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Reducing biocide concentrations for preservation of water-based paints

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Foreword

The project *Reducing biocide concentrations for preservation of water-based paints* was funded by the Environmental Technology Development Program (MUDP) under the Ministry of Environment and Food of Denmark in 2014.

This progress report covers the work carried out during the period November 2014 - October 2017. The appendix of the report contains confidential information and must not be published in its current form.

The project was carried out as a collaboration between Flügger A/S and Danish Technological Institute (DTI) and was headed by Michael Vedel Wegener Kofoed.

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Abbreviations

BIT:	Benzisothiazolinone
BPR:	Biocidal Products Regulation
CFU:	Colony Forming Units
CMIT:	Chloromethylisothiazolinone
CLP:	Classification, Labeling and Packaging
DDAC:	Didecyl dimethyl ammonium chloride
Li^+:	Lithium ion
MIT:	Methylisothiazolinone
PT:	Product type under the Biocidal Products Regulation
PVA:	Vinylacetate-ethene
ZnPt:	Zinc Pyrithione

Sammenfatning og konklusion

Vandbaseret maling består af en række forskellige råvarer, hvor af mange af disse kan bruges som næring af både bakterier og svampe, som herved kan vokse i malingen. Mikroorganismerne tilføres til malingen under produktionen bl.a. igennem råvarer, og tilføres desuden ved brug af malingen. Hvis væksten af disse mikroorganismer ikke hæmmes fordærves malingen og kan ikke længere bruges; således er holdbarhed af maling uden konserveringsmiddel kun ca. 2 uger før den er synligt fordærvet. Da de fleste vand-baserede malinger forventes at have en holdbarhed på mere en 3 år, selv efter åbning, er konservering derfor en nødvendighed. Konservering i emballagen (såkaldt *in-can* konservering) opnås i dag ved at tilsætte biocider – aktive stoffer der skal dræbe de mikroorganismer der tilføres malingen.
Gruppen af isothiazolinoner tilhører en bredt anvendt gruppe af biocider, der udover maling også har været anvendt i en lang række forbrugerprodukter bl.a. rengøringsmidler, kosmetik og plejeprodukter. Isothiazolinonerne har dog i flere tilfælde vist sig at være allergifremkaldende og anvendelsen af dem er derfor blevet begrænset og kræver mærkning ved anvendelser over fastsatte koncentrationer. De negative sundhedseffekter har øget fokusset på isothiazolinoner og udgjort en stramning og reduktion af den anvendelige koncentration af disse stoffer, især hvis producenter skal kunne deklarere deres produkter ift. fx Blomsten og Astma Allergi. Der er derfor et fokus fra producenterne af maling på at reducere brugen af isothiazolinoner. Specielt methylisothiazolinon (MIT) ønskes at undgås pga. dens allergene effekter. Fokus for indeværende projekt har været at reducere brugen af isothiazolinoner med specifikt fokus på MIT. Antallet af alternative effektive biocider er meget begrænset og en direkte substitution er derfor ikke mulig, men kræver nye tilgange – og endda kombinationen af flere tilgange - for at reducere koncentrationen af anvendte isothiazolinoner. Projektet har undersøgt fire forskellige tilgange til at reducere brugen af isothiazolinoner til konservering af maling:

(1) **Komponentsubstitution, hvor komponenter, der kan fremme bakterievækst, identificeres og substitueres til stoffer der ikke stimulerer vækst.**
Undersøgelser viste at mange af de råvarer, der i dag benyttes er kontaminerede med mikroorganismer og stoffer der øger vækstforhold for mikroorganismer i den færdige maling. I løbet af projektet blev der udviklet metoder til at identificere vækstfremmende råvarer. Ud fra udvalgte komponenter var det således muligt at formulere en maling, der kunne konserveres ved langt lavere koncentrationer af biocid, end en standard maling. Forsøgene viste at substitution af få udvalgte komponenter kan have stor indflydelse på hvor modstandsdygtig en malingen er ift. fordærv.

(2) **Forbedret biocidal effekt, hvor forskellige biocider kombineres for derved at nedsætte det samlede behov for biocider – specielt methylisothiazolinon.**
Forsøg viste at man ved kombination af forskellige biocider kunne opnå en konserverende effekt, der var på linje med den der kunne opnås ved brug af methylisothiazolinon. Der blev også fundet effektive kombinationer helt uden isothiazolinoner, der potentielt vil kunne bruges til konservering i fremtidige malinger.
Forsøgene viste dog at der var en overraskende interaktion mellem biociderne og de benyttede råvarer. Visse råvarer viste således helt at modvirke biocideffekten. Valget af råvarer er derfor potentielt lige så vigtigt som valget og kombinationen af biocider.

(3) **Brug af ikke-regulerede konserveringsmetoder som vandaktivitet og pH.**
Et produkts vandaktivitet er kritisk for mikrobiel vækst og benyttes i flere fødevare som konserveringsmiddel. Vandaktivitet kan justeres ved enten at reducere indholdet af vand, eller ved at tilsætte stoffer, der påvirker koncentration af vand der er tilgængelig for mikroorganismer: Begge tilgange blev undersøgt i dette projekt: (a) Sænk-

ning af vandaktivt ved at fjerne vand og herved formulere en high-solids maling, viste at vandaktiviteten ikke kunne sænkes til et niveau der var hæmmende for bakterievækst, selv ved meget lave koncentrationer af vand. (b) Sænking af vandaktivitet ved brug af forskellige stoffer, viste at væksthastigheden potentielt kunne reduceres ved tilsætning af visse salte så som urea. Den nødvendige koncentration, der skal benyttes for helt at hæmme væksten er dog så høj at den vil gå ud over den færdige malings egenskaber, bl.a. ift. vaskbarhed.
Hævet pH viste at hæmme den bakterielle vækst og forøge malingens holdbarhed. Selv pH >11 var dog ikke nok til helt at dræbe bakterierne i malingen.

(4) **Procesudvikling, hvor produktionsmetoder og tilgange undersøges og optimeres for at opnå produkter med færre mikrobielle kontaminanter.**
En omfattende ombygning af produktionsfaciliteterne og indførsel af nye produktionsrutiner på Flüggers produktionsfaciliteter i Kolding, viste et klart fald i antallet af reklamationer. Ved gennemgang af systemerne blev kritiske punkter identificeret med det primære mål at fjerne overskydende maling fra rør og tanke og hermed reducere mulighed for mikrobiel vækst i systemerne. Igennem ombygning af rørsystemer, indførsel af udstyr og processer til rengøring og desinfektion, kunne antallet af produktreklamationer reduceres.

Projektets resultater har klarlagt nogle af de muligheder og barrierer der er for forsat at udvikle maling med en reduceret koncentration af biocider. På basis af disse resultater ses forsat fokus på både formulering og produktionsprocesser som en absolut nødvendighed for forsat at have mulighed for at reducere behovet for biocider til konservering af vandbaseret maling. Resultaterne viste således at råvarerne spiller en større rolle ift. både mikroorganismer og biocider end man traditionelt har antaget i malerbranchen. Det var således muligt at formulere en maling der er mere modstandsdygtig over for bakteriel vækst, ud fra viden om de brugte råvarer, og projektet viste således vigtigheden af at screene og nøje udvælge sine råvarer. Resultaterne viste endvidere også at råvarerne har indflydelse på mere en blot den bakterielle vækst, og at nogle af dem faktisk direkte reducerer effekten af det tilsatte biocid.
Interaktionen mellem bakterier, råvarer og biocider er derfor noget mere kompleks end først antaget, og fremtidig indsats vil fokusere på at klarlægge denne sammenhæng. Denne nye tilgang til formulering vil gå hånd-i-hånd med forsat fokus på forbedret proceshygiejne, hvor man kommer tættere på de hygiejnestandarder der ses inden for fødevareindustrien. Både formulering og produktion ses som hjørnesten i den forsatte udvikling af vandbaseret maling med reduceret biocidkoncentration.

Summary and conclusion

Water-based paint consists of various raw materials, where many of these function as feed to both bacteria and fungi allowing them to grow in paint. Microorganisms are added to the paint during the production, among others, through raw materials and also while applying the paint. If the growth of these microorganisms is not restricted, the paint is spoiled and may no longer be used; this way the life of the paint without a preservative is only approx. 2 weeks before it is visibly spoiled. Since most of the water-based paints are expected to have a life of more than 3 years, even after opening, the preservation is very important. Today, preservation in packaging (the so-called in-can preservation) is achieved by adding biocides – active substances that kill microorganisms that are added to the paint.

The group of isothiazolinones belongs to a widely used group of biocides that besides the paint have also been used in a variety of consumer products, among others, cleaning detergents, cosmetics and personal care products. However, in many cases the isothiazolinones have proven to be allergenic, and their usage is thus limited and requires labelling if used more than the specified concentrations. The negative health effects have increased the focus on isothiazolinones and have resulted in tightening and reduction of the applicable concentration of these substances, especially if the manufacturer must declare their products according to Danish ecolabels e.g. Blomsten and Astma Allergi. As a result, paint manufacturers make an effort to reduce the usage of isothiazolinenes. There is a special focus on the omission of methylisothiazolinon (MIT) due to its allergenic effects. The aim of this project has been to reduce the usage of *isothiazolinones with a special focus on* MIT. The number of alternative effective biocides is very limited, and a direct substitution is thus not possible. This requires new methods, even a combination of many approaches, in order to reduce the concentration of the applied isothiazolinones. Four different methods have been investigated in this project with an aim to reduce the usage of isothiazolinones for paint preservation:

(1) **Substitution of components, where components facilitating bacterial growth are identified and substituted with substances that do not stimulate growth.**
The tests showed that many of the raw materials used today are contaminated with microorganisms in the final paint product. During the project, several methods were developed for the identification of growth facilitating raw materials. Based on the selected components, it was also possible to formulate paint that could be preserved with a considerably lower concentration of biocides compared to standard paint.
Tests showed that the substitution og selected components may have a great impact on how resistant the paint is to spoilage.

(2) **Improved biocidal effect, where different biocides are combined with an aim to reduce the full need for biocides, especially methylisothiazolinon.**
Tests showed that the combination of different biocides could achieve a preservation effect similar to what could have been achieved using methylisothiazolinone. Other effective combinations without isothiazolinones were identified that could be potentially used for preservation of future paints. However, tests showed a surprising reaction between biocides and the used raw materials: certain raw materials completely counteracted the biocide effect. Hence, the choice of raw materials is potentially as important as the choice and combination of biocides.

(3) **Use of non-regulated preservation methods as water activity and pH.**
The water activity of a product is critical for microbial growth and is used as a preservative in many food products. Water activity can be regulated by either reducing

the water content, or by adding substances that affect the water concentration available for microorganisms. In this project, both methods were investigated: (a) lowering water activity by removing water and thereby formulating a high-solids paint showed that the water activity could not be lowered to a level that inhibited the bacterial growth, even at very low water concentrations; (b) lowering water activity by using different substances showed that the growth speed could be reduced by adding certain salts, for instance urea. The necessary concentration for stopping the bacterial growth completely is so high that it will have an impact on the properties of the produced paint, e.g. washability. An increased pH showed to inhibit the bacterial growth and increase the life of paint. Even a pH >11 was not enough to kill the bacteria in the paint entirely.

(4) **Process development, where production methods and approaches are investigated and optimized to achieve products with fewer microbial contaminants.** An extensive reconstruction of production facilities and introduction of new production routines at Flügger production facilities in Kolding showed a clear decrease in the number of product returns. A review of their systems helped to identify critical points, where the main aim was to remove excess paint from pipes and tanks thereby reducing the chances of microbial growth in systems. The reconstruction of pipe systems, introduction of equipment and processes for cleaning and disinfection helped to reduce the number of product returns.

The results of this project have established some of the opportunities and barriers that would help to maintain the ongoing focus on developing paint with a reduced concentration of biocides. On the basis of these results, a continuous focus will be on both formulation and production processes, as an absolute necessity to ensure the reduction of the need for biocides for the preservation of water-based paints.

Project results also showed that raw materials play a more significant role in relation to microorganisms and biocides than traditionally assumed in the paint industry. It was also possible to formulate a paint that is more resistant to bacterial growth, based on the knowledge of the used raw materials. Furthermore, the project emphasized the importance of screening and careful selection of the raw materials. The results also showed that raw materials have an impact on aspects other than bacterial growth, and in fact some of them directly reduce the effect of the added biocide. The interaction between bacteria, raw materials and biocides is thus more complex than initially presumed, which means that the future activities will focus on identifying this connection. The new approach to formulations will go hand-in-hand with the continuous effort to improve process hygiene, gradually moving closer to hygiene standards applicable in the food product industry. Both the formulation and production are seen as milestones for the continuous development of water-based paints with a reduced biocide concentration.

