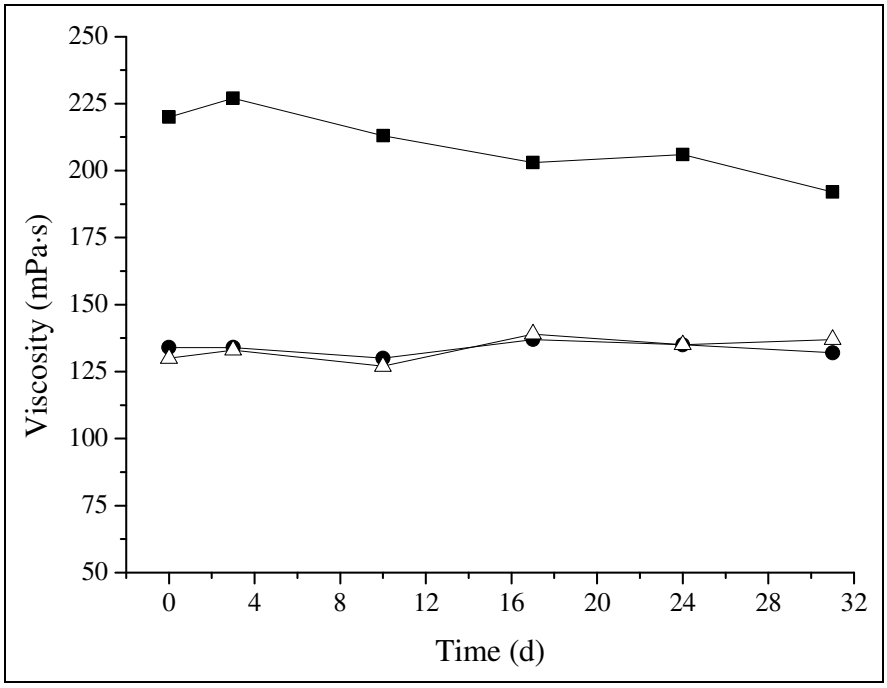


Figure 57. Viscosity course of Hydrocarb 90 slurry supplemented with lithium carbonate during the grinding process. (■) Standard H90 without lithium supplemented with 750 ppm (c/l) EDDM/CMIT/MIT biocide. H90 supplemented with Li_2CO_3 (1500 ppm lithium a/l) supplemented with (△) 750 ppm (c/l) and (●) 1500 ppm (c/l) EDDM/CMIT/MIT biocide.

The introduction of lithium via lithium carbonate during the wet grinding step showed additional advantages on the physical properties of calcium carbonate slurries. The viscosity of H90 slurry ground in the presence of lithium carbonate was even less compared to standard H90 slurries without lithium carbonate with a viscosity specification of 200 mPa·s. Besides, a higher solid content up to 79% was achieved (Personal communication M. Buri).

As a consequence of lithium showing an antimicrobial enhancing performance to different biocides against the relative resistant slurry bacteria it was assumed that other monovalent cations such as potassium or sodium might also be potential candidates to enhance the performance of the biocide. Sodium did not come into consideration since it is being used for neutralisation of the dispersant whereas potassium in the equivalent concentration to lithium (1500 ppm a/l) did not show any enhancement of the antimicrobial performance of the biocides EDDM/CMIT/MIT and GDA/CMIT/MIT against the relative resistant slurry cultures.

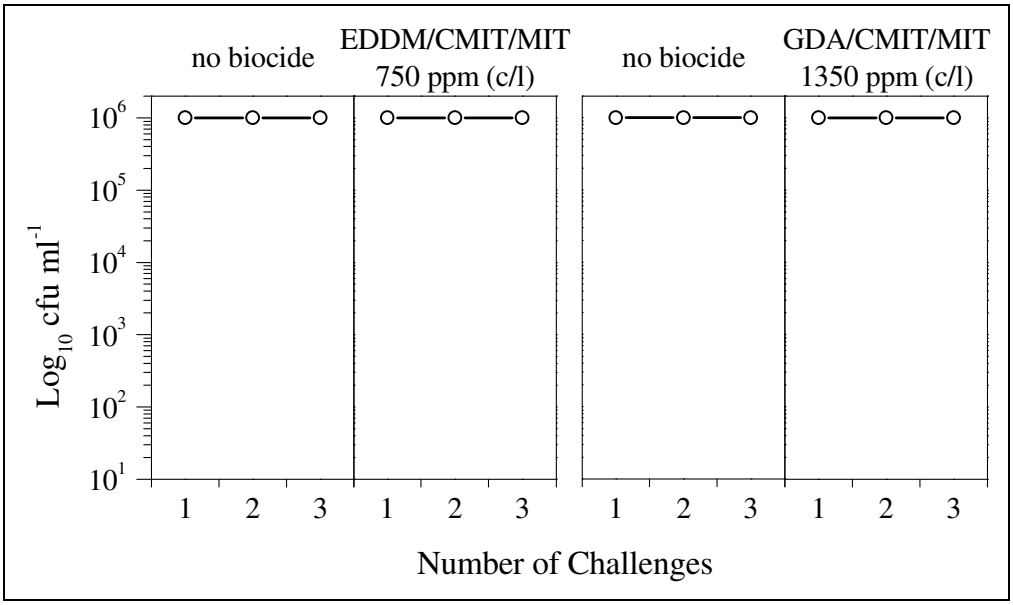


Figure 58. Enhancement of EDDM/CMIT/MIT and GDA/CMIT/MIT biocide formulations in Hydrocarb 90 standard calcium carbonate slurry by potassium carbonate (1500 ppm a/l potassium). Calcium carbonate samples were inoculated with the resistant bacteria rECM and rGCM, respectively. There were no more than three inoculations performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}.

Possible explanations for the differences in the mode of action compared to lithium are the different physical properties (higher atom mass, greater atom diameter and less electronegativity) of the potassium ion compared to the lithium ion. Potassium is an essential nutrient and plays an important physiological role in turgor pressure maintenance, growth and development of cells. An accumulation of potassium in the bacterial cytoplasm is the primary response to osmotic stress (Gutierrez*, et al.*, 1995).

Lithium has in fact been used since the 1950s to treat bipolar disorders in psychiatry (Cruceanu, *et al.*, 2009) and several clinical observations indicated that lithium might posses an antimicrobial and antiviral performance both *in vitro* and *in vivo* (Amsterdam, *et al.*, 1990, Lieb, 2002, Lieb, 2004, Lieb, 2007). There are a few studies that draw differing conclusions about the antimicrobial activity of lithium ions against bacteria. In other studies it has been found that the enzyme alcohol dehydrogenase of *Methylobacterium extorquens* was inhibited to 28% by 5 mM lithium (34.5 ppm) (Koutsompogeras, *et al.*, 2006). Others authors reported a toxicity of lithium toward *Escherichia coli* at a concentration of 4830 ppm and the arrest of cell division and differentiation of protozoa of the group trypanosomatidae by 200 mM LiCl (1380 ppm Li^+) (Spiegel and Soares, 1999). Some other authors have demonstrated that 100 mM LiCl (690 ppm Li^+) in the presence of various sole carbon sources inhibited the growth of *Salmonella thyphimurium* and *Escherichia coli* due to an accumulation of Li^+ in the cell cytoplasm that might cause inhibition of the enzyme pyruvate kinase (Niiya, *et al.*, 1980, Umeda, *et al.*, 1984), whereas others ruled out the enhancement of the antimicrobial performance of fluoride by lithium (Eisenberg, *et al.*, 1991). However, accumulation of high concentrations of lithium by certain gram-negative bacteria has been reported (Tsuruta, 2005).

In this context it is important to mention the fractional inhibitory concentration index (FICI) calculated according to Berenbaum (1978). It has been determined that in calcium carbonate slurries a lithium concentration above 3000 ppm (a/l) on its own is required to prevent microbial contamination with both biocide-susceptible and biocide-resistant bacteria over three bacterial challenges. Using this lithium concentration to calculate the FICI values of lithium combined with the biocides revealed a FICI value of 0.8 for the EDDM/CMIT/MIT biocide and 0.7 for the GDA/CMIT/MIT biocide, respectively. Synergism of drugs or antimicrobials has been described as occurring when the FICI is <1 (Berenbaum, 1978); nevertheless that interpretation was revised by the American Society of Microbiology, which has defined a synergy at FICI < 0.5. Controversially, other authors have defined a partial synergy as 0.5> FICI <1 (Botelho, 2000). Therefore, in view of the fact that lithium is not an antimicrobial yet enhances the antimicrobial performance of biocides in calcium carbonate slurries it is more appropriate to use the term “enhancement” rather than synergism.

5.2.1.4 Effect of lithium on *P. mendocina* cells isolated from WMD

Pseudomonas mendocina cells isolated from the EDDM/CMIT/MIT-resistant culture were used to investigate the effect of lithium on bacterial cells. The influence of lithium on the bacterial cells on its own and in the presence of biocide formulation EDDM/CMIT/MIT was studied by a choice of assays. The leakage of cytoplasmic constituents with an absorbance at 260 nm along with potassium were recorded over a time period of 60 minutes after exposing *Pseudomonas mendocina* isolated from the rECM slurry culture to lithium, biocide or combinations thereof (Figure 59a and b). Changes in cell morphology (lysis or swelling) were followed by monitoring the absorbance at 600 nm (Figure 59c).

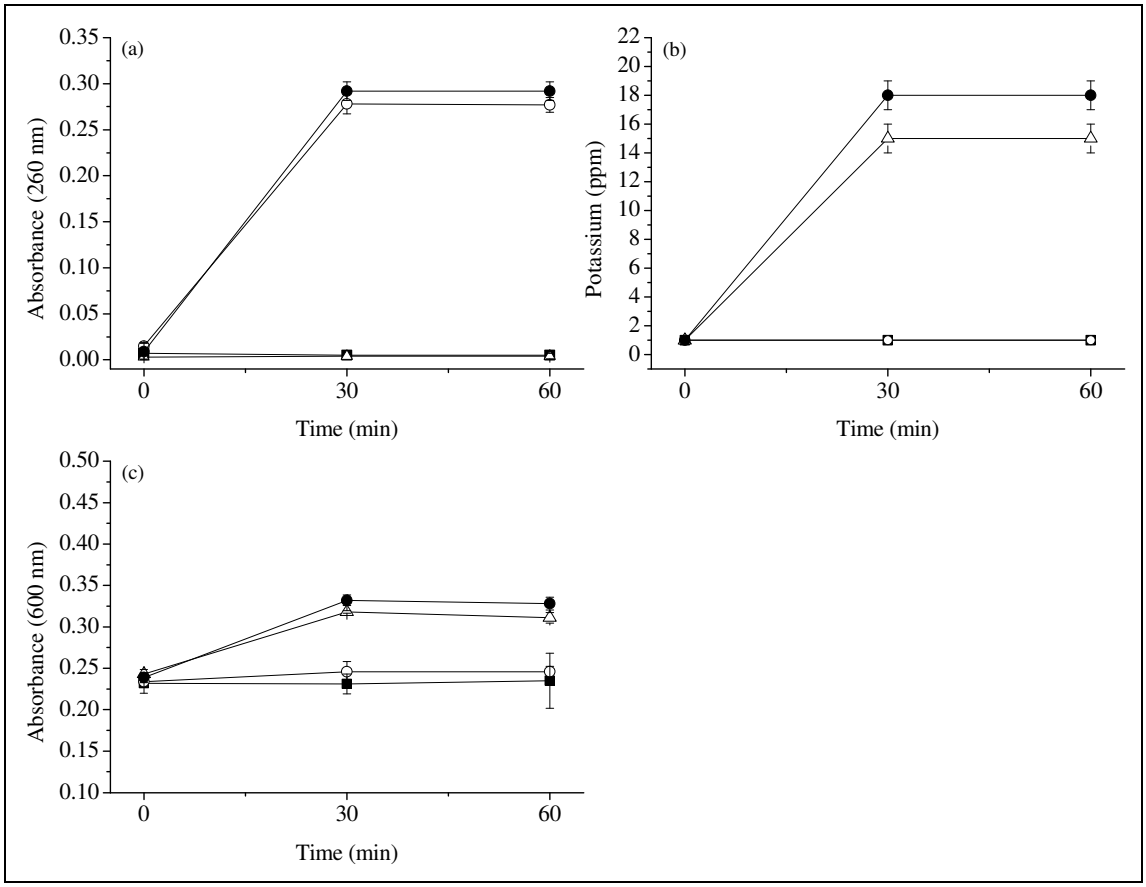


Figure 59. *Pseudomonas mendocina* leakage of cytoplasmic components at 260 nm (a) and potassium (b) as well as absorbance at 600 nm (c) measured 30 min. and 60 min. after exposure to the following combinations: No biocide/lithium control (■), 750 ppm (c/l) EDDM/CMIT/MIT (○), 1500 ppm Lithium (△) and 750 ppm (c/l) EDDM/CMIT/MIT + 1500 ppm Lithium (●). Values are presented as mean ± standard deviation (n = 4).

Firstly, in bacterial cells exposed to 750 ppm (c/l) of the biocide mixture EDDM/CMIT/MIT only, or in combination with 1500 ppm lithium, a significant leakage of cellular constituents absorbing at 260 nm (such as nucleotides, nucleosides and aromatic amino acids) was observed. In contrast, the same concentration of lithium on its own did not show any effect on the leakage of cellular constituents determined at 260 nm. 30 minutes after the treatment the absorbance (260 nm) increased to an average level of 0.28 units, which is equivalent to a secretion of 13.5 µg ml^{-1} of dsDNA (1.0 A260 unit = 50 µg ml^{-1}). On the other hand, 1500 ppm of lithium induced a rapid outflow of potassium ions either on its own or in combination with 750 ppm (c/l) EDDM/CMIT/MIT biocide, ranging from 14 to 18 ppm. Morphological effects monitored at 600 nm were observed in bacterial cells exposed to lithium or lithium combined with the biocide. An increase of absorbance at 600 nm probably indicated that the treatment with lithium resulted in a cellular swelling. Taking a closer look at the bacterial cells supplemented either with lithium or lithium combined with the EDDM/CMIT/MIT biocide by CFII showed a reduction of the bacterial cell volume whilst the Total cell count remained stable among the treatment time (Figure 60) for both lithium only and lithium+biocide treated cells.

The cell size distribution profiles of cells treated with lithium alone revealed lysis occurring between 30 and 60 minutes of treatment i.e. a reduction of the profile heights. A possible explanation for the slight increase in absorbance at 600 nm is the effect of lithium on bacterial cell division which results in the promotion of the cytokinesis within the bacterial population and thus to a shift of the average population size to smaller cells as well as to a change in the refractive index. However, because of the different measurement mode of the CFII instrument compared to the absorbance at 600 nm a comparison is limited and the CFII instrument is more sensitive to changes in the bacterial population since it takes into account cell size and cell number.

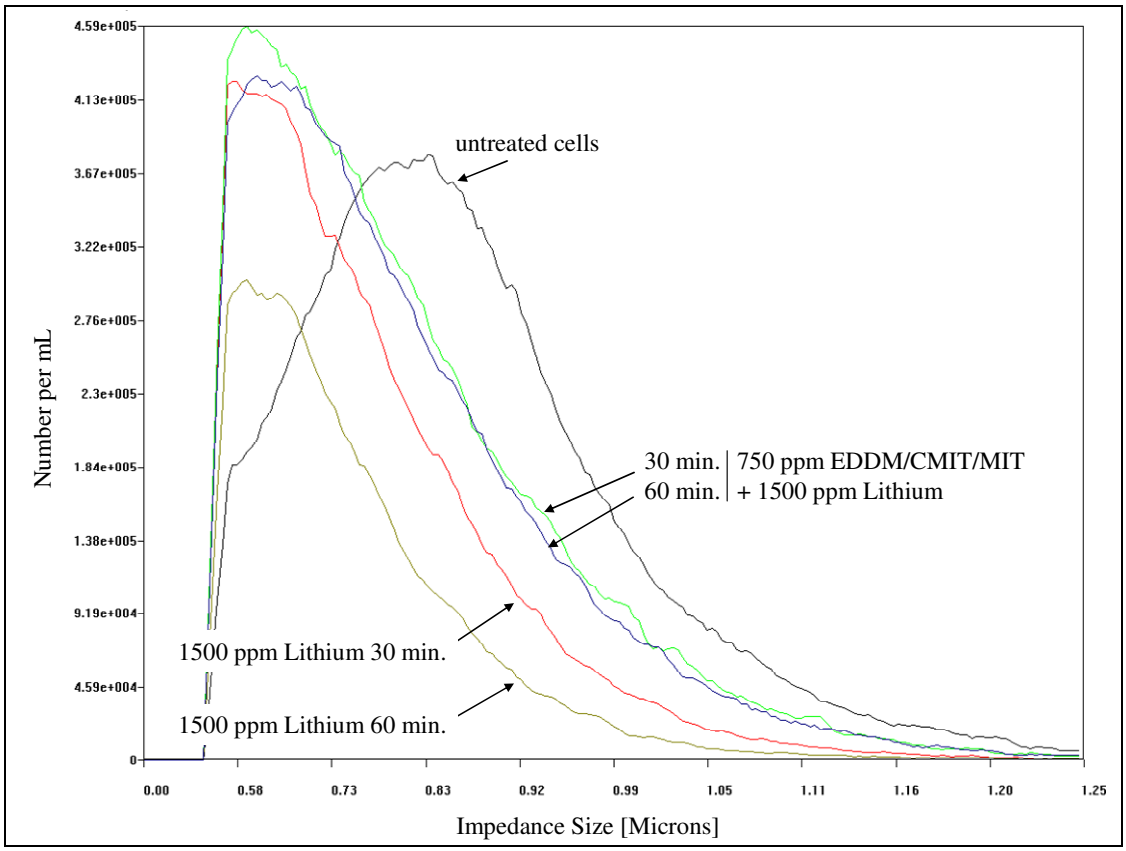


Figure 60. CellFacts II® profiles from *Pseudomonas mendocina* cells treated with 1500 ppm lithium alone or combined with 750 ppm (c/l) of the biocidal formulation EDDM/CMIT/MIT.

Secondly, the permeabilisation of *Pseudomonas mendocina* treated with lithium, EDDM/CMIT/MIT biocide blend or a mixture was assessed utilising the NPN uptake assay. The NPN uptake factors calculated are shown in Figure 61. Lithium on its own (1500 ppm) was significantly ($p < 0.01$) permeabilising *P. mendocina* cells as demonstrated by the enhanced uptake of NPN, whereas in combination with the biocide mixture EDDM/CMIT/MIT (750 ppm c/l) the uptake of NPN, and thus the permeabilisation of the cell membrane, was reduced but still significantly compared to the untreated control ($p < 0.01$). *P. mendocina* treated only with 750 ppm (c/l) of the biocide EDDM/CMIT/MIT mixture did not show significant uptake of NPN. Similar results were obtained by replacing the biocide/lithium volume with saline solution (0.9% w/w NaCl), indicating that the permeabilisation effect is fully attributable to the effect of lithium on the bacterial membrane.

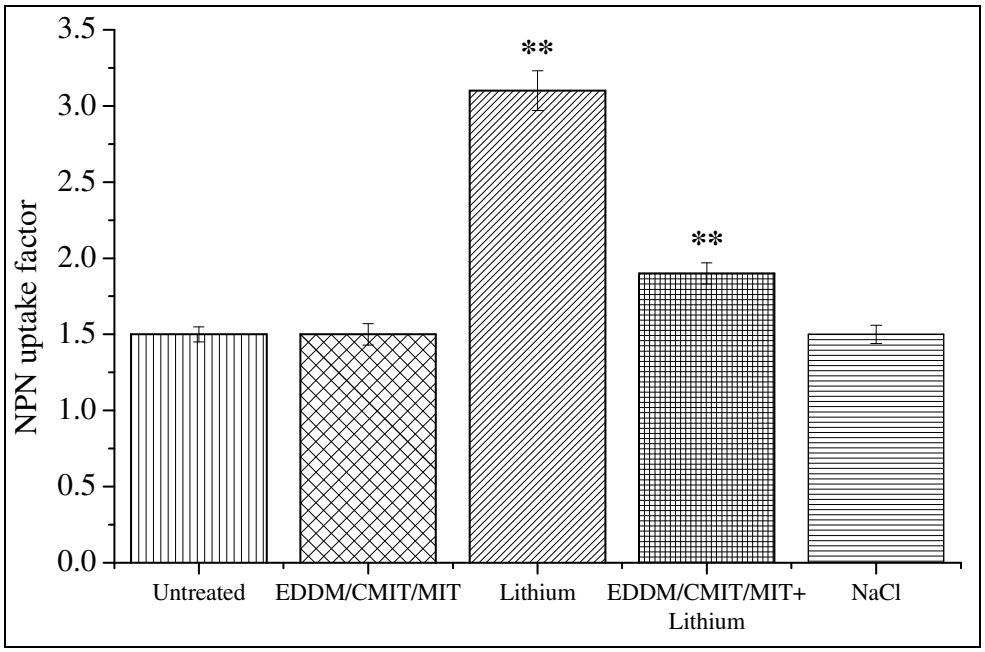


Figure 61. NPN uptake factor obtained after exposing *Pseudomonas mendocina* cells to different treatments. The following concentrations were used: 750 ppm (c/l) EDDM/CMIT/MIT, 1500 ppm lithium, 750 ppm (c/l) EDDM/CMIT/MIT + 1500 ppm lithium and NaCl (0.9% w/w). Values are presented as mean ± standard deviation (n = 16); ** $p < 0.01$ for student t-test (unpaired, equal variance) based on the untreated sample.

However, increased permeability did not enhance the uptake of biocide into the cell. The uptake of formaldehyde or lithium ions was determined for each compound on its own or in combination. No sequestration of lithium on the cell outer membrane or even uptake of lithium into the cell plasma was detected when cells were treated either with 1500 ppm lithium alone, or by combining lithium with 350 ppm formaldehyde (Figure 62).

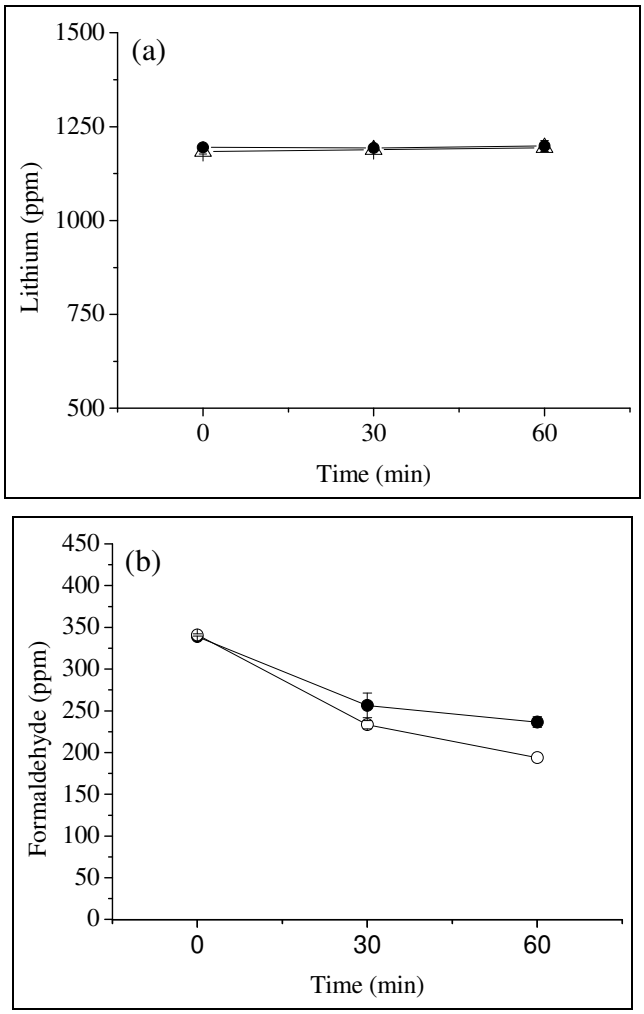


Figure 62. Uptake of lithium (a) and formaldehyde (b) by *Pseudomonas mendocina* measured 0, 30 and 60 min. after exposure to lithium, formaldehyde or a combination of both. The treatments applied in (a) were 1500 ppm lithium (△) and 350 ppm formaldehyde + 1500 ppm lithium (●), whilst the treatments applied in (b) were 350 ppm formaldehyde (○) and 350 ppm formaldehyde + 1500 ppm lithium (●), respectively. All concentrations are given in ppm based on the weight of the solution (a/l). Values are presented as mean ± standard deviation (n = 4).

With *Pseudomonas mendocina* cells treated with 350 ppm formaldehyde, only about 57% of the initial dosed formaldehyde was recovered after an exposure time of 60 minutes indicating an association of the formaldehyde with the bacterial cell outer membrane or even uptake of the formaldehyde. Combining 350 ppm formaldehyde with 1500 ppm lithium led to reduced association of formaldehyde with the bacterial cells, and 70% of the initial formaldehyde amount was retrieved after an exposure time of 60 minutes.

Within the scope of this investigation, attempts were made to hypothesise the enhancement mechanism of lithium in the presence of biocide. Experimental data showed that an outflow of cellular constituents detected at 260 nm only took place in the presence of the biocide blend (EDDM/CMIT/MIT) suggesting that the release is caused by interaction of the cell envelope with both formaldehyde and isothiazolinones. In fact, neither formaldehyde nor isothiazolinones are membrane-active biocides and do not disrupt the cell membrane and thus cause an outflow of cytoplasmic constituents (McDonnell and Russell, 1999). However, the release of cell wall components absorbing at 260 nm might be related to the initiation of cellular autolysis caused by formaldehyde (Denyer, 1995, Musterman and Morand, 1977) and the rapid association of CMIT/MIT with the cells (Diehl and Chapman, 1999). On the other hand, an outflow of potassium ions was observed only when lithium was present alone or in combination with the biocide (EDDM/CMIT/MIT). A leakage of potassium has been described as the first sign of an increase in membrane permeability, and biocide-induced membrane damage (Lambert and Hammond, 1973): bacterial growth is inhibited when K^+ becomes depleted (Padan*, et al.*, 2005). Apparently, the outflow of potassium ions results from exposure of the cells to lithium, even though the greatest efflux was observed with the combination of lithium and biocide. Additionally, the presence of lithium leads to an increase of absorbance at 600 nm, a swelling of the cells that is probably the result of water diffusing into the cell due to a rise in salinity. Previous studies described an increase of the absorbance at 600 nm to be related to an alteration of the refractive index, the release of light scattering material, or to swelling of the cells due to intracellular accumulation of biocide (Chun and Hancock, 2000, Sheppard*, et al.*, 1997).

In a similar fashion, the data provided by the NPN uptake test demonstrated that lithium significantly increased the permeability of the cell membrane to the hydrophobic NPN probe. It has been suggested that lithium permeabilises the bacterial cell membrane, hence increasing the biocide uptake into the cell. However, uptake kinetics demonstrated that lithium is not influencing the uptake/association of formaldehyde with the bacterial cells nor did lithium accumulate in the cells. Therefore, the biocide enhancing action of lithium is the consequence of interference with the $Na^+(Li^+)/H^+$-antiporter systems that in turn affects the cation balance of the bacterial cells. While the cytoplasmic membrane is not permeable to cations without

the involvement of membrane transport systems, the $Na^+(Li^+)/H^+$-antiporter exchanges a cytoplasmic Na^+ or Li^+ for an external H^+ (Inaba*, et al.*, 1994, Inaba*, et al.*, 1997). In an alkaline environment, like that found in calcium carbonate slurries, monovalent ions and especially Na^+ ions are key players in pH homeostasis, regulation of the Na^+ content, and cell volume (Hunte*, et al.*, 2005). Moreover, these cationic antiporters are also involved in the cationic detoxification of the cells since the cytotoxicity of excess sodium and lithium increases as the pH rises, and depends on the cytoplasmic K^+ concentration (Padan*, et al.*, 2005). An increased cationic load as a result of dosing lithium into the alkaline calcium carbonate slurry probably results in a toxic accumulation of Na^+ ions due to the competitive saturation of the antiporter system with Li^+. The elevated extrusion of Li^+ ions consequently influences the transmembrane electrochemical gradient of protons or even the transmembrane pH gradient. Other authors postulated that the efflux of potassium ions probably results from the dissipation of the transmembrane electrochemical gradient of protons (Kroll and Patchett, 1991). Similarly, using the membrane potential sensitive dye $Disc_3(5)$ a significant depolarisation of the bacterial membrane in the presence of lithium was observed in the calcium carbonate slurry bacterial culture, rECM (Figure 63), leading to increased permeability of the cell membrane or even to a loss of membrane integrity. As a consequence, dissipation of the proton motive force leads to the leakage of essential molecules (Friedrich*, et al.*, 1999).