1. Introduction

1.1 Background

Water-based paint for in-door painting is a complex formulation of different components that gives the paint its physical abilities, e.g., color, adherence, viscosity, etc. Water-based paint consists of a number of primary components, which include both organic and inorganic substances: Fillers, binders, pigments, emulsifiers, solvent (water) and other additives.
One or more of these components can function as nutrition for microorganisms hereby allowing these microorganisms to grow in the paint. A water-based paint where microbial growth is not inhibited in any way can only be stored at room temperature for a few weeks before it is visibly contaminated by either fungi or bacteria. These contaminating microorganisms have several detrimental effects, resulting in changed viscosity, discoloration, odorous smell, etc., which make the paint unfit for use. For this reason, an important part of a water-based paint includes the addition of biocides – substances that will inhibit or kill the microorganisms, which contaminate the paint. Therefore, the addition of biocides are an important part of water-based paints in connection with their preservation during storage (*in-can* preservation).

Although the biocides used for preservation of the water-based paints are essential for product preservation, biocidal substances can have some unwanted toxic effects on human health. The effects depend on the concentration and exposure time, but range from harmful to skinto eliciting allergic reactions.

To protect users from the unwanted effects of these biocides, the number of biocides that can be used for in-can preservation is regulated by the European Biocidal Products Regulation. Biocides for in-can preservation are listed in the product type group PT6 - “Preservatives for products during storage”[1] on BPR (EU) 528/2012.

Furthermore, the use of these biocides is regulated by the CLP regulation (Classification, Labeling and Packaging) from the European Chemical Agency (ECHA), which requires manufacturers of products that contain hazardous compounds above certain limits to classify, label and package their products before introducing them on the market. Manufacturers of water-based paints, like Flügger A/S, are therefore required to label their products according to CLP when they contain approved biocides from PT6 above a certain concentration. Both the number of approved biocides and the concentration at which they can be used safely without trigger a undesirable labeling are constantly decreasing to a level where their effective for use as in-can preservation is now severely limited.

Consequently, manufacturers of products that require in-can preservation such as Flügger A/S, constantly have to develop new solutions that will reduce the need for problematic biocides while obtaining the same product quality and shelf-life. At the moment, no easy solution exists.

Water-based paints have a very long shelf-life, and therefore new solutions that inhibit microbial growth and spoilage have to be very effective. Products from Flügger do not claim to have a specific shelf-life, but it is expected that they can be stored for at least 3 years. In addition, the products should be able to resist microbial spoilage after opening, use and subsequent storage of any residual volume of water-based paint. In many cases, the complaints that Flügger has accepted could be due to microbial spoilage after opening of the can that in reality could be more than 3 years old. The requirements to future formulations of paint and biocides are therefore rigorous and stress the need for an effective inhibition of microbial growth in the products through improved biocide systems, hygiejnic production and product formulations.

[1] https://echa.europa.eu/en/regulations/biocidal-products-regulation/product-types

The aim of the project called *Reduced Biocides in Water-based Paint* was to reduce the need for and use of problematic biocides for in-can preservation of water-based paint. The project was established because of new limits for CLP labeling of products containing a number of biocides of the group isothiazolinones (ratified in June 2015). The group of isothiazolinones mostly used for in-can preservation, includes MIT (Methylisothiazolinone), BIT (Benzisothiazolinone) and CMIT (Chloromethylisothiazolinone), which after June 2015 had to be labeled as either ""May produce an allergic reaction" (EUH208) or "May cause an allergic skin reaction" (H317) at a lower concentration (Table 1). The isothiazolinones are an effective group of biocides, which today are used in a wide range of consumer products. Especially the use of MIT is widespread within, e.g., paints, cosmetics, wet wipes and lubricants[2]. The all-round exposure to MIT from different products isproblematic as it increases the consumer's exposure to this biocide and is now known to be the cause of allergic skin reactions.

Table 1. Present classification according to CLP for labeling of products containing the biocides BIT, MIT and CMIT/MIT.

Active	Skin sensitizer, H317, specific concentration limit	EUH208 labelling, specific concentration limit
BIT (2634-33-5)	500 ppm	50 ppm
CMIT/MIT (55965-84-9)	15 ppm	1.5 ppm
DCOIT* (64359-81-5)	250 ppm	25 ppm
MIT* (2682-20-4)	1000 ppm	100 ppm
OIT (26530-20-1)	500 ppm	50 ppm

*Self classification only

The project focused on reducing the need for biocides - especially MIT - to below the limit requiring EUH208 labeling according to the CLP concentration limits, and also on investigating methods that completely would exclude the need for biocides without compromising the products in-can stability, quality and function.

The methods include substitution of components, which are prone to fuel microbial growth, the combination of biocides and other substances, and process optimization at the production facility at Kolding. The different approaches are addressed in different work packages (WPs) of the project (Figure 1).

[2] Sie Woldum Tordrup et al. *Kortlægning og eksponeringsvurdering af methylisothiazolinon i forbrugerprodukter*. Kortlægning af kemiske stoffer i forbrugerprodukter nr. 134, 2015. The Danish Environmental Protection Agency.

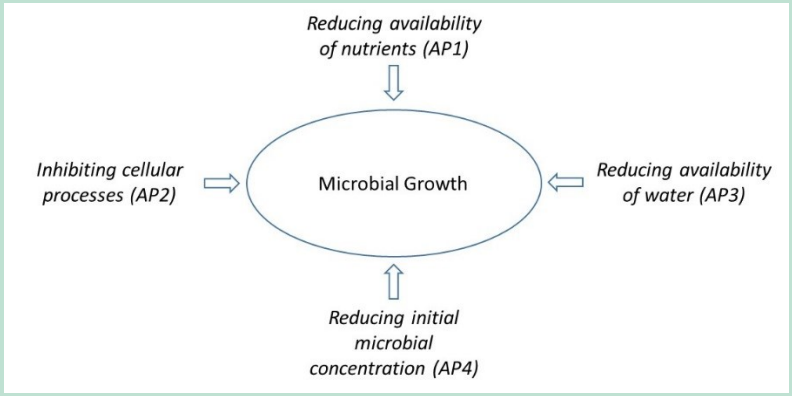


Figure 1. Illustration of the project's holistic approach where mulitple "pressure points" are applied to inhibit microbial growth.

Any of these approaches will be sufficient in themselves for quenching most microbial activity, e.g., complete inhibition of cellular growth by adding very high concentrations of biocide or reducing water activity (water availability) to below 0.6. However, a one-sided approach would potentially challenge product performance and handling and could also have undesired toxicological effects.

Therefore, the **hypothesis** of this project is that a multiphasic approach where microbial growth is challenged on multiple fronts is more effective and will result in the need for lower concentrations of biocide, than a one-sided approach where focus is on only one of these parameters.

2. Substitution of components as the means for reducing microbial growth in water-based paints

In the following chapter, the methods used for component substitution are described. Firstly, microorganisms were isolated and identified from contaminated paints. Different strings of fungus, and Gram-positive and Gram-negative bacteria were identified and it was determined, which bacterial reference culture should be used for the bacterial experiments.

The experiments disclosed that some of the components promoted growth. The growth was either due to the components themselves or impurities in the components. Focus was therefore on developing methods for quantitative analysis of the bacterial growth potential in the water-based paint and its components. Components such as fillers were in focus. The components that were identified to contribute to bacterial growth in the paint were evaluated and if possible removed to limit the growth in the optimized paint recipe.

2.1 Introduction

Most microorganisms that contaminate paint are chemoorganotrophic microorganisms – also known as heterotrophs. They degrade organic matter to obtain nutrients and energy to grow and multiply.

Although the rate of degradation may vary, microorganisms can degrade most organic molecules from simple fatty acids, large complex polymers like cellulose, or complex aromatic structures like polyaromatic hydrocarbons.

Paints consist of a complex mixture of organic and inorganic components where some of them will fuel microbial growth. The compounds can be divided into macro- and micronutrients, depending on whether they are required in large amounts or only in trace amounts.
Table 2 lists a range of different micronutrients known in microorganisms. Some of the listed metals are only needed in specific microorganisms.

Table 2. Macronutrients and micronutrients (trace elements) needed by microorganisms (Madigan et al., 2008, Chapt 5, 12th edt).

Macronutrients	Micronutrients
Carbon (C)*	Boron (B)
Hydrogen (H)*	Chromium (Cr)
Oxygen (O)*	Cobalt (Co)
Nitrogen (N)*	Copper (Cu)

Phosphorous (P)*	Manganese (Mn)
Sulfur (S)*	Molybdenum (Mo)
Potassium (P)	Nickel (Ni)
Magnesium (Mg)	Selenium (Se)*
Sodium (Na)	Tungsten (W)
Calcium (Ca)	Vanadium (V)
Iron (Fe)	Zinc (Zn)

* = Essential for all microorganisms

The two most important nutrients are carbon and nitrogen, constituting 50% and 12%, respectively, of the bacterial dry weight. Phosphorous and sulphur are also classified as macronutrients and constitute an important part of the microbial cell structures (Madigan et al., 2008, Chap 5, 12th Ed).

As listed in Table 3, carbon, hydrogen, oxygen, nitrogen, phosphorous and selenium are considered essential for growth of all microorganisms. Therefore, it is obvious to try to remove them when trying to limit bacterial growth. Unfortunately, these elements are also the main constituents of most chemically and biologically relevant compounds, including components in water-based paint (Table 3).

Table 3. Essential macronutrients and their sources in paints, based on information from suppliers.

Macro- and micronutrient	Main sources in water-based paints
Carbon (C) Hydrogen (H) Oxygen (O)	Organic molecules like polymers, biocides, surfactants, etc.
Nitrogen (N)	Organic polymers, biocides, surfactants, part of production process (NH3)
Phosphorous (P)	Chelator, part of production process of polymers as a surfactant (polymerization)
Sulfur (S)	Biocides, part of production process of polymers as a surfactant (polymerization)
Selenium (Se)	Water, part of production process

Carbon, hydrogen and oxygen are part of many organic polymers, e.g., cellulose-based thickers and acrylate-based polymers. Nitrogen is also included in polymers such as polyurethane, and in biocides such as BIT, CMIT and MIT, together with sulfur. Phosphorous is used as chelator in some paints.

Carbon, hydrogen and oxygen are ubiquitous in all molecules and cannot be substituted. At the most, they can be modified to polymers that are less prone to microbial degradation. This is the case for several of the used polymers such as the vinyl acetate polymers and acrylate-based polymers.

The challenge is that N,S and P is widely used for production of chemicals needed to obtain required properties of the paint and the content in the paint is sufficient for bacterial growth.. The production processes and the precise composition of the components are in many cases the proprietary right of each chemical supplier and are therefore not available.

Although selenium is not a direct part of the components used in paints, it is used in a wide range of chemical processes. The main source of selenium is probably one of the main ingredients in water-based paint – namely water.

In order to limit microbial growth it is not necessary to remove all constituents that fuel growth. In principle, only one limiting factor has to be removed. The principle was originally developed for agricultural science and is called *The Law of the Minimum* (or Liebigs Law of the Minimum). It states that "*the yield of any crop always depends on that nutritive constituent that is present in minimum amount*" (Hooker, 1917).

In this phase of the project the principle will be used to develop a paint in which growth conditions are severely hampered by removing a single nutrient.

Today, Flügger produces a wide range of products. As a reference model to water-based paints, a PVA based mat wall paint formulation was developed for this project.

2.2 Isolation and identification of microorganisms from contaminated products

A small selection of white water-based paints contaminated with either bacteria or fungi were retrieved from Flügger production facilities in Bollebygd (Sweden) and Kolding (Denmark). These paints were analysed to determine the bacteria or fungi that commonly are found in contaminated paint. Examples of contaminated paint are shown in Figure 2.

Figure 2. Water-based paints contaminated with microorganisms. Visible growth, discoloration and/or malodour are observed as contamination.

Microorganisms were isolated by using traditional laboratory media as well as customized agar plates based on the PVA based mat wall paint. Traditional agar plates contain a range of easy degradable components, which are very different from the ones present in a water-based paint. To isolate microorganisms that were actually able to grow on components in water-based paint, the fabricated agar plates were based on water-based paint.

Initial isolation took place by using regular PVA based mat wall paint adapted into agar plates by addition of agar. Only limited growth was obtained on the PVA mat wall paint-based agar plates (Figure 3, left). Microscopy of the bacterial growth from these agar plates revealed the presence of rod-shaped microorganisms – probably bacteria (data not shown).