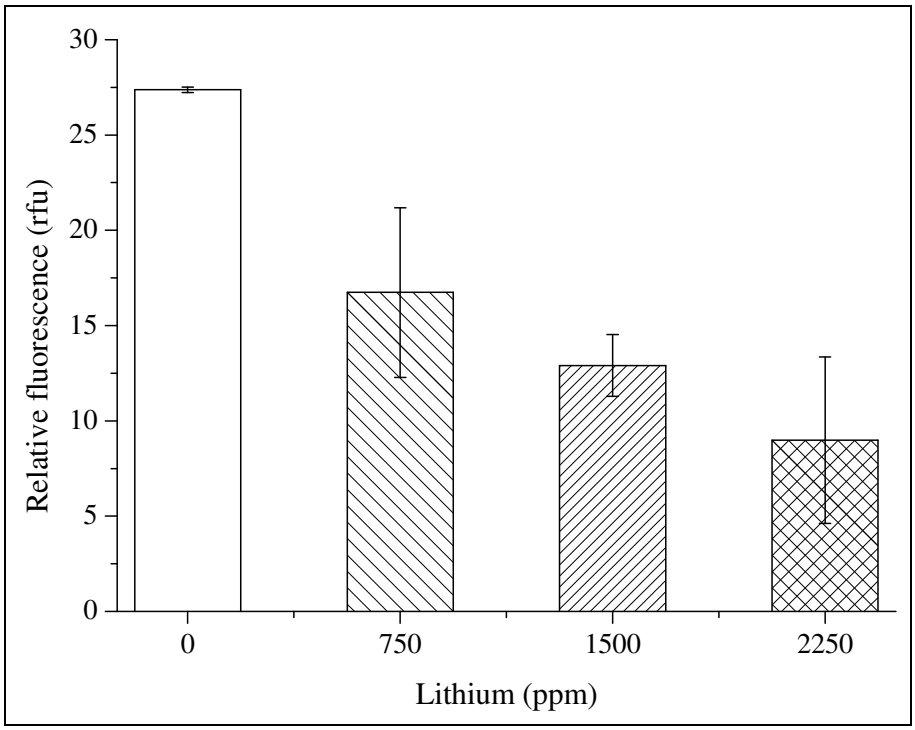


Figure 63. Influence of increasing lithium concentrations on the membrane potential of bacterial cells (rECM) in calcium carbonate slurry determined by means of the membrane potential sensitive dye $Disc_3(5)$ using the CellFacts II® device. Lithium was added to the slurry culture and fluorescence was determined after 30 min. Values are presented as mean ± standard deviation of two independent replicates.

Another possible explanation is that either the formaldehyde or the isothiazolinones deriving from the EDDM/CMIT/MIT biocide inhibited the antiporter system thus causing an inhibitory growth effect because of the disabled extrusion of surplus cations. Finally, lithium and magnesium are alike in some chemical and physical characteristics such as their atomic and ionic radii; these similarities are known as a diagonal relationship. Chemical resemblances might lead to an interaction between lithium and the magnesium ions and destabilise the lipopolysaccharide structure of the cell membrane in a fashion similar to the metal chelator EDTA consequently increasing the permeability of the outer cell membrane.

In calcium carbonate slurries the interference of lithium with the cationic antiporter is probably also supported by the increased pH observed after lithium carbonate is introduced. However, an enhancement of the biocide toward the biocide-resistant cells due to the alkaline stress caused by the pH shift of calcium carbonate slurry ground in the presence of lithium carbonate or supplemented with lithium

carbonate can be ruled out. On the one hand, the pH increase of lithium carbonate ground slurry and lithium carbonate supplemented slurry ranged from 0.4 to 0.8 compared to standard slurry and decreases over time. On the other hand, control experiments using potassium carbonate instead of lithium carbonate did not show any biocide enhancement activity (Figure 58). Finally, the effect of lithium on *Pseudomonas mendocina* cells isolated from WMD revealed a strong interaction with the cells even though the pH was maintained at 7.4 by the HEPES buffer system after the addition of lithium.

5.2.2 AMP – pH Stabiliser with Antimicrobial Properties

Due to microbial adaptation or resistance to the biocides used in the preservation of calcium carbonate slurry and the limited variety of suitable biocidal compounds both the slurry production plants as well as the customers tend to increase the biocide concentration with the intention of achieving efficient protection of the products against microbial contamination. To achieve long-lasting stability and shelf-life of certain biocidal compounds (e.g. glutardialdehyde) at lower pH values, the majority of the biocidal formulations utilised to preserve calcium carbonate slurries are rather acidic. As a result, the white mineral dispersions no longer have sufficient natural buffering capacity to stabilize the pH of the dispersions - especially under repeated exposure to acidic biocides or their acidic breakdown products (e.g. formic acid originating from formaldehyde degradation). Therefore, the aim of the following investigation was to evaluate a new preservation concept using a pH stabilising additive to quench the acidity introduced by the excessive or sequential addition of biocides to calcium carbonate slurries.

Alkanolamines are used as multifunctional additives in various products e.g. in paints and coatings as a source of alkalinity (Dow, 2009c). One of the more versatile applicable primary amino alcohols available on the market is 2-Amino-2-methyl-1-propanol (AMP). AMP has neutralising capabilities and co-dispersant characteristics as well as contributing to pH stability, low odour and anticorrosive properties (Dow, 2009b). Furthermore, in metal working fluids the presence of alkanolamines improves the biological stability as well as enhances the biocidal performance in these fluids and has been referred to as an off-diagonal effect

(Gernon and Dowling, 2006, Gernon*, et al.*, 2003). However, maintaining a high pH is important to sustain the rheology properties of white mineral dispersions thus the question arises whether AMP is compatible with the pH stabilisation of calcium carbonate slurries.

Figure 64 details the pH stabilisation of Hydrocarb 90 and Hydrocarb 60 slurries by AMP and demonstrates that independently from the dosed AMP amount (1110 to 1350 ppm a/l) the pH is stabilised in the range of 9.8 to 10. The difference in the pH between the coarse product Hydrocarb 60 and the fine product Hydrocarb 90 is due to the dispersion. Fine product use more dispersant than coarse product and therefore the base alkalinity is higher.

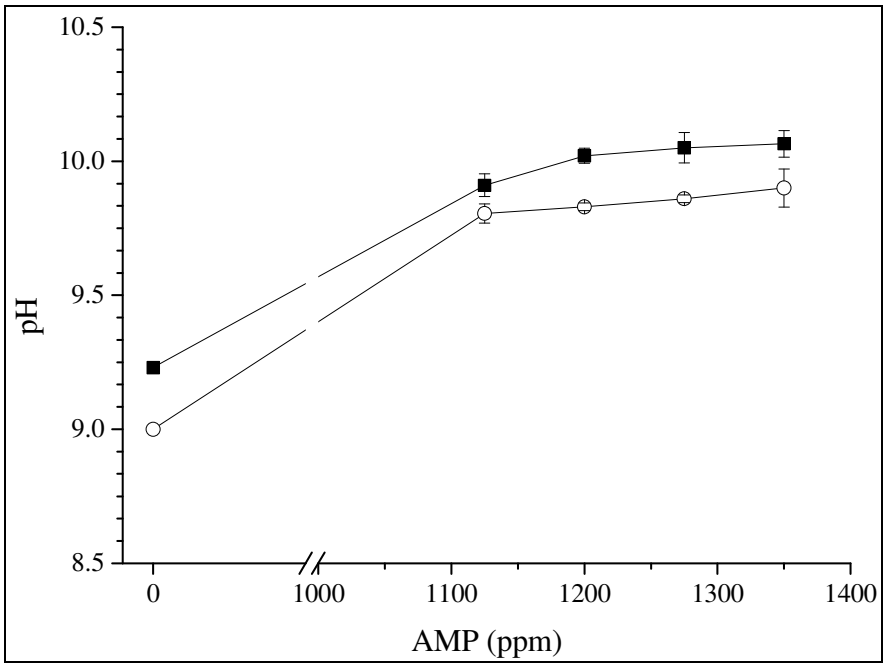


Figure 64. Correlation between the pH and the concentration of AMP in calcium carbonate slurries with different mean particle size. Hydrocarb 90 with 75% solids (■) and Hydrocarb 60 with 67% solids (○).

5.2.2.1 Formaldehyde Scavenger Efficacy of AMP

Alkanolamines are known to react with formaldehyde, often present as the biocide for the preservation of WMD, to form the oxazolidine 4,4-dimethyloxazolidine (DMO) and therefore to be a strong formaldehyde scavenger (Dow, 2009b, Dow, 2009a). The reaction of AMP with formaldehyde is reversible and consequently it can be used as a formaldehyde scavenger whilst the oxazolidine can be used as a formaldehyde-releaser. Temperature, pH and the amount of water are important parameters which influence the scavenge efficacy of AMP. In calcium carbonate slurry the formaldehyde scavenge performance of AMP was investigated by determining the concentration of formaldehyde released by EDDM (Table 31).

Table 31. Formaldehyde recovery rate from H90 slurry supplemented with either AMP, the formaldehyde-releasing agent EDDM (OmyAK) or combinations thereof.

AMP (ppm a/l)	OmyAK (ppm c/l)	Formaldehyde (ppm a/l)
1000	0	0
0	750	280
500	750	283
1000	750	287
1500	750	280

Increasing concentrations of AMP did not show any influence on the formaldehyde recovery rate from EDDM-supplemented calcium carbonate slurry.

These findings suggested that even though DMO is formed, in the alkaline calcium carbonate slurry environment this compound is not stable and directly dissociates to formaldehyde and AMP. However, because the derivatisation of formaldehyde to perform the chemical analysis is performed in an acid buffer, it cannot be ruled out that the formed DMO only dissociate to formaldehyde and AMP during the analysis.

The influence of AMP on the antimicrobial performance of the biocidal formulation EDDM/CMIT/MIT was investigated by means of challenge testing using both a EDDM/CMIT/MIT-resistant slurry culture (rECM) and biocide susceptible standard slurry culture retrieved from CC60 slurry (Figure 65).

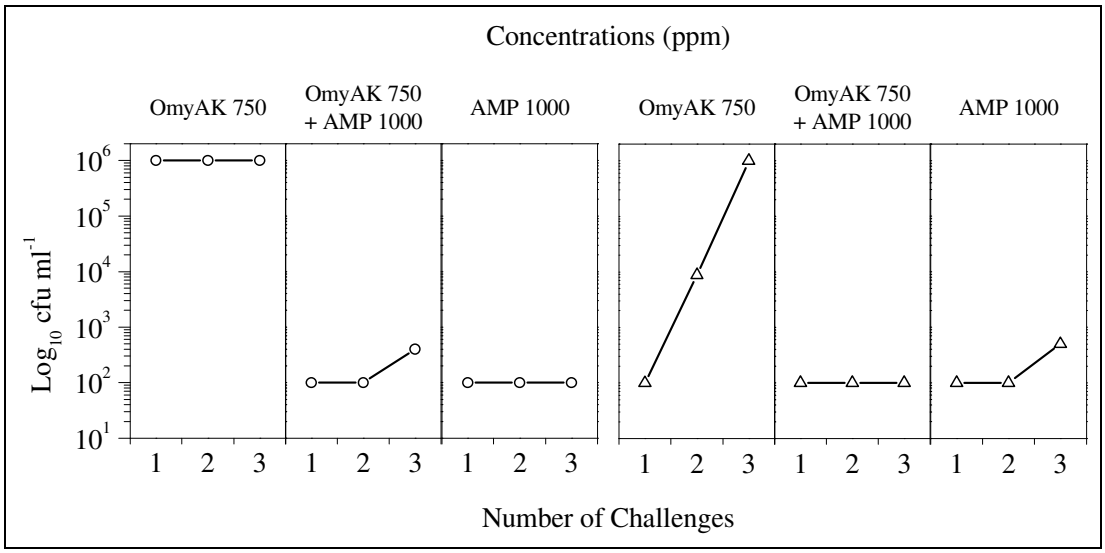


Figure 65. Preservation performance of EDDM/CMIT/MIT (OmyAK c/l) formulation in Hydrocarb 90 standard calcium carbonate slurry in the presence of AMP (a/l). Calcium carbonate samples were inoculated with the resistant bacteria rECM (○) or standard CC60 calcium carbonate slurry bacteria (△), respectively. There were no more than three inoculations performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}.

Surprisingly, AMP on its own or combined with 750 ppm (c/l) EDDM/CMIT/MIT showed an outstanding preservation performance in Hydrocarb 90 slurry. Even against the EDDM/CMIT/MIT-resistant bacteria a preservation performance over three challenge cycles was confirmed when 1000 ppm (a/l) AMP was applied. To some extent these findings are contradictory since AMP is a formaldehyde scavenger and the antimicrobial property of AMP is not pointed out in the technical descriptions of the AMP vendor. However, the present data clearly showed that AMP exhibits an intrinsic biocidal property in the alkaline environment of calcium carbonate slurry. In addition, a synergistic antimicrobial performance of the biocide EDDM/CMIT/MIT with AMP can be assumed.

5.2.2.2 Antimicrobial Activity of AMP in WMD – MIC

The preservation performance of AMP in calcium carbonate slurry was investigated against the EDDM/CMIT/MIT (rECM) and GDA/CMIT/MIT (rGCM) resistant slurry cultures by means of the challenge test. AMP at a concentration of 1350 to 1500 ppm (a/l) showed an outstanding preservation performance against both biocide-resistant bacteria used to perform the challenge tests (Figure 66a and b).