Figure 3. Microbial growth on agar plates based on PVA based mat wall paint including biocides. Limited growth could only be observed (left). Microbial growth on agar plates without biocide (right).

Figure 3. Microbial growth on agar plates based on PVA based mat wall paint including biocides. Limited growth could only be observed (left). Microbial growth on agar plates without biocide (right).

Although autoclaving was expected to deactivate MIT, the limited growth indicates that the added biocides in the PVA based mat wall paintagar plates were deactivated by autoclaving. Agar plates should be prepared with customized raw materials without added biocides for preservation of raw materials.

Therefore, subsequent isolation took place by using materials without the biocides normally added by suppliers for preservation of raw materials (growth medium, PVA based mat wall paint, Table 4). In addition to the use of the customized PVA based mat wall paint agar, bacteria and fungi were isolated on traditional laboratory growth media Plate Count Agar (PCA) and Malt Extract Agar (MEA).

The contaminated paints were diluted in a sterile physiological saltwater solution and streaked onto the different agar plates.

After growth at room temperature, visible growth could be identified on the agar plate (Figure 4, right). DNA was extracted from isolated colonies from agar plates based on PVA based mat wall paint, PCA and MEA. The isolated colonies were identified by subsequent molecular biological analysis by DNA-sequencing using primers specific for either bacteria or fungi.

Results of the DNA-based identification of isolated microorganisms are shown below. Nearest relatives to the retrieved DNA-sequences were identified using the Basic Local Alignment Search Tool[3]. Table 4 shows the nearest related organism identified from DNA sequence analysis of the genes encoding ribosomal RNA of 16S rRNA (Bacteria) and 18S rRNA (Fungi). Although the names of the nearest related species are listed, identification of the isolated microorganisms could only be done reliably to genus level.

Table 4. DNA based identification of the isolated microorganisms found in the paints. MP = PVA based mat wall paint, MP2 = Mat wall paint

Colony	Nearest related organism	Sequence identity	Organism type	Growth medium	Paint	Production facility
2	Enterococcus casseliflavus	99%	Bacteria	PCA	Paint B	Kolding
3	Enterococcus casseliflavus	100%	Bacteria	PCA	Paint C	Kolding
4	Pseudomonas aeruginosa	100%	Bacteria	MP	Paint C	Kolding
5	Enterococcus casseliflavus	100%	Bacteria	PCA	Paint D	Kolding
6	Pseudomonas sp.	99%	Bacteria	MP	Paint D	Kolding
8	Pseudomonas aeruginosa	99%	Bacteria	PCA	Paint D	Kolding
10	Pseudomonas aeruginosa	99%	Bacteria	MP	Paint D	Kolding
13	Pseudomonas sp.	99%	Bacteria	MP	MP	Kolding
14	Pseudomonas putida	100%	Bacteria	PCA	MP2	Kolding
15	Pseudomonas putida	100%	Bacteria	MP	MP2	Kolding
17	Citrobacter freundii	100%	Bacteria	MP	PVA	Kolding
19	Penicillium brevicompactum	99%	Fungi	MEA	Base 1	Kolding
20	Acremonium sp. / Sarocladium sp.	100%	Fungi	MEA	MP3	Kolding

[a] Isolate used for testing of new paint formulations

[3] blast.ncbi.nlm.nih.gov

Analysis showed that different organisms could be isolated from the same paint. It was furthermore found that some of the same organisms could be isolated on traditional agar-based growth-medium (PCA) and on paint-based medium (PVA based).

Although the paints are contaminated by different organisms, both fungi and Gram-positive and Gram-negative organisms, bacteria of the genus Pseudomonas, were isolated from several of the paints. As test strain for further testing in the project, we decided that *Pseudomonas aeruginosa* was a suitable test-strain for testing the effect of nutrients, salt-tolerance and biocidal efficacy in the subsequent studies and experiments. Strains of *Pseudomonas aeruginosa* constitute a metabolically versatile group of microorganisms known for their resistance towards different biocides (Paulus 2008 p. 7). In addition to its resistance to biocides, a test organism should be able to grow on the components present in a water-based paint. *Pseudomonas aeruginosa* DSM1253, a test strain used for plastic deterioration, was chosen for its potential ability to degrade long complex polymers. Therefore, it was expected to be able to grow on different components that were present in water-based paint.

2.3 Identification of macronutrients

2.3.1 Growth experiments in paint-based matrices without key nutrients

Based on the recipe for PVA based mat wall paint, different compounds were selected and tested for their growth enhancing ability. To this end, a range of paints were designed and produced with and without biocides. Special raw materials without biocide normally added by suppliers for preservation were furthermore procured and used for test paint production.

For further growth studies, test paints without biocides CMIT and MIT were chosen. Tests were made with:

(1) MP paint including all components in the PVA based mat wall paint (MP).
(2) MP NH3" without ammonia, a dominant source of nitrogen in the MP paint.
(3) MP Phosphor", without the phosphor-based chelator in the MP paint.
(4) MP CNP" without ammonia, phosphor-based chelator, antifoaming agent and thickener in the MP paint.

Growth on PCA plates was used as positive control for viability of the bacterial inoculum. The agar plates were inoculated by using microorganisms isolated from contaminated paints (Contaminant 4 & 8) and *Pseudomonas aeruginosa DSM1253.*

The effects of omitting nutrients were evaluated by rating colony size as a proxy for growth potential after 4 days at room temperature. Evaluation took place as a blind test with no prior knowledge on the composition of the different media (Table 5).

Table 5. Growth experiments on paint-based growth media without selected macronutrients. MP = PVA based mat wall paint. Growth was ranked by a score from 1 - 4: 4 Largest colonies/fastest growth, 1: Smallest colonies/reduced growth. PCA medium was used as positive growth control.

Growth medium	Contaminant 4	Contaminant 8	*P. aeruginosa*
PCA	+	+	+
MP	4	4	4
MP – NH3	3	3	3
MP – Phosphor	1	1	1
MP – CNP	1	1	1

The evaluation was very difficult and should only be considered as semi-quantitative. Nevertheless, the results showed similar effects on the different growth media with all three test organisms. The difference between MP - Phosphor and MP - CNP was considered to be negligible.

A repetition of the tests, using only *P. aeruginosa* DSM1253 as test organisms, showed a similar result (Figure 4).

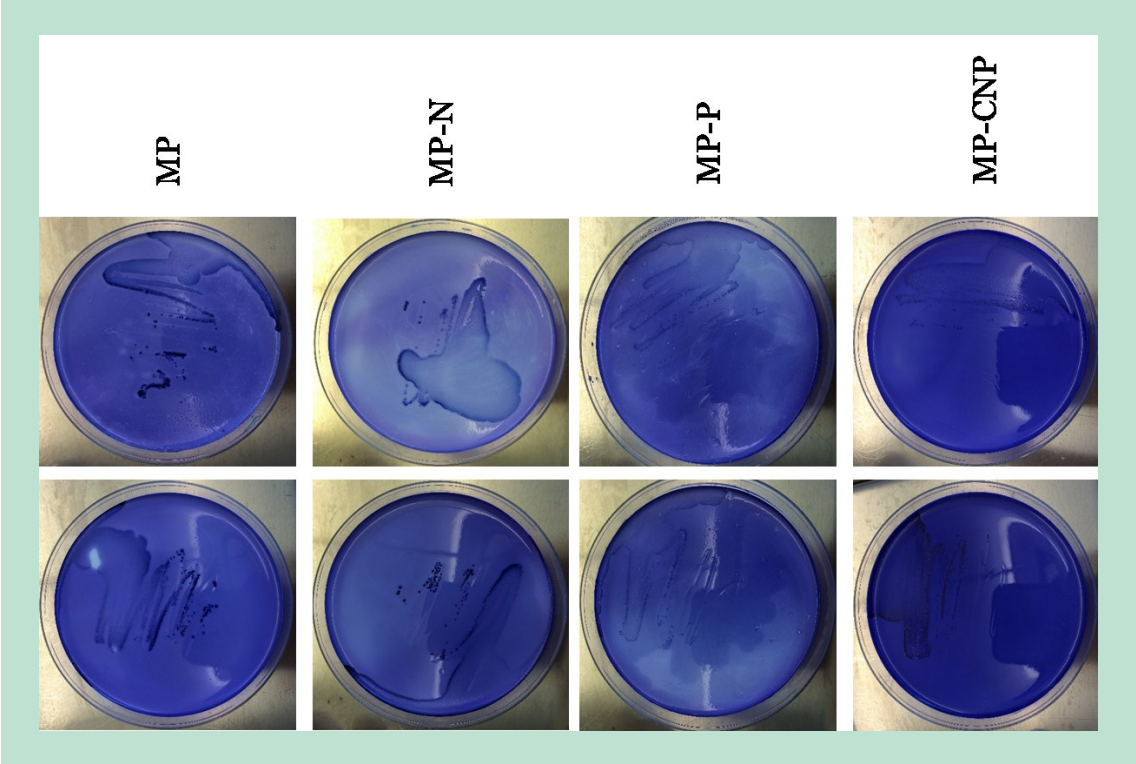


Figure 4. Growth experiments on paint-based growth media without selected macro-nutrients. The media were inoculated with *Pseudomonas aeruginosa* DSM1253, and subsequently they were stained by using methylene blue.

Although the removal of a phosphate-based chelator reduced growth of *P. aeruginosa* DSM1253 and the two isolated Pseudomonades (isolates 4 & 8 in Table 4), growth could still be observed. Furthermore, the non-preserved paint used as growth medium was spoiled by fungal growth after having been stored at room temperature. Therefore, the phosphor chelator is not the only source of phosphor in the paint.

A further surprising result was that the pure agar used to solidify the agar plates supported growth of *P. aeruginosa* DSM1253 on agar plates based on only demineralised water (dH_2O) and pure agar. The agar thus was shown to contain nutrients like phosphor which could support a small amount of growth. Further experiments showed the same results when using ultra-pure agarose as solidifying agent (data not shown). Although limited growth was observed due to these impurities in the agar, the difference in growth with and without the phosphor chelator show that it enhances growth of *P. aeruginosa* DSM1253 and isolate 4 & 8. Due to the challenges with the agar-based method, an alternative test method was developed for testing of bacterial growth on different raw materials (2.4.2).

2.4 Development of a new water-based paint with low nutritional value

Although the removal of single components did reduce the growth rate of bacteria in the PVA based mat wall paint, it did not inhibit growth completely. As a second approach to the development a new water-based paint with low(er) nutritional value, components were screened to form a new formulation based on raw materials, which have low impact on the concentration of nutrients and initial concentration of microorganisms in the paint.

2.4.1 Contamination in raw materials

Many of the raw materials and components used in the formulation of water-based paint either contain biocides to avoid spoilage during storage or are stored as solid powders, which do not require the addition of biocides. Many of these solid raw materials are produced by open mining, which can result in contamination with organic materials and microorganisms. These contaminating organics and organisms could function as nutrition for bacteria in the formulated paint. Therefore, the contamination level of different raw materials was studied.

The number of microorganisms was quantified by plating on agar plates for bacteria (PCA) and fungi (MEA). The number of microorganisms was quantified in dry materials directly spread on agar plates and in materials mixed with water to quantify the number of microorganisms released after mixing. The dry materials were tested by spreading 0.25 g of raw material on the agar plate. For the materials mixed with water, 1 g of raw material was mixed with 3 ml of water and 50 µl was spread on agar plates. The microbial growth on the agar plates was evaluated after 4 days of incubation. The results can be seen in Table 6.

Table 6. Contamination in raw materials in dry and wet form. Green: Low concentration of microorganisms; Yellow: Medium concentration of microorganisms; Red: High concentration of microorganisms; Grey: Very high concentration of microorganisms.

Type of raw material	PCA	PCA washed	Malt	Malt washed	Total evaluation	Troy's evaluation in relation to bacteria	Troy's evaluation in relation to fungi
CaCO3 A	●0	●0	●0	●0	●0	●0	0
Silicate A	●0	●0	●0	●0	●0		
TiO2 A	●0	●1	●0	●0	●0.3		
CaCO3 B	●1	●0	●1	●1	●0.5		
Dolomite A	●1	●0	●1	●1	●0.5		
Pigment A	●1	●1	●0	●0	●0.8		
CaCO3 C	●1	●1	●0	●0	●0.8		
Kaolin A	●0	●1	●2	●2	●0.8		
Talc A	●2	●2	●0	●0	●1.3	●2	●1
CaCO3 D	●3	●1	●2	●2	●1.8	●2	●1
Talc B	●3	●2	●2	●2	●1.8		
Talc C	●2	●3	●2	●2	●2.0		
SiO2 B	●3	●2	●2	●2	●2.0	●2	●0
Talc D	●3	●1	●4	●4	●2.3		
SiO2 C	●2	●4	●2	●2	●2.5	●3	●2
Dolomite B	●3	●2	●4	●4	●2.8		
Talc E	●4	●4	●4	●4	●4.0		
Thickener A						●0	●0
Thickener B						●0	●0
Dispersion agent A						●0	●0
Thickener C						●4	●2
Thickener D						●0	●1
Nepheline syenite						●1	●1
Dolomite C						●2	●1

The concentration of microorganisms varied between products. The same raw materials from different manufacturers had different contamination levels, e.g., when combinening different raw materials sources of $CaCO_3$, dolomite and talcum. For some of the raw materials, mixing with water released more microorganisms. Specifically Silicate C showed that the presence of

water had an impact on the release of bacteria, as there was a significant growth of bacteria after water addition.

2.4.2 Raw materials supporting growth

The concentration of microorganisms in the raw materials is part of the selection process for selecting the optimal raw materials. Although the initial concentration of microorganisms indicates if a certain raw material is contaminated, but it does not incidate if the component will support growth of added microorganisms. For these tests, a list of likely candidates for the formulation of a new water-based paint was selected and tested for their potential to support bacterial growth (Table 7).

Table 7. Candidates for further development of new paint system.

Type of raw material	Function	Conc (%)
Calcium Carbonate A	Filler	15
Magnesium Silicate A	Filler	15
Silicium oxide B	Filler	5
Kaolin A	Filler	10
Dispersion Agent B	Dispersant	0.5
Dispersion Agent C	Dispersant	0.5
Mineral oil	Antifoam agent	0.5
Co-polymer A	Antifoam agent	1
Thickener E	Anionic thickener	1
Thickener F	Nonionic thickener	2
Thickener G	Swelling thickener	2

Experiments were conducted to test which components support microbial growth:
Cells of *P. aeruginosa* DSM1253 were added to aqueous suspensions of different components. Before addition, cells were washed and resuspended in sterile saline solution (0.9%) to remove excess nutrients to ensure that growth was only supported by the added raw materials and not by the growth medium in which the bacteria were grown. Sterile tap water inoculated with *P. aeruginosa* was used as reference.
After incubation for two days at room temperature, growth was analyzed on agar plates. Results are shown in Table 8. '+' denotes growth of the added bacteria, '-' denotes survival of some of the added bacteria and "0" denotes complete absence of bacteria, showing bacterial death during incubation. The lack of growth (0) or very limited growth (-) in the liquid components could either be due to the presence of biocides added by the suppliers, or due to the fact that the liquid components are added in very small concentrations (0.5-2%), which only support very limited growth.

Components like silicium oxide, magnesium silicate as well as the tested anionic surfactant supported growth. That these components support growth indicate that they contain sufficient nutrients – both a carbon source as well as macronutrients like nitrogen and phospor. These nutrients could be a result of impurities in the components as some of these are procured through open mines, which makes it very likely that they contain contaminants. Micronutrients such as essential metals could also be added through the use of tap water. No bacteria could be detected in tap water (detection-limit, <1 CFU/ml).

Table 8. Growth of *P. aeruginosa* in liquid suspensions of different components used in water-based paint. The components were added in the same concentrations as used in the formulation of water-based paint. '+' denotes growth of the added *P. aeruginosa*; '-'

denotes detection of few bacteria equal or less than the original inoculum; '0' denotes no bacteria detected.

Type of raw material	Function	Concentration (%)	Added amount to 0.025 L water	Tube 1 (Growth)	Tube 2 (Growth)
CaCO3 A	Filler	15	3.75	0	0
Talc A	Filler	15	3.75	+	+
Silicium oxide B	Filler	5	1.25	+	+
Kaolin A	Filler	10	2.5	+	+
Dispersion Agent B	Dispersant	0.5	0.125	+	+
Dispersion Agent C	Dispersant	0.5	0.125	-	-
Mineral oil	Antifoam agent	0.5	0.125	-	-
Co-polymer A	Antifoam agent	1	0.25	0	0
Thickener E	Anionic thickener	1	0.25	0	0
Thickener F	Nonionic thickener	2	0.5	-	+
Thickener C	Swelling thickener	2	0.5	+	+
Tap water				0	0

The presence of growth supporting nutrients in many of the tested raw materials makes the production of a water-based paint with low nutritional value challenging. It was evaluated that it will be difficult to procure components of sufficient purity for use in industrial scale production when raw materials are produced through processes like open mining. As an alternative, the test platform used here makes it possible to screen future raw materials to select the best candidates for formulation.

Currently, it is not possible to test the contamination between different batches as they are not labeled with batch numbers from the suppliers. Routine screening of raw materials will reveal whether the quality of raw materials differ over time. Future efforts should focus on track and trace of raw materials to ensure their constant high quality.

2.4.3 Quantification of organic residues in raw materials

Growth-based tests can be laborious and time-consuming. Alternative approaches were tested to evaluate the growth potential of different raw materials and to quantify the concentration of organic material present in the materials that support growth. Two analytical approaches were tested: (1) Chemical Oxygen Demand (COD) and (2) Volatile Solid (VS) analysis; these standard methods are used to estimate the concentration of organic biodegradable material in waste water and biogas slurry, respectively.

Chemical Oxygen Demand: The method relies on chemical oxidation and subsequent spectrophotometric analysis. Although the method was tested with and without centrifugation to optimize the method for the solid particles, no reliable results could be obtained.

Volatile Solids: The mass of the fillers was determined before and after incineration at 550°C, and the relative loss of mass was calculated. The loss of mass will be an estimate of the concentration of organic material.

The incinerated components were used for growth experiments as described above. In Table 9, the results of weight loss and subsequent growth of the added *P. aeruginosa* are shown. The modified Smectite has the highest weight loss of the components and is an organic polymer. The fillers loose between 0.32-7.08% of the total mass, indicating that this is the concentration of organic substrate and impurities in these fillers. Not all the organic material is necessarily present as organic impurities. Some fillers are coated with different chemicals to ease their dispersion during production. These chemicals could potentially function as growth medium for microorganisms in the paint.

Incineration removes the basis for growth in two of the four components. Surprisingly, incinerated components such as silicium oxide and calcinated aluminum silicate still support growth even after incineration, which should have removed most of the organic substrate. Therefore, incineration at 550°C is not sufficient to completely remove the organic material supporting growth of the added microorganisms. That is why VS (and COD) analysis is not a suitable method for evaluation of the growth potential for microbial growth.

Table 9. Results of weight loss and growth of *P. aeruginosa* in a suspension of the incinerated components in sterile tap water.

Filler material	% loss	Growth before 550°C	Growth after 550°C
SiO_2 B	7.08	+	+
Talc A	0.45	+	0
Kaolin A	0.32	+	+
$CaCO_3$ A	2.47	0	0
Thickner C	52.2	+	0

2.4.4 Influence of components on biocide activity

2.4.4.1 General biocide mixture

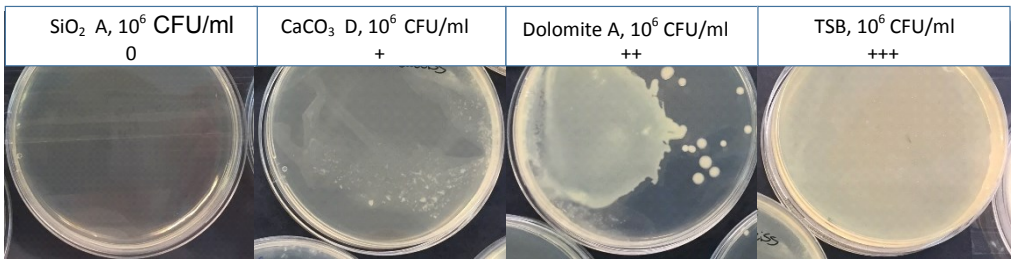
The impurities of the components can have other potential effects than supporting microbial growth. Organic material could potentially interact with the added biocides and thereby lower their biocidal effect. From the previous experiments, especially fillers have been identified as a group that might contain impurities - impurities which could potentially interact with added biocides.

To test if selected fillers interact with biocides, a test method was developed. Biocides were incubated with selected fillers, and the inhibitory effect of the supernatant was subsequently tested on the test strain *P. aeruginosa* DSM1253.

Initially, fillers were incubated with a mixture of BIT and an amine. To test the effect of selected fillers on the biocide activity, these fillers were incubated on a rotary shaker with sterile filtered tap water and the general biocide mixture for more than 48 hours. As a reference for pure biocide activity without the effect of the components, the general biocide mixture was added to sterile tap water with no other amendments and incubated along with suspensions. The general biocide mixture contains both an amine and BIT and was added in concentrations of 2 ppm BIT and 1 ppm amine.
After incubation the supernatant was removed and mixed with growth medium and with different concentrations of active culture of *P. aeruginosa* to a total biocide theoretic concentration of 1 ppm BIT and 1 ppm amine . To test two different levels of bioburden, cell-cultures were added to a concentration of 10^6 cells/ml or 10^8 cells/ml. The inhibitory effect of the added biocide was evaluated by measuring bacterial growth during overnight incubation (37°C). Bacterial growth was measured by optical density using a plate reader (Varioscan LUX Plate reader, Thermo Scientific). The bactericidal effect was evaluated growth-based analysis using agar plates (Table 10).

The reference samples, incubated with bacterial growth medium (Tryptic Soy Broth) containing the general biocide mixture, showed low to no growth. In contrast, many of the components - like talc and calcium carbonate - greatly reduced the biocidal effect to a level where it was no longer inhibitory or bactericidal.

Table 10. Growth experiments showing growth inhibition by the general biocide mixture combined with different components. Bacterial growth was evaluated and ranked from



zero to +++, where '0' = no growth and '+++' = substantial growth. 'NA' = not analyzed.

	Inhibitory (Plate reader)	**Bactericidal** (Agar)	**Inhibitory** (Plate reader)	**Bactericidal** (Agar)
Fillers	Bacteria: 10^6 CFU/ml		Bacteria: 10^8 CFU/ml	
Talc A	0	+	(+)	+++
Silicate A	0	0	++	+++
Dolomite A	0	++	++	+++
$CaCO_3$ D	0	+	++	+++
Dolomite B	0	++	0	+++
Kaolin A	0	++	++	+++
CaCO3 A	0	0	0	0
CaCO3 B	NA	0	NA	0
CaCO3 C	0	0	0	0
Water:TSB	+++	+++	+++	+++
Water+biocide mixture:TSB	0	+	0	0
Water	0	0	0	0

As the biocide is a mixture of two different biocides it is difficult to show the specific effect on the different biocides in BIT and the amine, which may react differently due to the presence of components. That is why each of the two biocides were tested in their pure form at different concentrations.

2.4.4.2 The biocidal effect of BIT, ZnPt and an amine

Following the same procedure as above, the effect of single biocides was tested. The biocides BIT, ZnPt and the amine were all tested in concentrations of 5 ppm. The results show that 5 ppm BIT alone was insufficient for growth inhibition. ZnPt mixed with TSB made a very opaque suspension making it difficult to measure the absorbance, and therefore it was difficult to evaluate the effect of ZnPt. There was no clear biocidal effect of BIT in any of the incubations. The amine is a very effective growth inhibitor even at 5 ppm compared to BIT and ZnPt, but components like CaCO3 A, B and C and Kaolin A interfere with the biocidal activity of the amine.