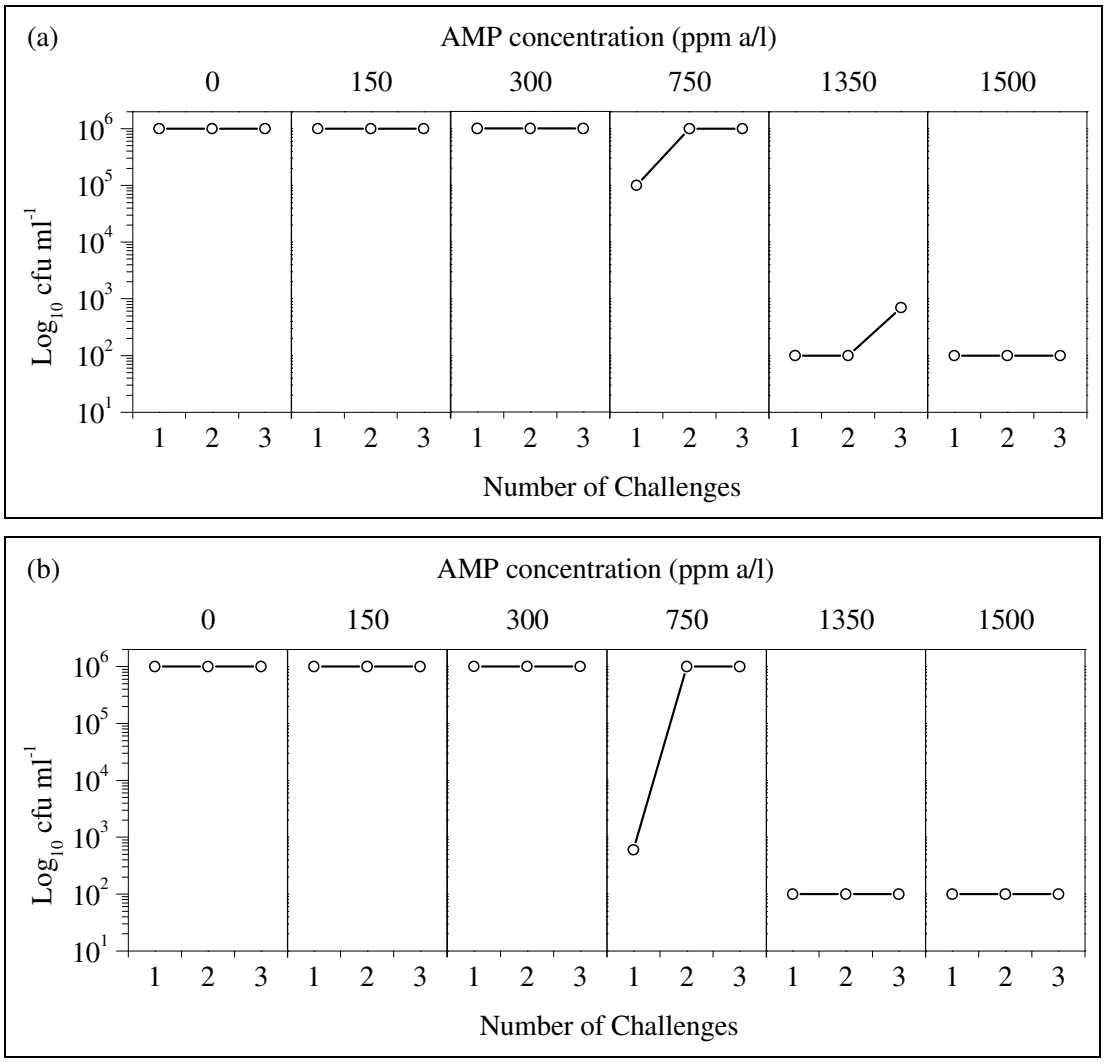


Figure 66. Preservation performance of AMP in Hydrocarb 90 standard calcium carbonate slurry. Calcium carbonate samples were challenged with the resistant bacteria rECM (a) or rGCM (b), respectively. There were no more than three inoculations performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}.

Moreover, the disinfection properties of AMP were investigated by means of the curative disinfection test. Calcium carbonate slurries contaminated with each biocide-resistant culture were supplemented with various concentrations of AMP and the TVC was monitored after 1, 2 and 3 days (Figure 67a and b). Besides of the preservation performance, AMP was an effective disinfectant in calcium carbonate slurries at concentrations of between 1050 and 1500 ppm (a/l).

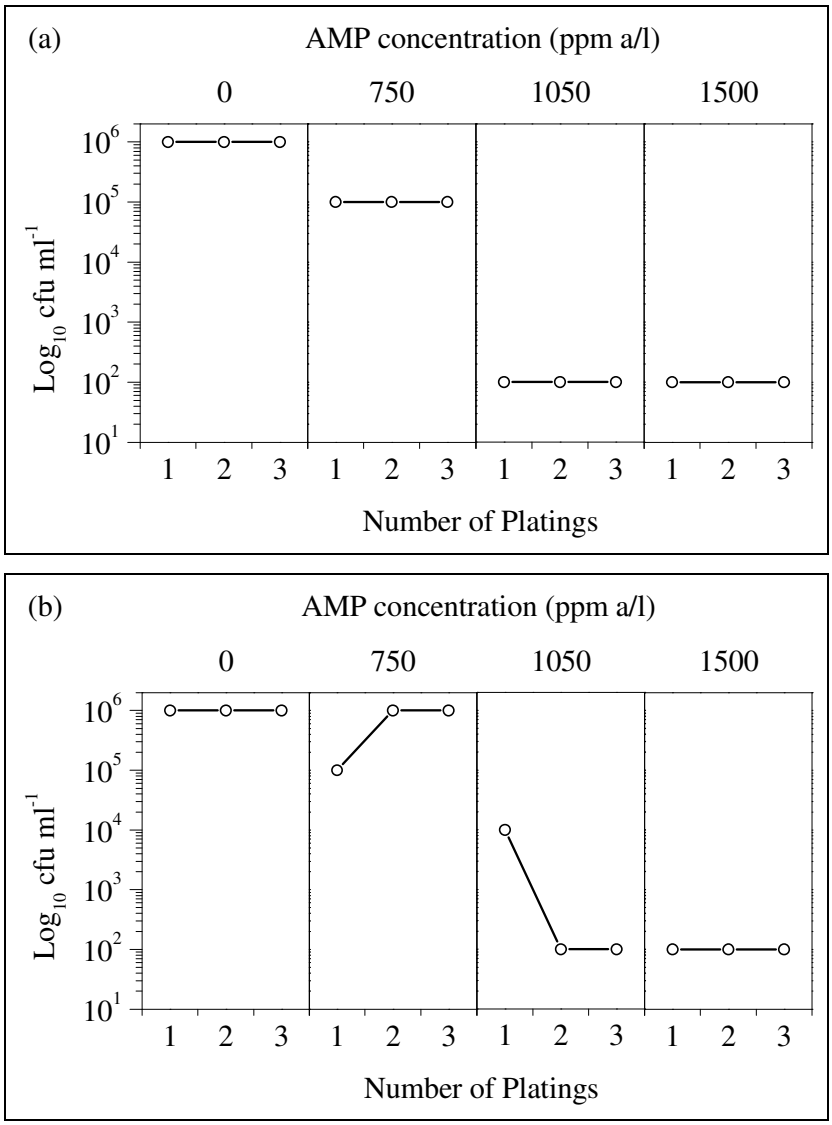


Figure 67. Disinfection performance of AMP in Hydrocarb 90 standard calcium carbonate slurry. Calcium carbonate samples were challenged with the resistant bacteria rECM (a) or rGCM (b), respectively. There were no more than three platings performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}.

The antimicrobial properties of alkanolamines as preservatives in metal working fluids were postulated many years ago (Bennett, 1979), however a few follow-up studies draw differing conclusions about their antimicrobial activity. Bennett (1979) investigated a wide collection of primary, secondary and tertiary alkanolamines used as corrosion inhibitors in metal working fluids for their antimicrobial properties and found that secondary alkanolamines (i.e. N-alkyl ethanolamines) exhibit potential antimicrobial activity. However, all investigated alkanolamines were applied at a concentration of 2000 ppm and a failure in the preservation performance was

considered once two consecutive total viable counts exceeded 10^5 cfu ml^{-1}. Even though the higher concentration of utilised alkanolamines and the high bacterial count threshold defining a preservation failure limit a direct comparison, in calcium carbonate slurries AMP showed a better preservation performance at 25% less concentration. Others reported an enhanced antibacterial activity of 2000 to 4800 ppm of butylethanolamines and dimethylamino-methyl-propanol at high pHs (8.5-10) in metal working fluids (Sandin*, et al.*, 1991). Gernon *et al.* (2003) emphasised an antimicrobial activity and biocide synergy for the alkanolamine product family Synergex™ (proprietary chemical structure, Taminco Inc., Belgium) but not for AMP at a concentration of 2000 ppm. In addition a recent study reported the incapacity of 100 ppm AMP to kill *Mycobacterium immunogenes* cells (Falkinham, 2009). In coolants AMP is employed at concentrations of 2500 ppm as an antifoam agent, corrosion inhibitor and as a co-dispersant because it prevents the precipitation of ingredients in the fluid (Bennett, 1979). Nevertheless, only a few patents about coolant formulations claim the antimicrobial properties of alkanolamines.

In a recent study it has been shown that the formaldehyde releaser 4,4-dimethyloxazolidine (DMO) has a superior preservation and disinfection performance compared to the N-formal-based formaldehyde-releaser TMAD (Giuliani, 2008). Using the challenge testing, 150 ppm (a/l) DMO instead of 250 ppm (a/l) TMAD have been shown to preserve calcium carbonate slurries over three cycles of bacterial inoculation. Similarly, 200 ppm (a/l) DMO compared to 300 ppm (a/l) TMAD were required to achieve disinfection. Furthermore, DMO led to a pH increase from 9.1 to 9.5 in calcium carbonate slurry whereas the other formaldehyde-releasers did not show any pH activity. Giuliani (2008) has suggested that this phenomenon results from the higher pH of the DMO biocidal formulations, however based on the data above the enhanced preservation and disinfection performance as well as the pH increase originates from the AMP released by the biocide DMO.

Finally, the results of this study showed that AMP is a potent triple-action compound (pH, viscosity and microbial contamination) for the stabilisation of calcium carbonate slurries. The industrial applicability of AMP represents an innovative new preservation system for the stabilisation and preservation of WMD.

5.2.2.3 Stability of AMP in WMD

The influence of AMP on the pH and viscosity of calcium carbonate slurries over a period of 11 days is shown in Figure 68. In calcium carbonate slurry, AMP showed a significant improvement of the pH and viscosity stability compared to untreated slurry. After the addition of either AMP or AMP combined with the biocide GDA/CMIT/MIT a significant decrease of the viscosity and an increase of the pH were observed. Both viscosity and pH remained stable over the entire period of investigation. Moreover, after the addition of 1350 ppm (a/l) AMP to the inherently contaminated sample the initial contamination of 10^6 cfu ml^{-1} was reduced to the detection limit of 10^2 cfu ml^{-1} as determined on PCA.

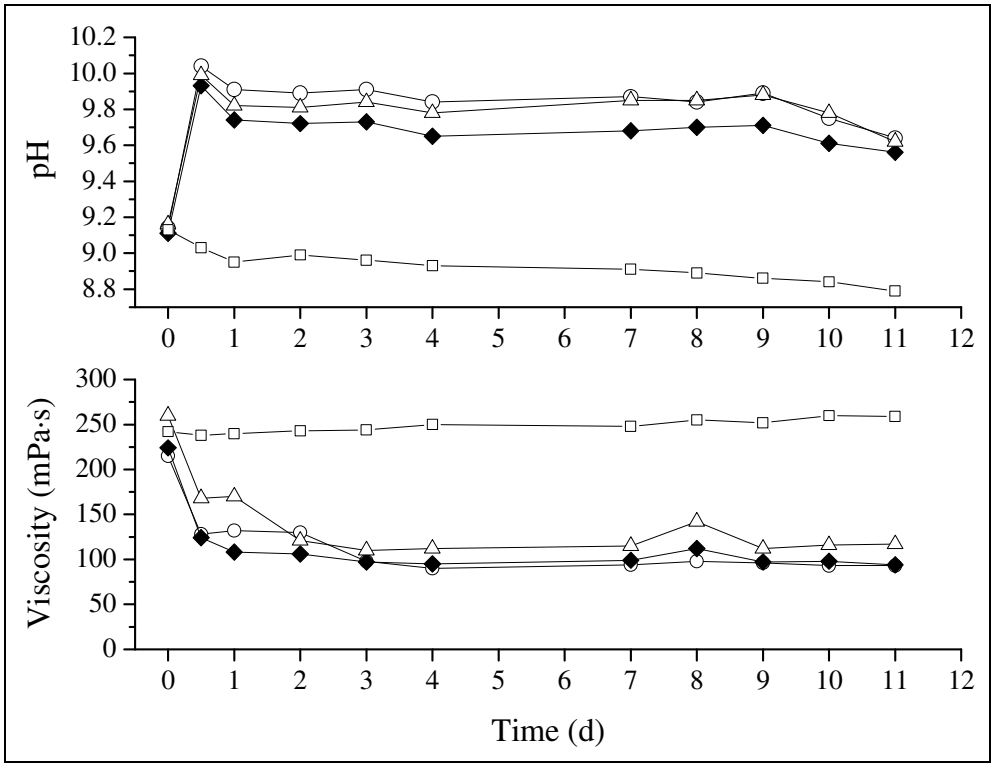


Figure 68. Improved pH and viscosity of H60-67% calcium carbonate slurry after the addition of AMP. (○) 1350 ppm (a/l) AMP to sterile slurry. (◆) 1350 ppm (a/l) AMP + 1350 ppm (c/l) GDA/CMIT/MIT biocide to sterile slurry. (△) 1350 ppm (a/l) AMP to inherently contaminated slurry. (□) control without AMP.

In this context it is important to highlight the poor recovery rate of active AMP in the aqueous filtrate of calcium carbonate slurries. The addition of 1350 ppm (a/l) AMP to calcium carbonate slurry returned a recovery rate of 85%. Possible explanation for

the differences between the expected and the measured amount of AMP is the reaction of the AMP molecule with the dispersant in the calcium carbonate. A complex formed between the dissociated polyacrylic acids of the dispersant and the protonated AMP might be responsible for the sequestration of AMP from the water phase to the carbonate particles. This assumption was confirmed since the addition of sodium ions lead to an increase of the AMP recovery rate in the slurry aqueous phase probably because the sodium ions have an enhanced affinity to neutralise the dissociated polyacrylic acids of the dispersant compared to AMP.

5.2.2.4 Synergy of AMP with Biocides used to Preserve WMD

A synergistic performance of alkanolamines with biocide has been proposed by Bennett (1979). The application of AMP in calcium carbonate slurries as a pH stabiliser does not necessarily require elevated concentrations such as 1350 ppm (a/l) which are necessary to achieve preservation. However, in the large-scale industrial process of calcium carbonate slurry manufacture the addition of biocides takes place either into the storage tank, at the load out before the product is supplied to the customer or at the customer site directly (post-treatment). Therefore, a primary stabilisation of the pH with AMP and the subsequent addition of biocide to achieve preservation are conceivable. Moreover, the enhancement of the biocide by AMP or in this specific case a synergistic performance of AMP with the biocide may bring about substantial preservation benefits.

The antimicrobial synergy between AMP and various biocides used to preserve calcium carbonate slurry was calculated by means of the Fractional Inhibitory Concentration Index (FICI) value according to the equation (a) of Berenbaum (1978):

$$\text{(a)} \qquad \mathrm{FICI} = \Sigma \mathrm{FIC}_{A+B} = \frac{\mathrm{MIC}\left[A_B\right]}{\mathrm{MIC}\left[A\right]} + \frac{\mathrm{MIC}\left[B_A\right]}{\mathrm{MIC}\left[B\right]}$$

Whereas $[A_B]$ and $[B_A]$ represent the new MICs of [A] combined with [B] and *vice versa* and [A] and [B] represent the MIC of the single compounds. The FICI were determined by means of the challenge test (single challenge) and checkerboard tests were performed for all combinations of AMP and biocides (Table 32).

Table 32. FICI values calculated for a range of combinations of AMP with different biocides. Samples were challenged once with the relevant biocide-resistant calcium carbonate slurry culture and the TVC was determined after 24h and 48h. FICI values were calculated from AMP/biocide combinations achieving preservation defined as a return to a TVC < 10^2 cfu ml^{-1} after 5 days of incubation.

AMP (ppm a/l)	**Biocide concentrations**										
	GDA/CMIT/MIT (ppm c/l)				**EDDM/CMIT/MIT (ppm c/l)**				**OPP (ppm a/l)**		
750	450	300	200	130	400	265	175	120	300	200	130
	0.63	*0.61*	*0.59*	*0.58*	*0.70*	*0.65*	*0.62*	*0.60*	*0.66*	*0.62*	*0.60*
600					450	300			300	200	130
					0.61	*0.56*			*0.54*	*0.51*	*0.49*

Overall, the data indicated that in calcium carbonate slurries AMP in combination with the in-use biocides exhibited a significant synergistic effect against the biocide-resistant slurry cultures. The FICI values ranged from 0.7 to 0.49. For the biocide resistant slurry cultures rOPP the lowest reported FICI value was 0.49 arising from the combination of 600 ppm (a/l) AMP with 130 ppm (a/l) OPP. In a similar fashion, the combination of EDDM/CMIT/MIT and AMP showed comparable synergistic patterns toward the relevant biocide-resistant culture. The employment of the FICI to quantify synergism originates from the estimation of a drug's synergism in pharmacology and was accordingly adapted to quantify the synergistic effect of antimicrobials such as antibiotics and biocides (Berenbaum, 1978). As mentioned above the interpretation of the FICI value and therefore of the synergistic affect is a matter of definition: Berenbaum (1978) defined a synergism to occur when the FICI is <1 whereas the American Society of Microbiology has defined a synergy at FICI < 0.5 and other authors have also defined a partial synergy as 0.5> FICI <1 (Botelho, 2000). A further problem inherent in a study of this kind is that in order to enlarge the numbers of samples of the checkerboard test, 25 combinations were tested for each biocide, the elucidation of biocidal synergism in calcium carbonate slurry derives from a mono-inoculation challenge test. Therefore, elucidation of the preservation synergism of AMP with the biocides over three bacteria challenges would possibly have generated a different picture in terms of FICI values.

5.2.2.5 Effect of AMP on *P. mendocina* cells isolated from WMD

In a similar fashion to the lithium investigations in section 5.2.1.4, *Pseudomonas mendocina* cells isolated from the EDDM/CMIT/MIT-resistant culture were used to investigate the effect of AMP on bacterial cells. The leakage of cytoplasmic constituents with an absorbance at 260 nm along with potassium were recorded over a time period of 240 minutes after exposing the bacterial cells to AMP or to its amine-less analogue 2-methyl-1-propanol (MP) (Figure 69a and b). Changes in cell morphology (lysis or swelling) were followed by monitoring the absorbance at 600 nm (Figure 69c).

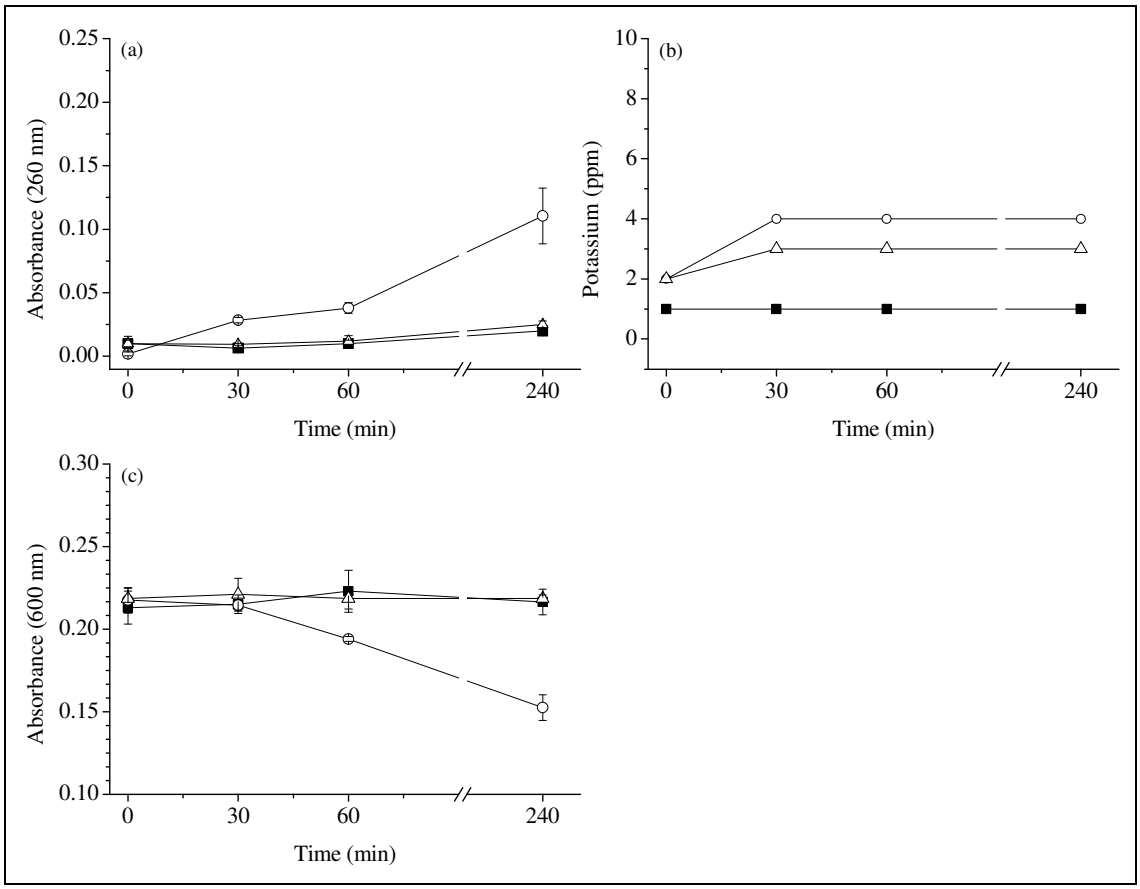


Figure 69. *Pseudomonas mendocina* leakage of cytoplasmic components at 260 nm (a) and potassium (b) as well as absorbance at 600 nm (c) measured 30 min. and 60 min. after exposure to the following combinations: Untreated control (■), 1350 ppm AMP (○), 1350 ppm MP (△). All concentrations are given in ppm (a/l). Values are presented as mean ± standard deviation (n = 4).

The leakage of cellular constituents absorbing at 260 nm (such as nucleotides, nucleosides and aromatic amino acids) was observed in the AMP-treated cells only and the same concentration of MP on its own did not show any effect on the leakage of 260 nm absorbing materials. Furthermore, a very low outflow of potassium ions either for AMP as well as for MP was induced compared to the data above for lithium ranging from 14 to 18 ppm (section 5.2.1.4). A decrease of absorbance at 600 nm indicated that the treatment with AMP resulted in cell aggregation or even in cell lysis. Based on the low levels of potassium and 260 nm absorbing material found in the supernatant the evaluation whether cell aggregation or cell lysis occurred is ambiguous and cell aggregation may have occurred rather than lysis. Taking a closer look at the AMP-supplemented bacterial cells with the CellFacts II® instrument revealed that the decrease of absorbance at 600 nm is also related to a reduction in the average cell size i.e. a reduction in the size of the individual bacterial cells. In fact, after 30 and 60 minutes respectively, the bacterial population treated with AMP shifted to the left whilst the total cell count remained stable (Figure 70).

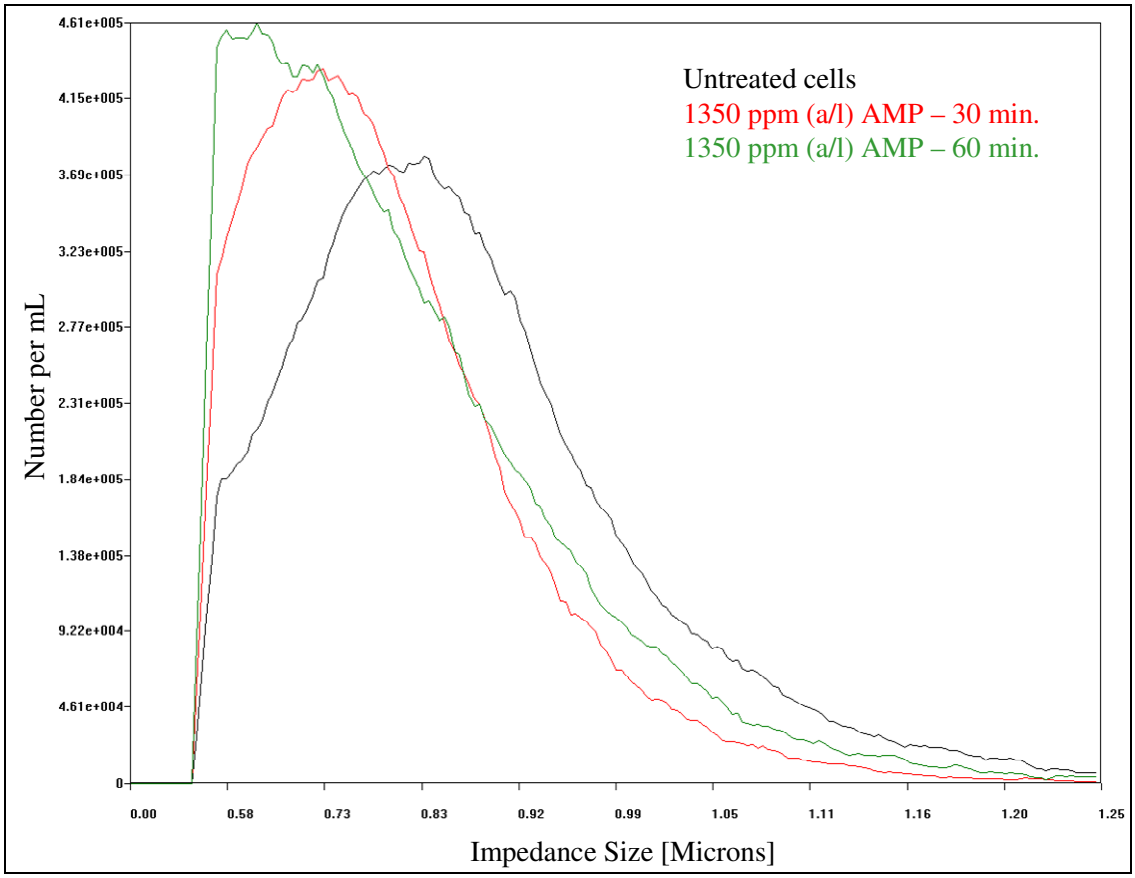


Figure 70. CellFacts II® profiles from *Pseudomonas mendocina* cells treated with AMP.

However, a previous study investigating the antimicrobial action of *N*-octylethanolamine reported a decrease of absorbance at 600 nm whilst the release of u.v.-absorbing material increased significantly and the lytic effect of this alkanolamine was subsequently confirmed on synthetic liposomes (Shepherd*, et al.*, 1998).

In a similar fashion to lithium, AMP caused a significant increase of the cell permeability ($p < 0.01$), whereas MP did not influence the membrane permeability as assessed utilising the NPN uptake assay (Figure 71). An even higher permeability was obtained by using EDTA as the positive permeabilisation control ($p < 0.01$) thus indicating that the permeabilisation effect is fully attributable to the effect of AMP on the bacterial membrane.

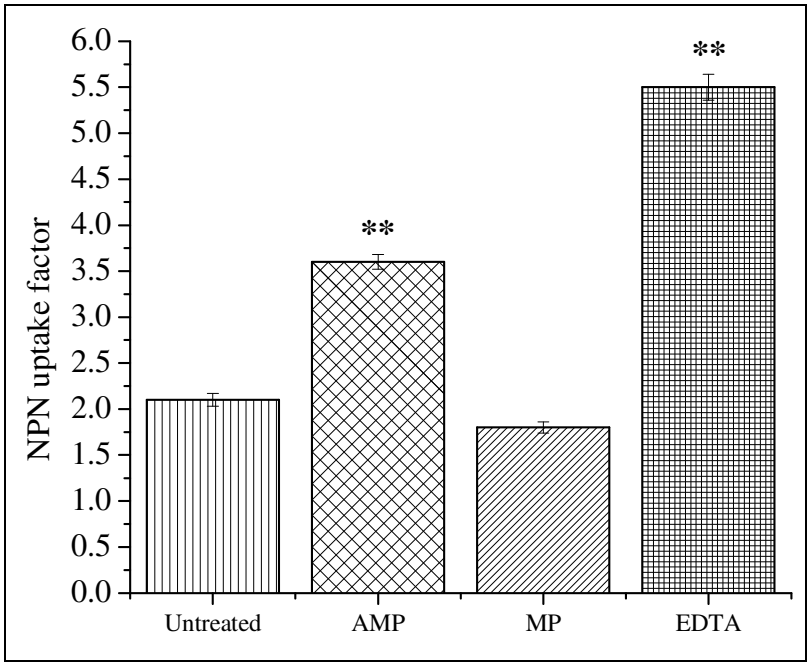


Figure 71. NPN uptake factor obtained after exposing *P. mendocina* cells to different treatments. The following concentrations were used: 1350 ppm AMP, 1350 ppm MP and 180 ppm EDTA. Values are presented as mean ± standard deviation (n = 16); ** $p < 0.01$ for student t-test (unpaired, equal variance) based on the untreated sample.