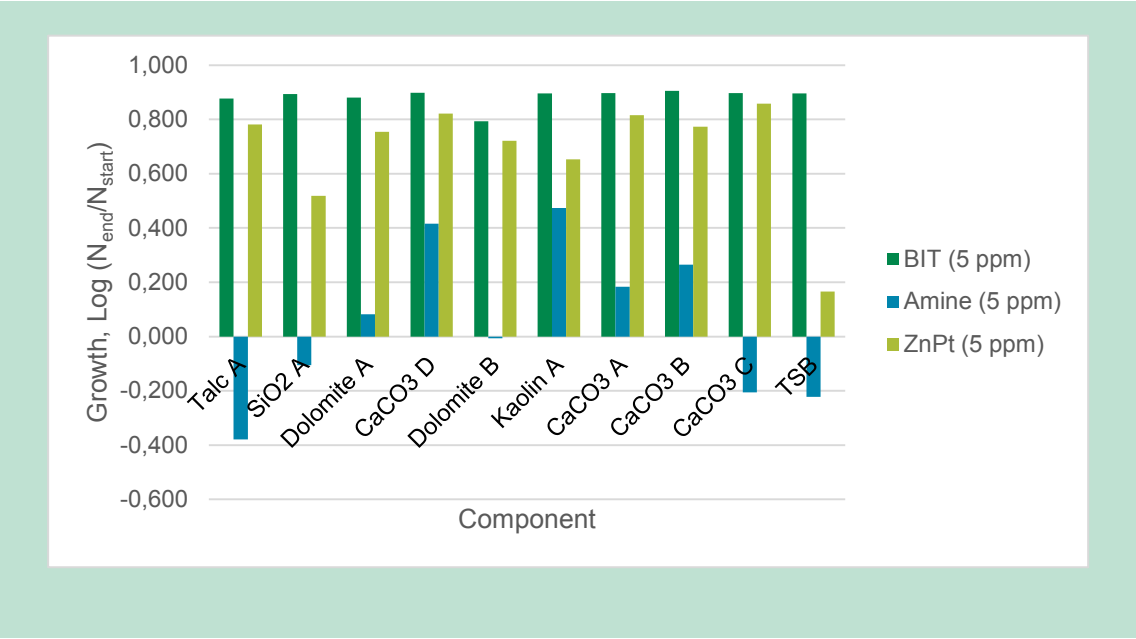


Figure 5. The biocidal effect of three biocides with different paint components.

The data show a slightly different picture than the previous test, where Talc A and SiO_2 A reduced the biocidal activity of the general biocide mixture.

2.5 Effect of binders on biocide activity

A large part of a water-based paint consists of binders, components that bind the pigments in the dried paint. The binders are therefore crucial for the correct function of a paint system.

In tests similar to those made for the fillers, the effect of binders on biocide activity were investigated: Binder was mixed in water to a concentration of 40%. Two different binders based on either Vinylacetate-ethene (PVA) or Styrene Acrylate (SA) were tested. The binders were supplied without added biocides from the suppliers. Biocide (BIT) was then added in different amounts (1, 3, 5, 10, 25, 50, 100 ppm). For comparison, water amended with BIT was used. *S. aureus* DSM799 was added to the solutions and incubated for 24 hours. Viability of *S. aureus* was checked by spreading on agar plates. Bacterial growth on the agar plates was evaluated after 1 and 5 days of incubation (Table 11).

No viable bacteria could be detected in BIT-Water with concentrations of 25 ppm and above. But for both Incubations with binders, even 100 ppm BIT was not enough to kill the added bacteria. The results are similar to the PVA and SA binder.

Table 11. Effect of binder on biocidal activity of BIT. PVA: Poly vinyle acetate binder. SA: Styrene acrylate binder. Green: No or low concentration of microorganisms; Yellow: Medium concentration of microorganisms; Red: High concentration of microorganisms; Grey: Very high concentration of microorganisms.

	Concentration	Evaluation	Evaluation
Medium	BIT (ppm)	Day 1	Day 5
40% PVA	1	●3	●4
	3	●3	●4
	5	●3	●4
	10	●3	●4
	25	●3	●4
	50	●2	●4
	100	●1	●4

40% SA	1	●4	●4
	3	●4	●4
	5	●4	●4
	10	●4	●4
	25	●3	●4
	50	●3	●4
	100	●2	●4
Water	1	●4	●4
	3	●4	●4
	5	●3	●4
	10	●1	●2
	25	●0	●0
	50	●0	●0
	100	●0	●0
Water		●4	●4
40% PVA		●3	●4
40% SA		●4	●4

To validate these results, and to further elucidate the effect of the binder, a similar experiment was conducted, but with increased BIT concentrations of up to 300 ppm.

Here, the bacteria were killed already at 10 ppm BIT. The difference from the previous test could be due to some variation in the concentration of the inoculum. Despite some potential variation, the effects of the binders were again similar with high or very high concentrations of surviving *S. aureus*. No significant biocidal effect was seen even with concentrations as high as 300 ppm BIT when binder was added. The PVA binder might have less effect on the concentration of BIT, but the test is not sensitive enough to give a reliable quantification of these small differences.

Table 12. Effect of binder on biocidal activity of BIT. PVA: Poly vinyle acetate binder. SA: Styrene acrylate binder. Green: No or low concentration of microorganisms; Yellow: Medium concentration of microorganisms; Red: High concentration of microorganisms; Grey: Very high concentration of microorganisms.

	Concentration	Evaluation	Evaluation
Medium	BIT (ppm)	Day 1	Day 5
40% PVA	1	●2	●2
	3	●2	●2
	5	●2	●2
	10	●3	●3
	25	●3	●3
	50	●2	●2
	100	●2	●2
	200	●2	●2
	300	●2	●2
40% SA	1	●4	●4
	3	●4	●4
	5	●4	●4
	10	●4	●4
	25	●4	●4
	50	●4	●4
	100	●4	●4
	200	●3	●3
	300	●2	●2
Water	1	●4	●4
	3	●4	●4
	5	●2	●2
	10	●0	●0
	25	●0	●0
	50	●0	●0
	100	●0	●0
Water	0	●4	●4
40% PVA	0	●3	●4
40% SA	0	●4	●4

The results are both surprising and alarming. The reason for this de facto inactivation of the effect of BIT is unknown, and for the time being it is only speculative. The binder is not of natural origin as most of the fillers, and therefore natural contamination of microorganisms and other organics is not believed to be possible. A more likely explanation could be that the binders themselves bind or shield the reactive BIT molecules. The exact structure of the binders is unknown, but reaction or binding to the BIT molecules could be a possible explanation.

Although the exact mechanisms are unknown, the fact that the biocidal effect of BIT is quenched by the two tested binders, poses a challenge to the formulation of water-based paint with low concentrations of biocide. It also stresses the need for further development and knowledge within the field of formulation and interaction of biocides with different components.

2.6 Summary of component vs biocide activity

Likely candidates for minimal biocide inhibition for the general biocide mixture and the amine could be selected from the results decribed above. Dolomite B and $CaCO_3$ C show no effect on biocide activity with the general biocide mixture or the amine. If a second filler is needed, SiO_2 A could be combined with the amine.

Table 13. Comparative tests of different component's effect on the general biocide mixture and the amine. Potential candidates for a new formulation are marked in black.

Type	Function	Growth – General biocide mixture	Growth – Amine
Talc A	Filler	+	-
SiO_2 A	**Filler**	**++**	**-**
Dolomite A	Filler	++	(+)
$CaCO_3$ D	Pigment	++	++
Dolomite B	**Filler**	**-**	**-**
Kaolin A	Filler	++	++
$CaCO_3$ A	Pigment	-	+
$CaCO_3$ B	Pigment	ND	+
$CaCO_3$ C	**Pigment**	**-**	**-**
Growth Medium	-	-	-

Combined with the results in Table 8 where growth support was studied a list of compounds could be identified for use in a new formulation in combination with the amine (Table 14).

Table 14. Potential candidates for a new paint formulation.

Type	Function
SiO2 A	Filler
Dolomite A	Filler
$CaCO_3$ C	Pigment
Co-polymer A	Antifoam agent
Thickener E	Anionic thickener
Dispersion Agent C	Non-ionic dispersant

2.7 Full-concept evaluation of component substitution

Employing knowledge from the previous experiments, a full-concept evaluation was done on two new water-based paints. In these products, selected components were substituted with candidates less likely to support bacterial growth. The following products were challenge-tested by the addition of bacteria:

- 81-012: Best case product, where potentially problematic products were substituted candidates less likely to support bacterial growth.
- 81-013: Best case product where potentially problematic products were substituted with candidates less likely to support bacterial growth. 81-013 is almost identical to 81-012, except that an alternative binder was used.
- 81-014: Standard product.

The products were formulated in the laboratory with biocide concentrations reduced to 1/4 of normal biocide-levels in order to have better options for visible responses and clear differences in the results.
Before the start of the test, the products were diluted 1:1 in sterile saline to ease homogeneous mixing of bacteria into the products. The final biocide concentration was therefore 1/8 of normal biocide-levels.

The diluted products were tested by addition of a mixture of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to a total concentration of $1.25 \cdot 10^5$ CFU/ml at day 0, 7, and 21. Samples of the inoculated paints were analyzed after 7 days of incubation by plating on Tryptic Soy Agar. Growth on the agar plates was evaluated on a scale from 0-4 after 3 days of incubation at 37 °C. The samples were tested in four replicates per product. Samples with no added bacteria was used as reference samples.

All reference samples remained uncontaminated during the test period. The products with added bacteria did on the other hand handle the bacterial load very differently. The standard product 81-014 killed the added bacteria at day 7, but had very high bacterial concentrations at day 14 and day 21. The product was thus not able to handle the load of bacterial contamination.

The new formulation with a new binder 81-013 performed even worse and contained high bacterial concentrations already at day 7. Substituting the binder therefore seemed to have the opposite effect and increased the bacterial growth in the product.

Tests on the product 81-012, which was almost identical to 81-013 except that it contained the same binder as 81-014, resulted in a product, which even after three inoculations (day 21) did not contain viable bacteria. Substitution of the identified components thus resulted in a product which was very resilient to bacterial growth even at low biocide concentration.

Table 15. Results from bacterial challenge tests on three different water-based paints. Product contamination was evaluated on a scale from 0-4, where 0 shows no growth and 4 shows maximum growth.

Recept	Sample	Start	Day 7	Day 14	Day 21
81-012	Ref1	0	0	0	0
	Ref2	0	0	0	0
	12.1	0	0	0	0
	12.2	0	0	0	0
	12.3	0	0	0	0
	12.4	0	0	0	0
81-013	Ref1	0	0	0	0
	Ref2	0	0	0	0
	13.1	0	3	4	4
	13.2	0	3	4	4
	13.3	0	3	4	4
	13.4	0	2	4	4
81-014	Ref1	0	0	0	0
	Ref2	0	0	0	0
	14.1	0	0	4	4
	14.2	0	0	4	4
	14.3	0	0	4	4
	14.4	0	0	4	4

The results showed that it is possible to formulate a product which is less prone to bacterial spoilage, by use of knowledge of bacterial growth and raw materials. The test also indicate that substitution of a single component can have substantial effect on bacterial growth in the product.

As the focus on biocides and in-can preservation continues, formulation of new products will require not only knowledge on the components physio-chemical abilities but just as importantly knowledge on the components effect on microbial growth.

3. Improved biocidal effect

Two approaches were taken to boost the effect of the biocides in the water-based paint. The first approach was to investigat the effect of combining different biocides. The second approach was to let the biocides work more efficiently by combining them with other additives that can weaken the microorganisms.

The effect of the biocides in water (MIC-values) does not correspond to the needed concentration when used in a paint formulation, which paved the way for a new set of experiments that could determine how the biocides interact with the components in the paint.

By combining additives such as lithium and other non-biocidal compounds with biocides, it was possible to increase the killing effect of BIT. Also by altering the pH in the paint, the microorganisms were weakened and the effect of the biocides was amplified.

By combining biocides it appeared that the most promising results were achieved with two biocides that were not isothiazilones. It was possible to develop a recipe that was MIT free.

3.1 Introduction

According to the Biocidal Products Regulation (BPR) a biocide is defined as a substance that has an effect on or is harmful to organisms (Regulation 528/2012/EC). These biocides (also occasionally referred to as “Microbiocides”) are used for inhibiting growth of microorganisms.

Microbiocides function by reacting with microbial cells and thereby infer with vital cellular processes. Microbiocides can be classified through their mechanism of action (Paulus, 2008 p. 10-14, p. 34):

(1) *Membrane-active microbiocides.*
Their first mode of action is by adsorbing to the microbial cell wall. This adsorption causes changes in the cell wall and outer membrane, prompting loss of cell wall and membrane integrity. This causes disorder in the semi-permeable properties of the membrane and inhibition of enzymes localized here. Desintegration of the cell wall and membrane can furthermore prompt the escape of essential components from cytoplasm, precipitation in periplasm and finally disintegration of the cells. *Examples: alcohols, quaternary ammonium compounds.*

(2) *Electrophilically active microbiocides.*
These compounds react with molecules with heightened electron density hereby resulting in electrophilic addition or substitution. In and on microbial cells, these nucleophilic reaction partners are thiol, amino and amid groups.

Examples: aldehydes, organometallic compounds, compounds with activated N-S bonds like BIT and MIT.

(3) *Chelating agents*
Many enzymes contain metallo-catalytic sites where divalent metal ions like iron or copper are essential for enzymatic function. Chelating agents have strong affinity for divalent metal ions, making these ions unavailable for the microorganisms and thereby removing these vital micronutrients. In addition to their competition for divalent metal ions, chelators are also reported to have membrane activity.
Examples: zinc pyrithione, thiohydroxamic acids

(4) *Inorganic bactericides*
Metal ions have different modes of mechanism: They can influence the electrochemical potential between internal and external parts of the cells, and inside the cell where they compete with other ions and aggregate with thiol groups of proteins.
Examples: silver, copper, zinc

Examples of different electrophilic or membrane active biocides are shown in Figure 6.