The antimicrobial activity of alkanolamines in calcium carbonate slurry is enhanced due to the alkaline environment in view of the fact that with increasing pH the antimicrobial activity of these compounds increases (Sandin*, et al.*, 1990). Using

various secondary and tertiary amines, Sandin and co-workers (1990) suggested that the antimicrobial activity of alkanolamine is associated with membrane diffusion of the unprotonated form of these molecules at high pH. A study investigating the antimicrobial activity of octyl ethanolamine suggested that after the alkanolamines penetrate the bacterial cell membrane, the lower pH and the proton-rich environment of the cytoplasm lead to a protonation of the amine group within the cell (Sandin*, et al.*, 1992b) and the pH within the cells increases. The positively charged amine group within the cell acts similar to cationic detergents and results in damage to the cell membrane, leakage of cytoplasmic constituents and cell death. In addition, in this study it has been suggested that the binding of uncharged alkanolamine molecules on the bacterial cell membrane leads to a shift of the equilibrium and hence to an accumulation of protonated alkanolamines at pH 7.1, i.e. below the pKa value of octyl ethanolamine of 9.6.

The antimicrobial activity of AMP is most likely to occur because of the analogue action mechanism as described above. AMP has a pKa value of 9.72 and consequently the alkaline environment of calcium carbonate slurries favours the antimicrobial performance. Bacterial challenge tests in calcium carbonate slurries supplemented with the amine-free analogue 2-methyl-1-propanol did not prevent bacterial growth after the first inoculation cycle and have corroborated that the antimicrobial activity arises from the presence of the amine group (data not shown).

Finally, prediction of the antimicrobial activity of alkanolamines on the basis of the molecule size as well as the number and properties of substitutions in the amine group would help to make a primary selection of alkanolamines with potential antimicrobial activity. However, Bennett (1979) showed that monoethanolamines are more affective antimicrobial agents than di- and triethanolamines and another study reported that with increasing alkyl chain length the antimicrobial performance of diethanolamines decreased (Sandin*, et al.*, 1992a).

5.2.2.6 AMP On-site Study

A pilot trial to determine the industrial applicability of AMP was conducted on-site at the Avenza plant in Italy. As from January 2009 the calcium carbonate slurry product Hydrocarb 60 was supplemented in the storage tank with AMP only (1350

ppm a/l). The microbial contamination was monitored by means of both the CellFacts II® analyser and the Easicult® dipslide system (Bode Chemie, Hamburg) before (Figure 72a) and after (Figure 72b) the change to AMP for a period of six weeks.

Before the implementation of AMP at the slurry production plant, the biocidal formulation CMIT/MIT was used to preserve to the product after the flash cooler and in the occurrence of contamination the biocidal formulation GDA/CMIT/MIT was dosed directly into the storage tank and/or at loadout. During the time in the storage tank, the total cell count remained stable between 10^5 and 10^6 cells ml^{-1} and an average total cell count of $3.3 \cdot 10^5$ cells ml^{-1} was determined. The relative fluorescence was for the most part above a value of 10 indicating the presence of metabolically active cells. In week 3 (5-12 Oct) the fluorescence peaked at a value of 36 and moved afterwards to between 20 and 30. The average relative fluorescence was determined to be 21 (± 1.4). Concomitantly, the dipslides used to determine the culturable fraction of the bacterial population reported contamination in weeks 2, 3 and 5.

According to the biocide dosage rules at that time, an in-tank treatment with biocide was performed after contamination was recognised with dipslides. The biocide treatment caused a decrease in the fluorescence indicating an impact of the biocide on the average metabolic activity of the bacterial population. On the one hand, shortly after the biocide treatment the fluorescence increased again indicating that the biocide treatment was not efficient at inhibiting microbial growth. On the other hand, the fluorescence measured by CFII increased prior to the detection of a contamination by means of the dipslides, thus would have been useful in predicting microbial contamination. This trend is reinforced when taking into account that the dipslides TVCs were read after 48h into incubation but plotted retrospectively on the date of sampling. Using the CellFacts II® analyser to predict microbial contamination by means of trend analysis of the fluorescence has lead to prediction of the first biocide treatment and probably to a more efficient inhibition of the cells at an earlier physiological stage. In addition, the subsequent treatments would have been avoided.

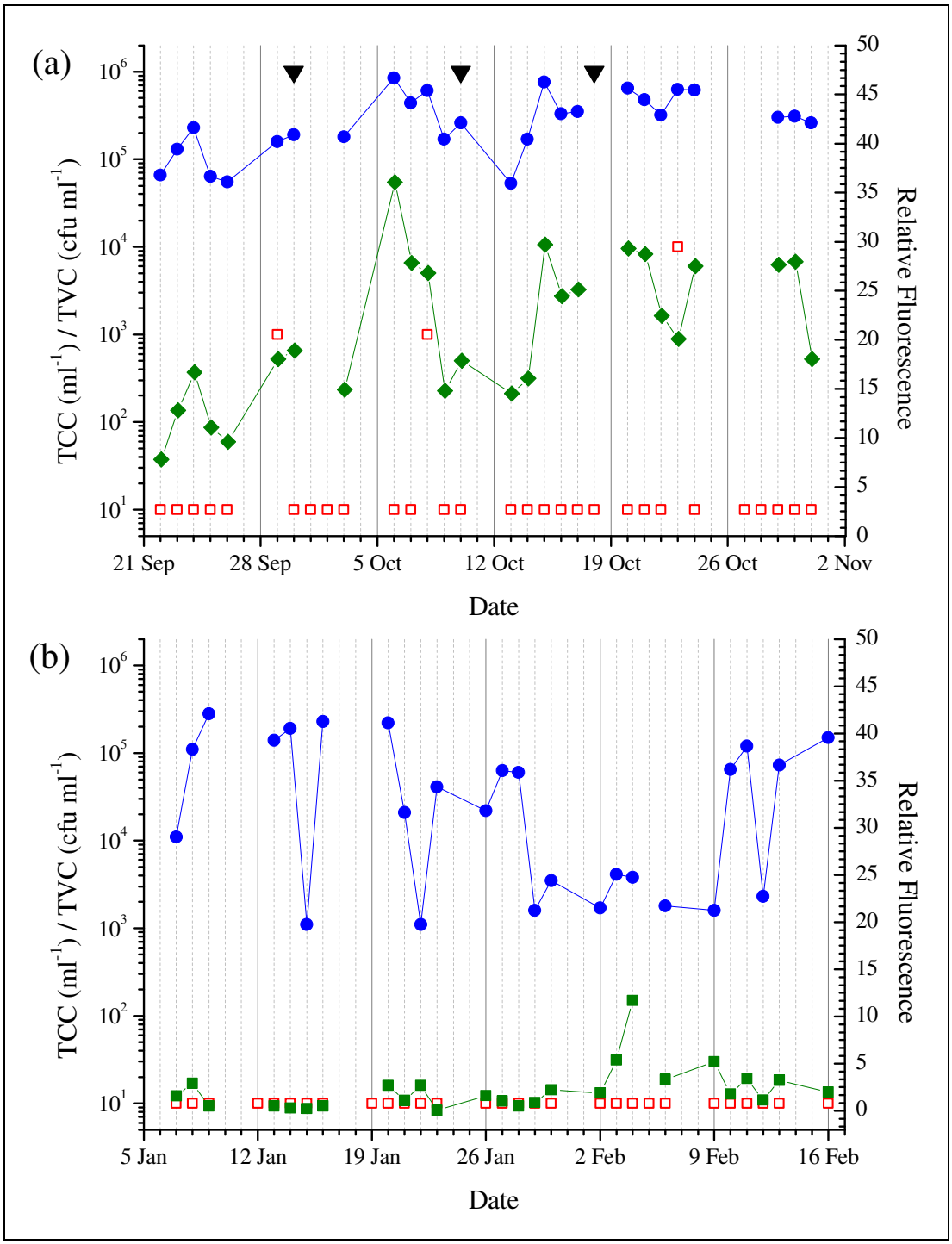


Figure 72. Microbiological profile of Hydrocarb 60 calcium carbonate slurry determined by means of CellFacts II® and Easicult® dipslides system in the storage tank before the implementation of AMP (a) and afterwards (b). (●) Total cell count and (■) relative fluorescence determined by means of the CellFacts II® analyser. (□) TVC determined with Easicult® dipslides. Note: The Easicult® dipslides were read after 48h into incubation, but plotted retrospectively on the date of sampling. (▼) biocide treatment (1350 ppm c/l GDA/CMIT/MIT).

After the change to AMP, the microbiological profile in the storage tank of Hydrocarb 60 slurry changed considerably. The total cell count moved between 10^3 and 10^5 cell ml^{-1} and the average cell count decreased to $6.9 \cdot 10^4$ cells ml^{-1}. The fluorescence was reduced significantly ($p < 0.05$, paired t-test) and the mean fluorescence was determined to be 2.2 (±0.5). Dipslide analysis did not reveal a bacterial contamination during the entire period of investigation. Above all, taking a closer look at the pH and viscosity of the AMP supplemented calcium carbonate slurries showed a considerable improvement of the rheological properties (Figure 73). The average pH shifted from 9.45 (±0.03) to 9.88 (±0.01) and the average viscosity shifted from 78 (±4.5) mPa·s to 53 (±1) mPa·s. Both the differences in pH and viscosity were determined to be significant ($p < 0.05$, paired t-test).

Summing up, the results of this pilot trial are in line with the previous findings confirming that AMP is a potent triple-action compound (pH, viscosity and microbial contamination) for the biological and rheological stabilisation of calcium carbonate slurries. Nonetheless, the industrial implementation of AMP as a pH stabiliser of WMD with biocidal synergistic or even antimicrobial properties depends on the cost-benefit ratio. Additionally, the compatibility with the customer down-stream processes, the customer acceptance and the regulatory requirements are also of importance. To date in a few Omya plants, AMP has been successfully implemented as pH stabilisation agent with the added value of having an antimicrobial performance as side effect. The arising expenses of the AMP application are comparable to the preservation costs of WMD with the biocidal formulation GDA/CMIT/MIT. However, using synergistic combinations of AMP and the in-use biocides would offer the possibility of maintaining the actual preservation costs.

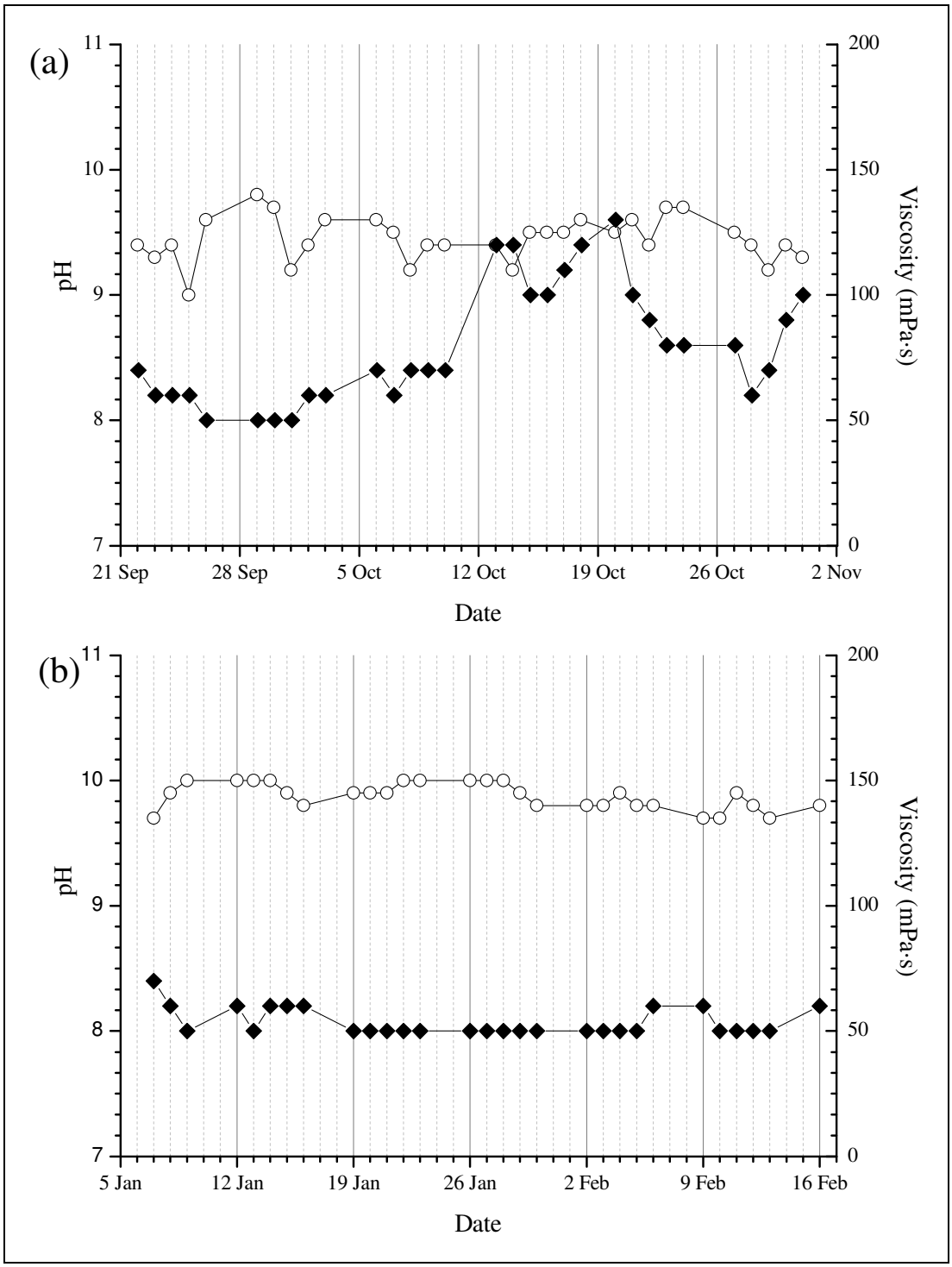


Figure 73. Viscosity and pH profiles of Hydrocarb 60 calcium carbonate slurry determined before (a) and after (b) the implementation of AMP. (○) pH and (◆) viscosity.