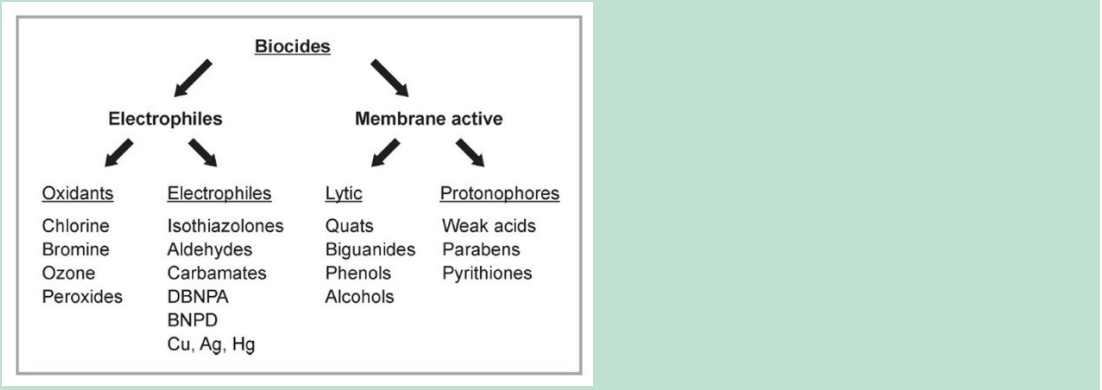


Figure 6. The mechanism of different industrial biocides (Williams, 2007).

Some of the most commonly used microbiocides in water-based paint are the electrophiles isothiazolones like 1,2-benzisothiazolin-3-one (BIT) and 2-methyl-4-isothiazolin-3-one (MIT) (Figure 7).

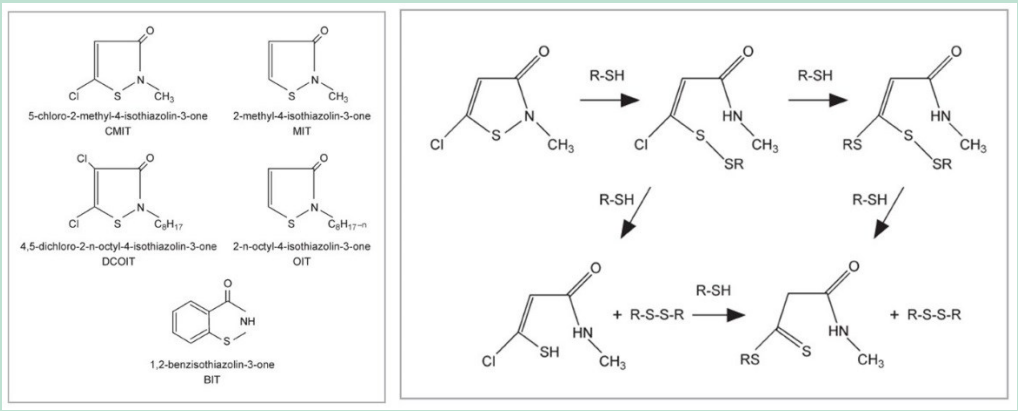


Figure 7. Commonly used isothiazolones for preservation of products (left) and reaction of CMIT with a thiol group (right). (Williams, 2007).

Part of this project is to study the effect of combining biocides with other biocides or compounds with no biocidal activity. The biocidal effect can be classified as: (1) the lowest concentration required for inhibition (Minimum Inhibitory Concentration, MIC), and (2) the lowest concentration required for cell death (Minimum Biocidal Concentration, MBC). MIC and MBC were not determined in the experiments described in this chapter, but the effect of the biocides are studied with regard to either their inhibitory effect (plate reader tests) or biocidal effect (challenge test on products).

These concentrations will depend on biocide type, and identity and functionality of the contaminating microorganism (Williams, 2007).

The biocides and other compounds tested in this chapter are all evaluated by Regulatory Affairs with regard to their impact on labelling of the final paint including the hazards. The environmental department at Flügger A/S has evaluated the type, concentration and combination of biocides used in the project. The biocides are evaluated from their Safety Data Sheet (SDS). This evaluation is based on classification of the individual components and on the MAL-kode (Måleteknisk Arbejdshygiejnisk Luftbehov), aiming at achieving the MAL code 00-1. These evaluations are carried out in relation to current regulations, but also in relation to expected future regulatory issues.

The concentration of biocides used in the test programmes shown in this chapter include two scenarios: (i) where biocide concentrations are below concentrations requiring EUH208, and (ii) in concentrations where EUH208 is required, but below the limit requiring H317 labeling (May cause an allergic skin reaction).

3.2 Effect of pH on bacterial growth

Creating a milieu that is very unfavorable to microorganisms will potentially omit the need for biocides. A water-based paint normally has a pH within the range of pH 8-9, which is not enough to inhibit bacterial or fungal growth.

To study the effect of pH on bacterial growth, bacterial paint systems of mat ceiling paint were mixed without added biocides, i.e., containing only the low concentration of biocides present in the raw materials.

The experiments were conducted without adding any bacteria to the different paints. Any microbial growth in the pH adjusted products is therefore a result of microorganisms introduced during production.

Products with pH ranging from neutral to alkaline were tested: 7.46, 8.17, 8.91, 9.47, 10.35, and 11.35. The samples were incubated for up to 5.5 weeks at 25°C.

The microbial spoilage of the paints was monitored through bacterial analyses of viable bacteria along with visible signs of spoilage such as malodour and discoloration of the paint.

After one week, the samples with pH 7.46, 8.17 and 8.91 showed signs of malodour. After 11 days, pH 7.46, 8.17 and 8.91 had a distinct malodour and discoloration of the paint surface.
After 4 weeks, pH 9.47 also showed malodour and discoloration.
After 5.5 weeks, paint with pH 10.35 showed weak discoloration.
Bacterial analysis showed growth in all samples at all time points – although the number of bacterial colonies is lower at pH 10.35 and 11.35.

Even though microbial spoilage is retarded at high pH 10.35 and 11.35, this elevated pH does not have a complete bactericidal effect, since viable bacteria can still be retrieved even at pH 11.35. Although bacteria are still present at pH 11.35, the product shows no visible signs of spoilage and an approved preservation may be expected.

Therefore, pH alone is not sufficient to effectively inhibit microbial growth, but might be sufficient to slow microbial growth rates to a level were the products can be preserved by a lower concentration of biocide. Biocide tests at elevated pH are shown in section 3.6.

3.3 Bacterial inhibition by addition of lithium

It has previously been demonstrated that lithium increases the effect of biocides. The effect of lithium alone is limited, but combined with the use of biocide, lithium concentrations above 1000 ppm boosts the bactericidal effect even when using a biocide-resistant strain (Figure 8, Maiuta et al, 2001). The effect is therefore not considered synergistic, but as a booster of the biocidal effect (Maiuta et al, 2001).

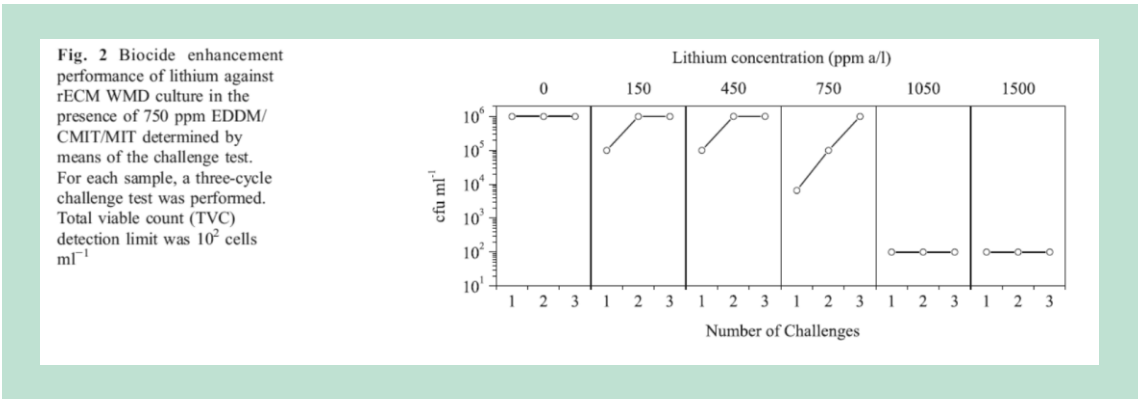


Fig. 2 Biocide enhancement performance of lithium against rECM WMD culture in the presence of 750 ppm EDDM/CMIT/MIT determined by means of the challenge test. For each sample, a three-cycle challenge test was performed. Total viable count (TVC) detection limit was 10^2 cells ml^{-1}

Figure 8. Enhancement of 750 ppm biocide (EDDM/CMIT/MIT) concentration by addition of lithium (Maiuta et al, 2011). The biocide is a combination of (Ethylene dioxy) dimethanol, 5-chlor-2-methyl-2H-isothiazolin-3-one (CMIT) and N-methyl-siothiazaolin-3-one (MIT). The detection limit of the method is 10^2 cells. The effect is tested on a biocide-resistant strain identified as *Pseudomonas spp.*

Although lithium is an ion, the effect is not considered to be linked to a decrease in water activity as concentrations of lithium only have a limited effect on water activity (Figure 9).

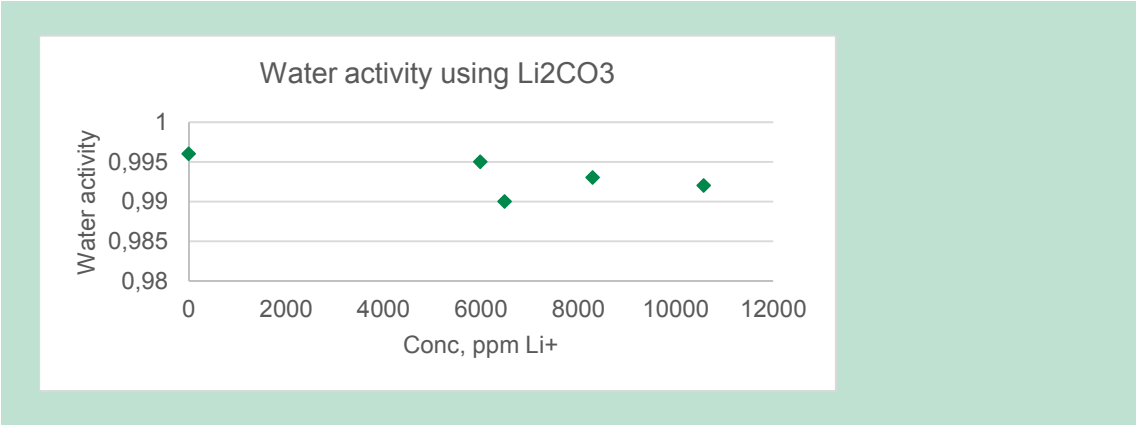


Figure 9. Measurements of water activity as function of lithium concentration. No or very limited effect on water activity is observed with concentrations of >10,000 ppm lithium.

In order to investigate lithium's effect in combination with BIT, bacterial growth was tested as function of different BIT and lithium concentrations to obtain the Minimal Inhibitory Concentration (MIC) for *Pseudomonas aeruginosa* DSM1253.

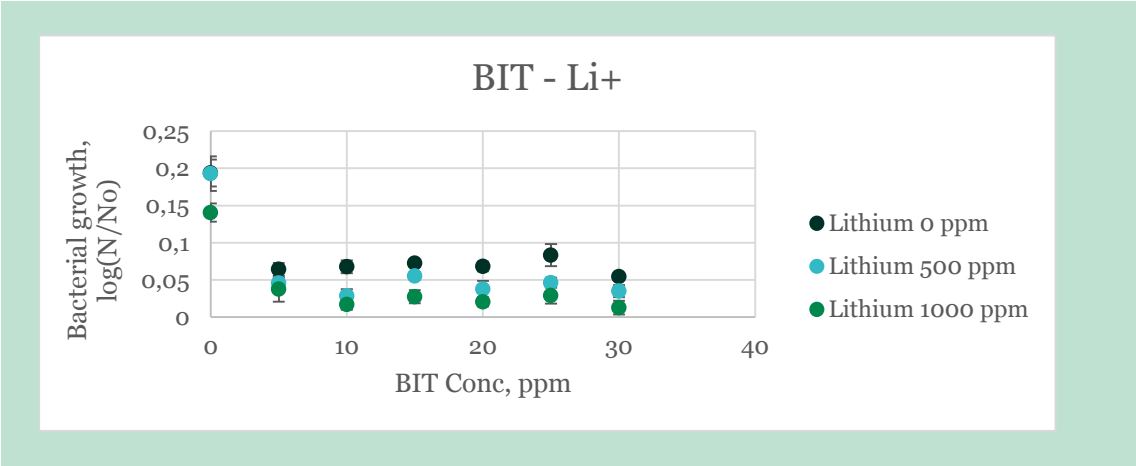


Figure 10. Measurement of bacterial growth as a function of different BIT and lithium concentrations.

Two concentrations of lithium (500 and 1000 ppm) and seven concentrations of BIT (0-30 ppm, i.e., well below the limit of 50 ppm before CLP labeling with EUH208 is required) were tested for their inhibitory concentrations in bacterial growth medium. The biocidal effect was measured using a multi-well plate reader (Varioskan Lux, Thermo Scientific), which measures bacterial growth using optical density of the growth medium.

The tests with 0 ppm BIT showed bacterial growth as expected. When testing the highest amount of lithium ions (1000 ppm) in combination with BIT it appeared that the biocidal effect was increased compared to incubations without lithium ions (Figure 10). The growth rate using 0 and 500 lithium with no BIT was the same, whereas the growth rate was reduced when using 1000 ppm lithium.

All measurements for 0 ppm lithium-ion showed an increase in absorbance within the first two hours, where the cell density became stable. This indicates that the full effect of the biocides is not achieved until two hours and until that time the culture can grow before all cells are inhibited (Figure 11). This growth phase is reduced or absent when Li-ions are added.

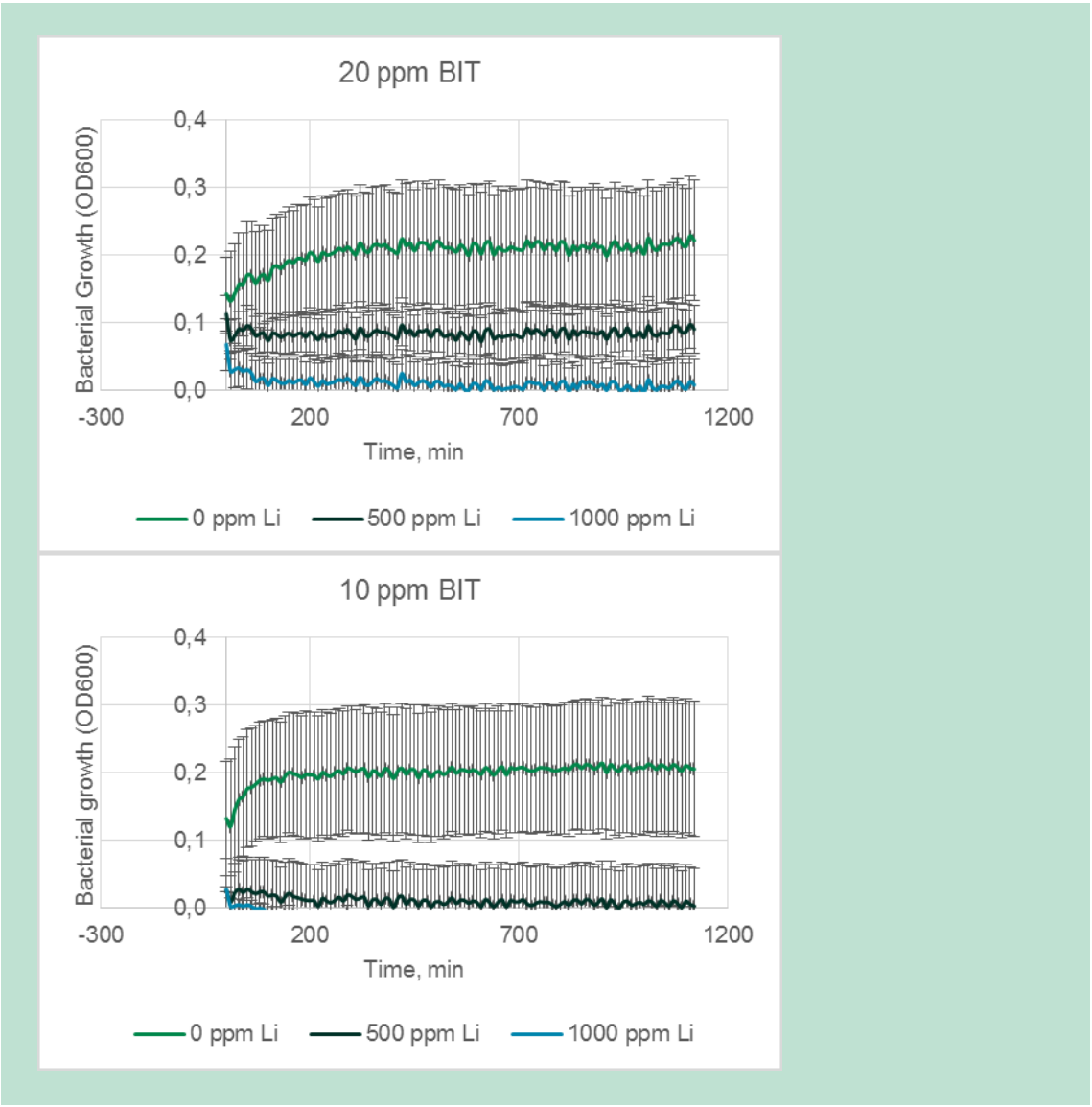


Figure 11. Growth curves for incubation with 10 or 20 ppm BIT with concentrations of lithium 0, 500 or 1000 ppm.

The results above show that lithium have some effect on bacterial growth. To study the effect in water-based paint further studies were carried out (see section 3.6.2).

3.4 Combination of Biocides and complementing compounds

As previously described, membrane-active compounds interact with both membrane and cell walls of the microorganisms. Our hypothesis is that the action of electrophilic biocides can be enhanced by combining the use of membrane active compounds. The membrane active compounds will facilitate the transport of the electrophiles into the cell where they can react with vital bio-molecules – like enzymes – and inhibit metabolic processes.

3.4.1 Tests with two biocides in combination with non-biocidal components

Some non-biocidal components can enhance the effect of biocides by making them more chemically adequate to attack the bacteria by, e.g., disrupting the outer membrane of Gram-negative bacteria.

In the cosmetic industry, substances known as “multi-functional ingredients” or “boosters” are used to enhance the effect of the added preservatives (Varvaresou *et al*, 2009). These substances are not biocides and are primarily used for their beneficial effect on skin and product property, e.g., their effect as moisturizer or emulsifier.

To test the use of different boosters, two biocides with different functionality were tested in combination with five compounds that are suspected to boost the effect of biocides (Table 16). These boosters were selected based on literature and knowledge from other industries. The biocide effect was measured as the concentration needed to inhibit the growth of the added test bacteria (MIC).

Table 16. Overview of biocides and boosters used for test.

Name	CAS No.	REACH regulation	Classification	MAL code	TEB	VOC	Function
Benzisothiazolinone (BIT)	2634-33-5						Biocide
Amine	-						
Amine coco alkyl ethoxylate	61791-14-8	no	Acute tox 4; (H302) Skin Corr. 1B; (H314) Eye Dam. 1; (H318) Aquatic Acute 1; H400 (M=1)	0	>1% => -4	No	
Urea (Carbamide)	57-13-6	01-2119463277-33	IK	0 (P = 0 mmHg v. 25 °C)	>0% => -1	No, it decomposes before the boiling point is reached	Booster
Caprylyl glycol (Octane-1,2-diol)	1117-86-8	01-2119966905-22	Eye Irrit 2; H319	0 (P = 0,00113 mmHg at 20 °C)	>2% => -3	No (boiling point 267 °C)	
Ethylhexylglycerine	70445-33-9	no	Eye Dam. 1; (H318) Aquatic chronic 3; (H412) (industry)	0	>2% => -3	No	
Sodium caprylyl lactylate	977067-37-0						

The biocide-booster combination was tested using two different levels of bacterial inocula (10^6 and 10^8 CFU/ml).

To test the effect of the boosters it was necessary to use biocide concentrations, which were not completely inhibitory when not combined with boosters. Previous tests have shown that 5 ppm biocide mixture of BIT and an amine can inhibit 10^8-10^9 bacteria in a water-based growth medium (Section 2.4.4.1). Therefore, the biocides were tested in low concentrations: BIT 5 ppm and 1 ppm amine, respectively. The boosters were tested at the same fixed concentration of 300 ppm to make a it possible to compare the efficiency of the boosters, and at a level that makes it possible to integrate them in paint recipes without compromising the functionality of the paint.

Results showed some variation, and although some of the booster incubations had reduced growth rates, no consistent effect could be seen for any of the boosters (Figure 12).

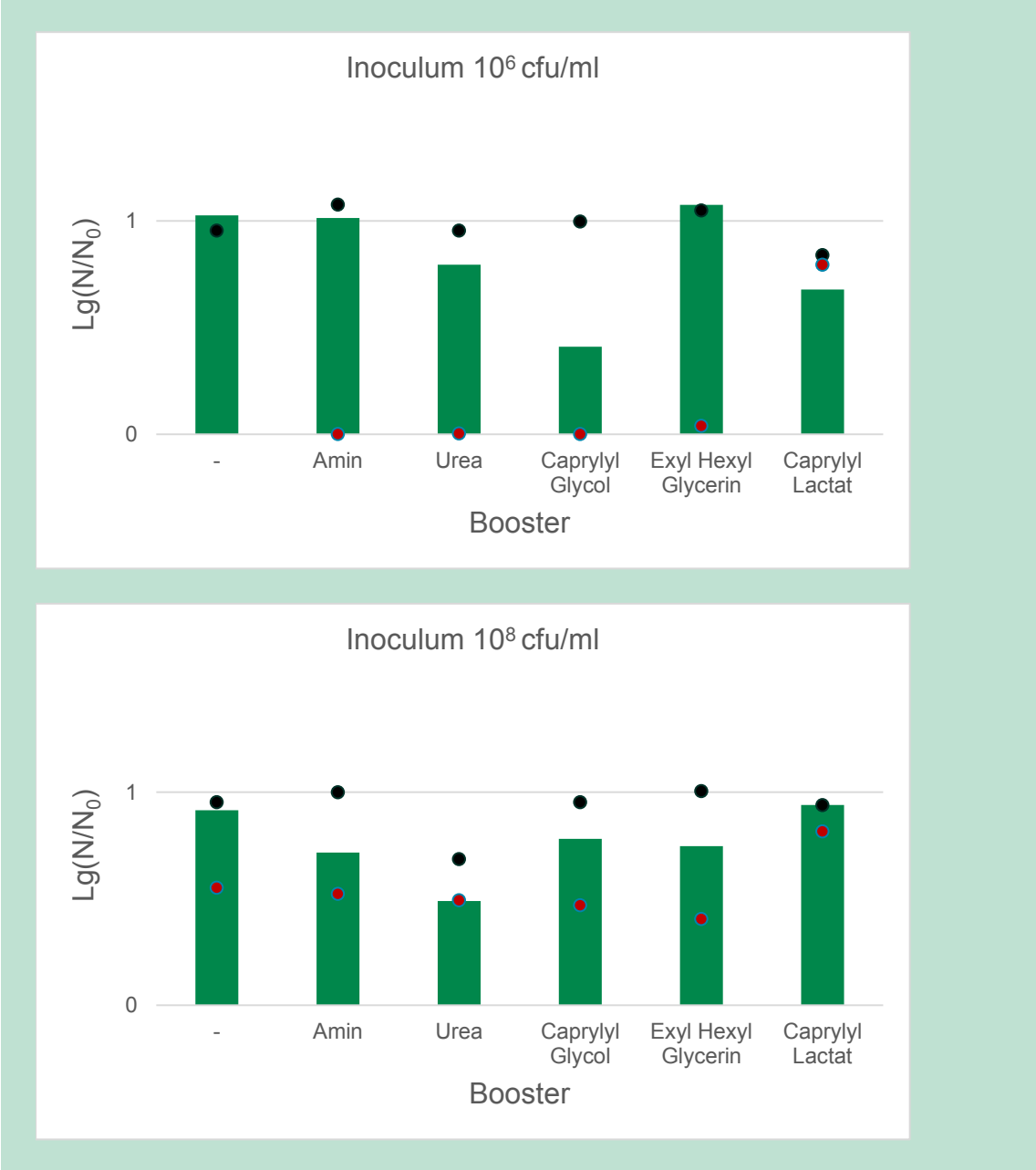


Figure 12. The effect of boosters combined with the amine biocide and BIT on bacterial growth. Growth was evaluated after 17 hours incubation at 37 °C: '-' no booster added;

Boosters without biocide (green bars); Boosters + amine (Red); boosters + BIT (Black).Inoculum 10^6 CFU/ml: Caprylyl glycol was the only booster that in it-self had some impact with more than 50% reduction in growth rate within the 17 hours of incubation.