5.3 Conclusions

The aim of this chapter was to investigate alternative preservation systems and protocols useful to control microbial contamination of calcium carbonate slurries. Summing up, experimental data provided evidence that lithium enhances and boosts the biocidal activity of various biocides used for the preservation of WMD and provides a novel technology to minimise biocide resistance. WMD bacteria with resistance to the in-use biocide concentration were susceptible to the relevant biocide in the presence of lithium ions. The major advantage is that lithium can be introduced into calcium carbonate slurry simply via dispersant neutralisation. No difference in the biocide enhancer properties of lithium was observed whether it was introduced into the calcium carbonate slurry during the grinding procedure, or afterwards into the final product. Additionally, lithium can be introduced into calcium carbonate slurries via the dispersant efficiently and without any additional dosage steps. This approach may solve the emerging problems of bacterial resistance or adaptation by using an enhancer compound to assure the effectiveness of the biocide. However, a possible scenario for the industrial applicability of lithium in WMD would not imply a consistent use of lithium in WMD but rather would offer the opportunity to make use of this new technology temporarily to combat biocide-resistance after they had been detected. The described concentration of lithium in the down-stream product of calcium carbonate slurries as determined in this study neither causes an adverse effect on the final product itself nor infringing any regulatory limitation values even for products having food contact (personal communication C. Northfleet). Nevertheless, with the increasing global demand the price for lithium is increasing (e.g. lithium batteries, computer industry) and thus the use of lithium in WMD would lead to a rise in the preservation costs. The application forms and the value in biocide enhancement of lithium are under current patent rights (Di Maiuta*, et al.*, 2009b).

Furthermore, it has been shown that the alkanolamine AMP in the alkaline environment of calcium carbonate slurries is a potent preservative. AMP turned out to be a triple-action compound which enjoys the advantages of being an antimicrobial agent with concomitantly pH and viscosity stabilising properties. In addition, the results of this study provided a first indication of antimicrobial synergism between AMP and the in-use biocides at the laboratory scale. Despite

from a conservative antimicrobial synergism point of view, the determined FICI might not be of significance, the employed synergistic combination of AMP and biocides in calcium carbonate is effective in respect to the preservation costs. However, whether this would be applicable in a large scale process at the calcium carbonate production sites has to be evaluated. The on-site pilot trial performed under regular production conditions were not only promising because of the improved properties of the slurry product stability in terms of pH and viscosity in the presence of AMP. Microbial contamination monitored by means of the CellFacts II® instrument revealed a significant reduction of the total cell count and metabolic activity of the bacterial cells thus corroborated the preservation performance of AMP. A patent application disclosing the forms and the value of the enhancing performance of AMP to various biocides was filed recently and is under current patent rights (Di Maiuta and Schwarzentruber, 2009).

Finally, the application of biocide enhancers represents a breakthrough that offers a potential tool to revolutionise the consumption of biocidal agents in the WMD producing industry. Two final items of particular importance for the future discovery of new biocidal enhancer are the specifications of these compounds, which must be chemically compatible with WMD, and the economic and ecological sustainability of their deployment.

CHAPTER 6

Discussion

6.1 Review of the Results

The findings presented in this work are not only of particular importance for the WMD (white mineral dispersions) manufacturing industry but also provide valuable information to the down-stream branches of those industries processing WMD to various products. The application of these findings make a more effective preventive treatment of white mineral dispersions possible and helps to avoid biocide overdosing, and thus save both environmental as well as financial resources. Particularly, due to the development and implementation of new preservation protocols to enhance the performance of the limited biocides available and to counteract the development of biocide-resistant microorganisms in WMD.

The application of multiple culture-independent molecular methods to elucidate the microbial diversity of white mineral dispersions disclosed a greater number of phylotypes than previously estimated by culture-dependent techniques or screening of clone libraries. In recent years *Pseudomonas* spp. have been assumed to be the main contaminant of WMD, however, due to the great microbial diversity and the physiological heterogeneity the influence of other bacterial species on the deterioration of WMD can no longer be ignored. The microbial diversity of WMD is plant specific, rather than product specific, and fluctuations are of a seasonal nature most likely lead by the seasonal dynamics of the storage tank temperatures as well as the naturally occurring microbial dynamics in the open manufacture system of WMD and the raw materials used (water, marble). Arranged according to abundance, the majority of bacteria found in calcium carbonate slurries are represented by the bacterial genera *Pseudomonas*, *Delftia*, *Achromobacter*, *Propionibacterium*, *Hydrogenophaga* and *Pannonibacter*.

Even though the microbial diversity is greater in biocide-free than in biocide-supplemented WMD, the addition of biocides causes a perturbation in terms of bacterial species abundance ratios of the predominant species. Nevertheless, highly contaminated samples tend towards a narrow diversity in that a single species tends to dominate. By development of the propidium monoazide (PMA) agent to assess bacterial species with integral membranes, a molecular method to study the bacterial species able to withstand a biocide challenge has become available. The use of this

new technique enables the culture-independent investigation of persistent bacterial cells and promotes the understanding of preservative performance at the level of the heterogeneous bacterial population in WMD.

There is clearly a risk of biocide-resistance development in the application of biocides for the preservation of WMD and this gives cause for serious concern. The permanent use of certain biocidal compounds and the incorrect use of biocides (suboptimal dosage) combined with poor house-keeping results in the development of biocide-resistant bacteria. The biocide-resistant bacteria occurring in WMD are in most cases able to decompose the relevant biocidal compounds and, depending on their chemical nature, the by-products cause a destabilisation of the mineral dispersion characteristics such as the pH and viscosity as well as discoloration or the development of malodour.

In this context, one has to mention the extraordinary ability of WMD bacteria such as *Pseudomonas putida* and *Methylobacterium extorquens* in metabolising formaldehyde to formic acid synergistically. Even though combinations of biocidal actives have been more common in the recent past the incidence of biocide-resistant bacteria in WMD has not been circumvented. The resistance of the WMD bacteria against the relevant biocide is stably maintained and a 20-fold increase of the minimal inhibitory concentration (MIC) compared to susceptible WMD bacteria was found. In addition, the incidence of WMD bacteria with multiple biocide-resistance to almost all available formulations of biocidal compounds for the preservation of WMD gives rise to concerns. Therefore, the development and implementation of new preservation protocols is indispensable to maintain the high hygiene and quality requirements of WMD.

The choice of suitable biocides for the preservation of WMD is reduced to a minimum because of the regulatory situation created by the BPD (Biocidal Products Directive 98/8/EC) and the customer acceptance for certain compounds. As a result, once a lack of biocidal activity has been recognised it is almost impossible to substitute the in-use biocidal formulation with another as was done in the past. Therefore, enhancement of the in-use biocides with non-biocidal compounds or the implementation of new preservation techniques is of high value for the future. The

rising costs of preservation by increasing the in-use biocide concentration not only impacts on the economic efficiency, but nowadays the regulatory limitations, health and safety requirements as well as the impact on the environment demand compliance.

Lithium has been shown to be a potent and universal biocide enhancer which in calcium carbonate slurries reconstitutes and boosts the biocidal activity of various biocides. Therefore, it provides a novel technology to improve the preservation and restrict the development of resistant bacteria. The addition of lithium is easily performed via the dispersant thus a production plant can transiently change to the lithium neutralised dispersant without any investment in production facilities or equipment.

The implementation of new preservation technologies such as alkanolamine 2-amino-2-methyl-1-propanol (AMP) exceeds expectations in respect to the properties of a preservative intended for use in WMD. In addition to the well known dispersing and buffering properties, AMP exhibits a triple action and stabilise the pH, the viscosity and the microbial contamination of WMD. The additional synergistic preservation performance of AMP combined with the prevalent biocidal formulations used in WMD, enables compilation of tailor-made preservation protocols for individual production sites and improves the flexibility in combining various biocidal agents whilst preserving the cost effectiveness.

Finally, the application of these findings revolutionises the consumption of biocidal agents in the WMD industry. A better understanding of the biochemical interactions between biocide-resistant microbes and the biocides is the basis to revisit the previous biocide strategies and permit the development of novel preservation strategies to effectively control bacterial contamination of white mineral dispersions in the future.

6.2 Outlook and Further Work

The microbial diversity of WMD elucidated in this work is primarily based on the characterisation of mixed bacterial populations. The assessment of microbial diversity and the discrimination of viable (metabolically active) from compromised or dead bacteria will be essential to evaluate the efficiency of biocide treatments in WMD. The increasing cost-effectiveness and rapidity in the development and application of new sequencing technologies such as deep amplicon sequencing certainly will revolutionise the molecular characterisation of microbial communities. At the same time, the real-time assessment of microbial contamination in respect to the metabolically activity population will allow optimisation and control of biocide dosage and thus save economic and environmental resources. However, it is important to emphasise the challenges of managing microbial contamination in large-scale industrial processes and the fact that WMD storage tanks are difficult to manage in respect to the continuous tank level fluctuations. Therefore, it can be postulated that the management of bacterial contamination in WMD involves five factors: (i) the species occurrence in mixed bacterial populations, (ii) the cell number and their metabolical activity, (iii) the concentration of "active" available biocidal compound, (iv) the product turnover as well as (v) the delivery and storage strategy.

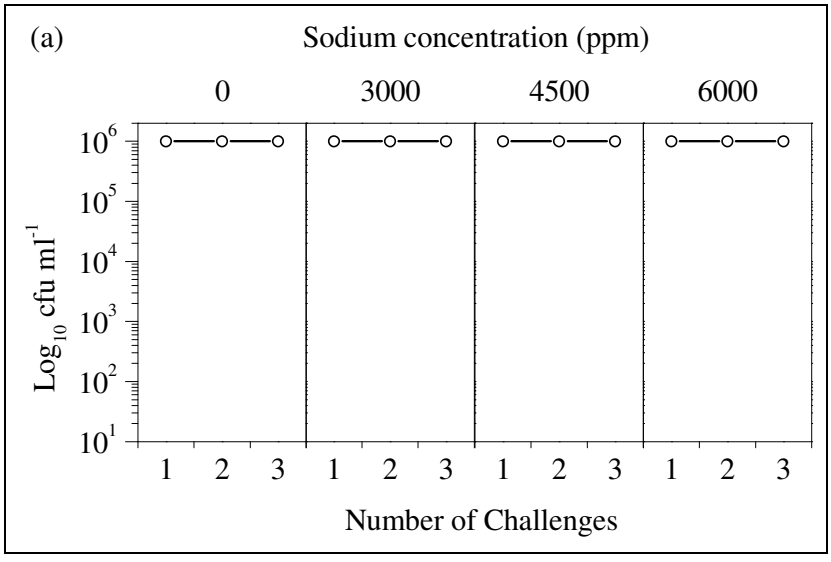
Furthermore, due to the small number of biocidal agents available and the limited antimicrobial performance in WMD, the research in the field of new preservation protocols and strategies needs to be intensified. Whether the enhancement of biocides or the use of multifunctional additives is the road to success is subject of future investigation also with regard to their implementation in large-scale industrial processes. The range of available alkanolamines to be tested for their versatile activity in WMD is numerous and other innovative approaches (e.g. antimicrobial enzymes and bacteriophages treatment) should not remain unexplored.

6.2.1 Biocide-free preservation of WMD

As explained above in section 5.2.1.4, the ability of bacteria to survive in alkaline environments such as calcium carbonate slurries involves the maintenance of the intracellular pH at levels to guarantee optimal functional and structural integrity of the cytoplasmic proteins (Padan*, et al.*, 2005). In bacterial cells, intracellular pH

regulation is driven by the uptake of protons (H^+) and simultaneous extrusion of Na^+ ions via $Na^+(Li^+)/H^+$-Antiporters and therefore a route that recycles the Na^+ ions is essential to maintain the antiporter activity. This process is better known as pH homeostasis. Consequently the concentration of both sodium and lithium in calcium carbonate slurries might negatively influence the pH homeostasis of bacteria and hence reduce the susceptibility of WMD to bacterial contamination. Therefore, the antimicrobial performance of sodium and lithium was investigated in calcium carbonate slurry.

In standard Hydrocarb 90 calcium carbonate slurry (H90-MgNa5050) increasing concentrations of sodium added in the form of sodium carbonate did not prevented bacterial contamination (Figure 74a). In contrast, the addition of 2000 ppm (a/l) sodium in the form of sodium carbonate to H90-gLi ground in the presence of lithium carbonate (1500 ppm lithium a/l dosed) prevented microbial contamination over three challenge cycles (Figure 74b) whereas no effect was observed in H90-MgLi5050 calcium carbonate slurry containing less lithium introduced via the dispersant (920 ppm a/l) (Figure 74c). Apparently the inherent magnesium concentration introduced via the dispersant had no influence on the preservation performance of sodium which rather depends on the concentration of lithium in the calcium carbonate slurry.



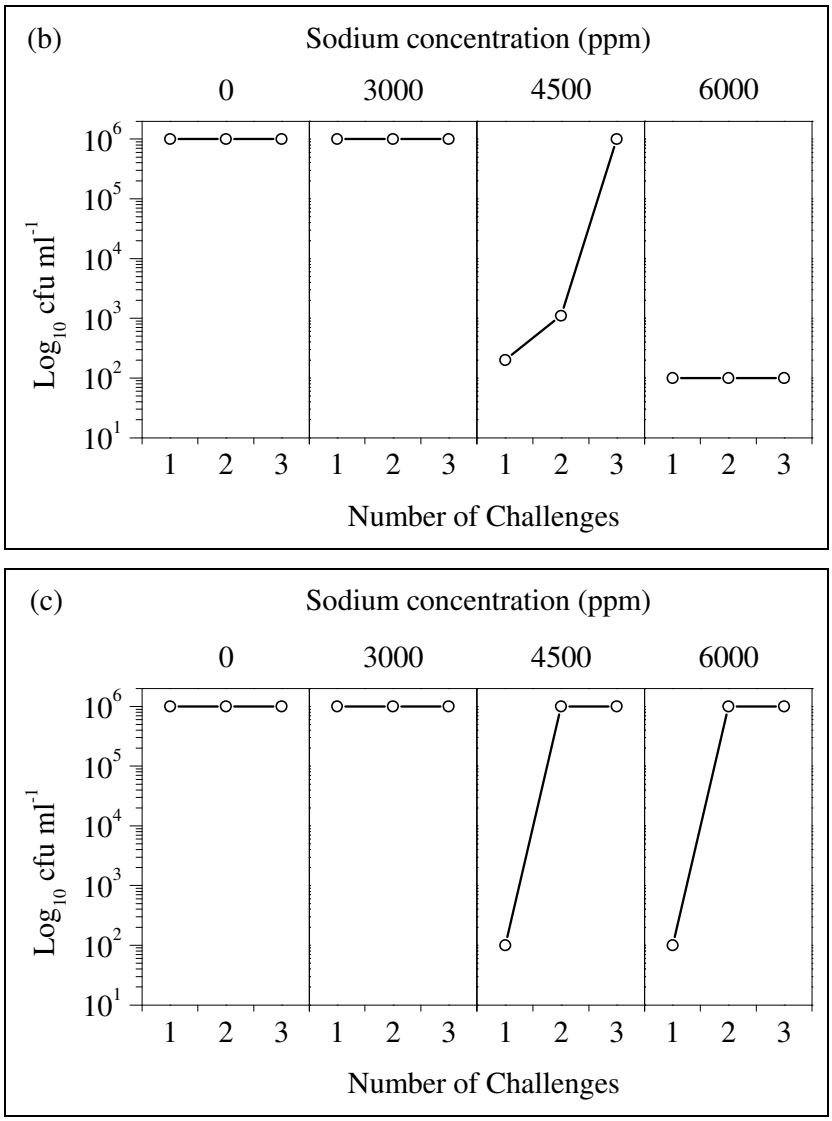


Figure 74. Preservation performance of sodium (supplemented in the form of sodium carbonate) in (a) Hydrocarb 90 standard calcium carbonate slurry (H90-MgNa5050), (b) H90-gLi calcium carbonate slurry ground in the presence of lithium carbonate (1500 ppm a/l lithium) and (c) H90-MgLi5050 calcium carbonate slurry (Li via dispersant: 920 ppm a/l). Samples were challenged with the resistant bacterial culture rGCM. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}. There were no more than three inoculations performed.

Moreover, the addition of both lithium and sodium via the carbonate salts to Hydrocarb 90 standard calcium carbonate slurry (H90-MgNa5050) indicated that 1050 ppm (a/l) lithium was required to prevent microbial growth in the Hydrocarb 90 slurry containing 6000 ppm (a/l) sodium (Figure 75).

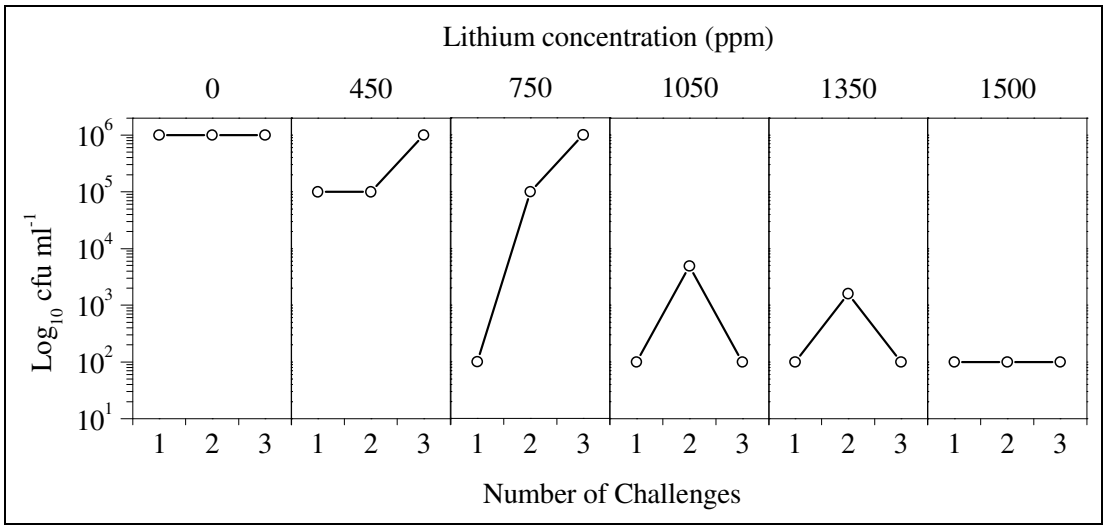


Figure 75. Preservation performance of increasing lithium concentration (added in form of lithium carbonate) in Hydrocarb 90 standard calcium carbonate slurry (H90-MgNa5050) supplemented with 2000 ppm (a/l) sodium in the form of sodium carbonate. Samples were challenged with the resistant bacterial culture rGCM. There were no more than three inoculations performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}.

Control trials with potassium instead of sodium showed similar findings when 1350 ppm (a/l) lithium and 6000 ppm potassium (a/l) were used. Additionally, in H90-Li slurry manufactured with 100% lithium-neutralised dispersant (1580 ppm a/l lithium dosed) a concentration of 4500 ppm (a/l) sodium or 2000 ppm (a/l) potassium were sufficient to achieve preservation. In this context it is important to point out that standard Hydrocarb 90 calcium carbonate slurry inherently contains a concentration of 930 ppm (a/l) sodium, 36 ppm (a/l) potassium and 21 ppm (a/l) magnesium in the aqueous phase which originates from dispersant neutralisation. Finally, both sodium and potassium in combination with lithium efficiently prevents the growth of microorganisms in calcium carbonate slurry. Furthermore, a shift of the pH to 10-10.3 was measured in all calcium carbonate slurries after the addition of sodium and lithium carbonate, therefore an influence of the pH on the microbial susceptibility of the slurries cannot be ruled out. This is the first description of a “biocide-free” self-preserving calcium carbonate slurry system which has recently been registered by a current patent application (Di Maiuta and Schwarzentruber, 2010). Whether this technology is applicable in the WMD field with the high amounts of ions causing an increase in conductivity so impairing the slurry down-stream processes is subject to future investigation.

The idea of preserving products, above all food, has been used for thousands of years (Gutierrez, *et al.*, 1995), e.g. desiccation or the addition of high amounts of osmotically active substances such as salts and sugars leading to a decrease of the water activity (a_w) so preserving the product from microbial spoilage (Russell, 2003a, Wijnker, *et al.*, 2006). A previous study has reported the inhibition of bacteria and yeast by 1% (10,000 ppm) sodium carbonate and attributes the antimicrobial activity to the pH increasing activity of the bicarbonate ion since equimolar preparations of sodium chloride do not show osmotic or sodium mediated inhibition (Corral, *et al.*, 1988). Other authors have noted an enhancement of the antimicrobial performance of agents such as ovotransferrin, lactoferrin and clavanins by sodium in the form of sodium carbonate or sodium chloride (Al-Nabulsi and Holley, 2006, Ko, *et al.*, 2008, Lee, *et al.*, 1997). Preservative-free (self-preserving) systems are well described for cosmetic formulations and result from the physiochemical composition of the formulations in relation to the high or low pH, low a_w value as well as the presence of chelating agents, surfactants, alcohols and multifunctional ingredients (Orth, 2010).

6.2.2 Resistance to AMP

A recent study has shown that ethanolamine can be used as a carbon and nitrogen source by various phylogenetically distinct bacteria (Tsoy, *et al.*, 2009) and due to the adaptive acclimatisation potential of bacteria in harsh environments the application of AMP to support the preservation of calcium carbonate slurry is not a final solution. Recently the bacterial strains *Bacillus cohnii* and *Ochrobactrum anthropi* have been isolated from calcium carbonate slurries preserved with 1350 ppm (a/l) AMP. In addition, challenge tests demonstrate that these strains are resistant to 3375 ppm (a/l) AMP in calcium carbonate slurries (2.5-fold in-use concentration). *Bacillus* strains are well known to be alkaliphilic and the strain *Bacillus cohnii* has been described to possess ornithine and aspartic acid instead of diaminopimelic acid in the cell wall (Spanka and Fritze, 1993). Therefore, it can be assumed that the modification of the cell wall might prevent the uptake of unprotonated AMP into the cytoplasm thus inhibiting the antimicrobial activity of AMP against *Bacillus cohnii*. The bacterium *Ochrobactrum anthropi* (former

*Achromobacte*r sp.) is a gram-negative rod-shaped bacillus which has been considered to cause opportunistic infections (Duran*, et al.*, 2009). A recent study has reported that *O. anthropi* was one of the main bacterial species found in metal working fluids which contains high amounts of alkanolamines such as AMP (Gilbert*, et al.*, 2009). Schwarzentruber (2003b) demonstrated that in calcium carbonate slurries *Bacillus* species cannot compete with the other bacterial species and their growth is impaired because of the dominance of the latter. Bacteria affiliated to both *Bacillus* and *Ochrobactrum* genera were previously found to be present in AMP-free calcium carbonate slurries (section 3.2.4.2 Table 18). Therefore, it can be assumed that the application of AMP in calcium carbonate slurries might significantly influence the microbial diversity of calcium carbonate slurries and cause a shift of the microbial communities toward alkaliphilic spore-forming bacillus species.

6.2.3 Antimicrobial performance of other amines

The antimicrobial performance of alkanolamines such as AMP was deduced from the primary amino group since the amine-less analogue MP did not show any activity. The alkaline polycationic compound polyethylenimine (PEI), the polymerisation product of the monomer ethylenimine, is available in two forms: linear PEI containing secondary amines only and branched PEI containing primary, secondary as well as tertiary amino groups. PEI has been described to exhibit extensive antimicrobial activity (Pasquier*, et al.*, 2008).

The antimicrobial performance of low molecular weight PEI (Mw 800 Da) was investigated in Hydrocarb 90 slurry and based on the results it was concluded that 3000 ppm (a/l) PEI are sufficient to preserve Hydrocarb 90 calcium carbonate slurry over three bacterial challenges with the biocide resistant culture rECM. Furthermore, analyses by means of the CellFacts II® revealed that PEI in calcium carbonate slurries caused lysis of the bacterial cells (Figure 76).

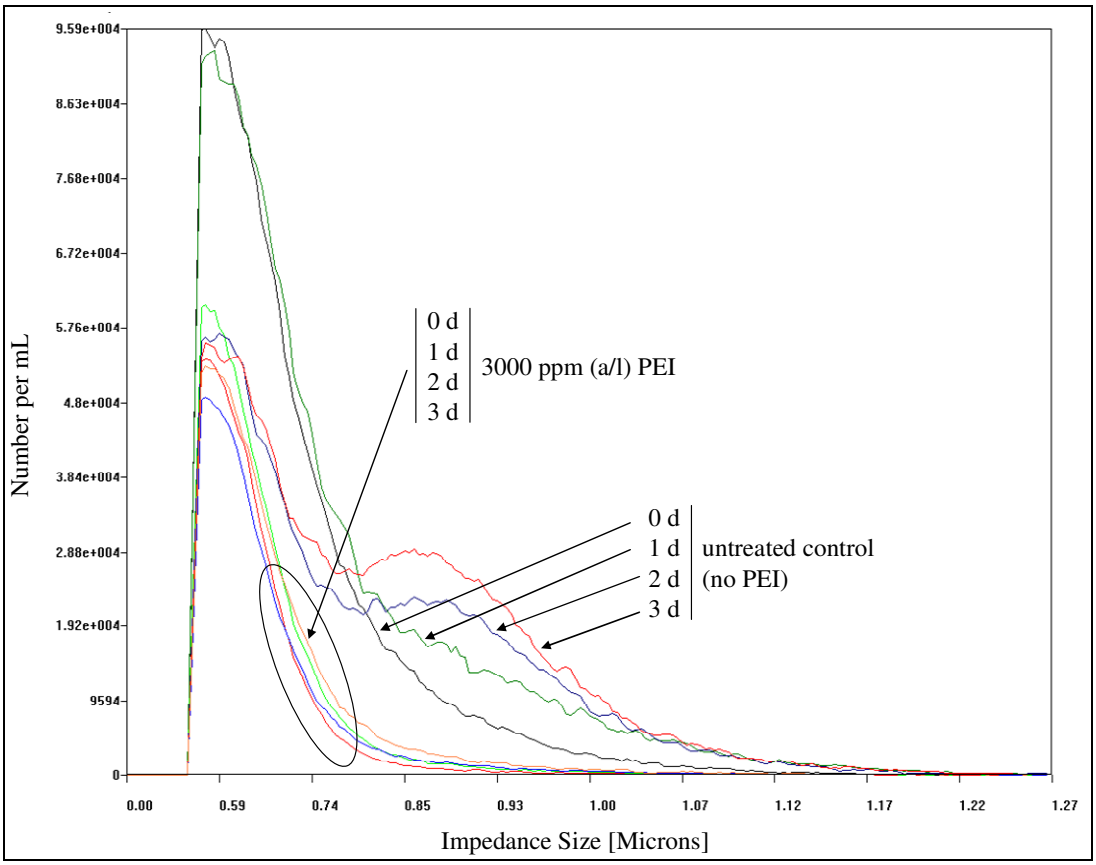


Figure 76. CellFacts II® profile of bacterial cells (rECM culture) in calcium carbonate slurry supplemented either with 3000 ppm (a/l) PEI or without compounds (no PEI control).

Other studies have noted that PEI is an effective permeabiliser of the outer membrane of gram-negative bacteria and involves the disruption of the membrane (Helander, *et al.*, 1998). Even though PEI has been demonstrated to sensitise bacterial cells to the lytic action of sodium dodecylsulfate, no inhibition of bacterial growth by PEI on its own has been observed (Helander, *et al.*, 1997). However, other authors have demonstrated a significant synergism of PEI in combination with various antibiotics (Khalil, *et al.*, 2008). So far, in calcium carbonate slurries a concentration of 1350 PEI (a/l) in the presence of 750 ppm of either EDDM/CMIT/MIT (c/l) or OPP (a/l) biocide resulted in a significant enhancement of the antimicrobial performance over the two bacterial challenges with the respective resistant bacteria. Nevertheless, the industrial implementation of PEI as a biocide enhancer in calcium carbonate slurry is at present unlikely because of the uncompetitive price with regard to other new preservation technologies (lithium / AMP).

CHAPTER 7

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CHAPTER 8

Publications