1 ppm amine was sufficient to inhibit growth in all incubations even without the use of boosters.
5 ppm BIT did not result in growth rate inhibition with or without boosters. In fact growth with BIT + Caprylyl glycol was as high as growth without booster or biocide.

Inoculum 10^8 CFU/ml: Only urea reduced growth rate by more than 50%. In combination with BIT, urea also had some effect on growth rate. The other boosters had no effect in combination with the amine or BIT.
Increasing the bioburden from 10^6 CFU/ml to 10^8 CFU/ml alleviated the inhibitory effect of the amine, but the effect of the amine could not be increased by adding more booster.

The concentration of booster used in these tests is probably too low to show any effect in combination with the amine or BIT. Increasing the concentration of booster could potentially increase their effect, but not to a level that could have a significant effect. Under the rigorous conditions that are used when testing the biocide effect in complete paint systems, repeated inoculation of bacterial pure cultures or contaminated paint is used (see sections below).

3.4.2 Test of biocides and non-biocidal compounds in contaminated paint

As an alternative to the boosters used in, e.g., cosmetics, a range of different compounds used in the paint industry were evaluated for their potential boosting effect.

Water-based paints contain a wide array of different compounds included to stabilize the paint and to obtain the desired properties (fitness-of-use). Some of these compounds include amphiphilic compounds that have (just as the membrane active compounds) a hydrophobic as well as a hydrophilic part. That would potentially allow them to interact with microbial membranes and cell walls and partly have a function similar to that of the membrane active biocides.

Based on their molecular structure, combinations of biocides and compounds with other functionalities were selected, and their efficiency for inhibiting microbial growth was tested (Table 17).

Table 17. Non-biocidal additives tested in combination with biocides.

Name	Abbreviation	Function	CAS No.
2S)-Alanine, N,N-bis(carboxymethyl)-, trisodium salt+ (2R)-Alanine, N.N-bis(carboxymethyl)-trisodium salt	AA salt	Chelating agent	164462-16-2
2,2',2",2'"-(Ethane-1,2-diyldinitrilo)tetraacetic acid	EDTA	Chelating agent	60-00-4
bis(triethanolamine) titanium di-isopropoxide	TET	Crosslinking agent	36673-16-2
Didecyl dimethyl ammonium carbonate/ Didecyl dimethyl ammonium bicarbonate	DDAC-DDAB	Rust Inhibitor	148788-55-0 / 148812-65-1
$LiCO_3$		Filler	554-13-2
Alkyl dihydroxyethyl amine oxide	ADHAO	Surfactant	61791-47-7
Alkyl dimethyl amine oxide	ADMAO	Surfactant	73502-08-6
Dodecyl guanidine hydrochloride	DGH	Surfactant	13590-97-1
Didecyl dimethyl ammonium chloride	DDAC	Preservative	7173-51-5

An extensive test programme was initiated, and the additives (Table 17) were tested in combination with different biocides shown in Table 18. The table includes isothiazolinones as well as biocides not related to isothiazolinones.

Table 18. CAS numbers, abbreviations and IUPAC names of the tested biocides.

IUPAC name	Abbreviation	CAS number
Methylchloroisothiazolinone/Methylisothiazolinone (3:1)	CMIT/MIT	55965-84-9
Benzisothiazolinone	BIT	2634-33-5
Methylisothiazolinone	MIT	2682-20-4
2-bromo-2-nitropropan-1,3-diol	Bronopol	52-51-7
bis(2-pyridylthio)zink 1,1'-dioxid	ZnPt	13463-41-7
Iodopropynyl butylcarbamate	IPBC	55406-53-6

The biocides were tested at concentrations that is known to be at the lower limit to prevent microbial spoilage in combination with different components. The test programme is described in Table 19.

The different formulations were inoculated with 6% spoiled paint, incubated at 30°C and subsequently re-inoculated with spoiled paint. The concentration of bacteria and fungi was enumerated 72 hours and 1 week after each inoculation.

In order to identify the potential enhancing effect of biocide combinations, each test included a paint containing only one biocide. Selected parts of the test programme are summarized in Table 19.

Table 19. Test programme specifications. T = Test series. No baceriocidal effect after two times inoculation and 1 week incubation is red, limited bacteriocidal effect where bacterial growth is observed in some of the replicates is orange and clear bacteriocidal effect where no growth was observed is Green.

	Non biocides:	None	AA salt 0.25 % Active	EDTA 0.25 % Active	TET 0.25 % Active	ADHAO 0.3 % Active	ADMAO 0.3 % Active	DDAC/DDAB 1 % Active
	Biocides:							
	CMIT/MIT 15 ppm	CMIT/MIT-0	CMIT/MIT-1	CMIT/MIT-2	CMIT/MIT-3	CMIT/MIT-4	CMIT/MIT-5	CMIT/MIT-6
T1	BIT 300 ppm	BIT-0	BIT-1	BIT-2	BIT-3	BIT-4	BIT-5	BIT-6
	MIT 50ppm BIT 50ppm	MIT-0	MIT-1	MIT-2	MIT-3	MIT-4	MIT-5	MIT-6
T2	Bronopol 100 ppm	Bronopol-0	Bronopol-1	Bronopol-2	Bronopol-3	Bronopol-4	Bronopol-5	Bronopol-6
	ZnPt 100 ppm + BIT 380ppm	ZnPt-0	ZnPt-1	ZnPt-2	ZnPt-3	ZnPt-4	ZnPt-5	ZnPt-6
T3	DDAC 3000 ppm	DDAC-0	DDAC-1	DDAC-2	DDAC-3	DDAC-4	DDAC-5	DDAC-6
	IPBC 50 ppm	IPBC-0	IPBC-1	IPBC-2	IPBC-3	IPBC-4	IPBC-5	IPBC-6
T4	Amine 100 ppm	Amine-0	Amine-1	Amine-2	Amine-3	Amine-4	Amine-5	Amine-6
	Biocides:	DGH 115ppm	$LiCO_3$ 1000ppm	No addition				
T5	CMIT/MIT 1,5ppm	DGH-CMIT	Li-CMIT	CMIT				
	BIT 50ppm	DGH-BIT	Li-BIT	BIT				

Bronopol 100 ppm	DGH-Bron	Li-Bron	Bron
BIT 300ppm + ZnPt 100ppm	DGH-ZnPt	Li-ZnPt	ZnPt
IPBC 50ppm	DGH-IPBC	Li-IPBC	IPBC

The only component that clearly boosted the effect of the conventional biocides was didecyl dimethyl ammonium carbonate/ didecyl dimethyl ammonium bicarbonate (CAS no. 148788-55-0 / 148812-65-1). Further investigation showed that the component has recently been registered as a wood preservative on the PT 8, but not on PT6. However, didecyl dimethyl ammonium chloride (CAS No. 7173-51-5) is a similar compound in relation to the chemical structure, but it has a chloride-ion as the counter-ion instead of a carbonate ion. It is approved as an in-can preservative on the PT6 list in Annex V of the Biocidal Product Regulation. It is expected that the two components will have similar biocidal effect as the only difference is the counter-ion. Therefore, didecyl dimethyl ammonium chloride is used in further tests in water-based paint systems. The component is a quaternary ammonium compound and is named DDAC in the following sections.

3.5 Biocide tests in a simplified paint system

Based on the tests on the PVA based mat wall paint, shown in Chapter 2, we found that PVA based mat wall paint is a very complex system with many different raw materials potentially influencing both bacterial growth and biocide activity. To reduce the complexity of the formulation, a simplified test paint (TM) was developed (Table 20). The biocides were tested in both the PVA based mat wall paint and TM.

Table 20. Formulation of the simplified paint system.

Function	Concentration (%)
Water	32.55
Dispersing agent B	0.16
TiO_2 B	32.55
Binder A	32.55
Thickener H	2.18
	99.99

The used binder systems in the test paints contain different biocides for preservation of the binder, which are not included in the tables. The biocide concentration in the binders is shown in Table 21.

Table 21. Biocide concentration in the binders used in the paint formulation.

Product	Binder	CMIT/MIT(PPM)	MIT(PPM)	BIT(PPM)
Test paint	Binder A	15	100	100
PVA based mat wall paint	Binder B	14	0	184

The previous biocide tests included the addition of contaminated paint. The tests described below included the test organisms *Pseudomonas aeruginosa* DSM1253 and *Staphylococcus aureus* DSM799, as representative of Gram-negative and Gram-positive bacteria, respectively. The cell-walls of Gram-negative and Gram-positive bacteria differ a lot, and so do their potential responses to the different biocides. Although no staphylococci was found in the contaminated paint analyzed in Chapter 2, *S. aureus* DSM799 is a test-strain used to test disinfection agents. It is believed to be a good representative for testing biocidal activity.

The bacterial suspensions used for inoculation of the paint-biocide formulations were prepared as follows: The bacteria were cultured on agar, a colony was harvested, suspended in MH medium and grown overnight (ON). ON culture was added to the different paint-biocide formulations and incubated at 37°C for 7 days. After 7 days, paint was sampled after stirring and diluted 1/10 before spreading the solution on agar plates. The bacterial growth on the agar plates was evaluated after 3 and 6 days of incubation at 37°C. The growth on the plates ranked from none (-) to completely overgrown (+++).

Bacterial suspension of either *P. aeruginosa* or *S. aureus* was added (inoculated) to the 50 g paint-biocide formulations 16 times during the incubation period. Bacterial suspension was added in different volumes: 1st inoculation, 100 µL, 2nd inoculation, 100 µL, 3rd inoculation 1000 µL, 4th-16th inoculation, 3000 µL. The 16 inocaluations are considered a very harsh treatment as they resulted in a total inoculum volume of 40.2 ml, and they also diluted the concentration of the biocide system of the paint by more than 40%. However, the harsh treatment allowed us to estimate the strength of each tested biocide system.

The different biocides that were tested appear in Table 22. Note that DDAC = Didecyl dimethyl ammonium chloride.

Table 22. Biocide concentrations in the tested products. All concentrations are given in ppm. DDAC = Didecyl dimethyl ammonium chloride. Test medium: TM = simplified test formulation; MP = PVA based mat wall paint. P.A. *Pseudomonas aeruginosa* DSM1253 / S. A. *Staphylococcus aureus* DSM799.

Sample no.	Test medium	g. test m.	Bacteria		Amine	ZnPT	Bronopol	CMIT/MIT	MIT	DDAC	BIT
			P.A.	S.A.							
TM	TM	50									
MP	MP	50									
PATM	TM	50	X								
SATM	TM	50		X							
PA7	MP	50	X								
SA7	MP	50		X							
1	TM	50	X		100	100	50				
2	TM	50		X	100	100	50				
3	TM	50	X		100	100	50	15			
4	TM	50		X	100	100	50	15			
5	TM	50	X		100	100	50				300
6	TM	50		X	100	100	50				300
7	TM	50	X		100	100	50	15			300
8	TM	50		X	100	100	50	15			300
9	MP	50	X		100	100	50				
10	MP	50		X	100	100	50				
11	MP	50	X		100	100	50	15			300
12	MP	50		X	100	100	50	15			300
13	TM	50	X		100			15	50		300
14	TM	50		X	100			15	50		300
15	TM	50	X			100	50				
16	TM	50		X		100	50				
17	TM	50	X		100					1000	
18	TM	50		X	100					1000	

The results from the tests are shown in Table 23 (*Pseudomonas aeruginosa* DSM1253) and Table 24 (*Staphylococcus aureus* DSM799).

In general, *P. aeruginosa* had a higher resistance than *S. aureus* to the tested combinations of biocide.

Using a model system with the biocide package of CMIT/MIT, MIT and BIT, viable bacteria can be detected in low numbers in the control paint without added bacteria, but at the end of the test they are present in high numbers (row 1). Addition of bacteria challenged the activity of the biocide combination of isothiazolinones: A high number of bacteria (score 3) could be detected already at 4th or 5th inoculation for *P. aeruginosa* and *S. aureus*, respectively (row 2). Similar results with insufficient biocidal effect could be observed by using a combination of CMIT/MIT and BIT in the PVA based mat wall paint as test medium (row 10 & 11).

Using a formulation with no added isothiazolinones, but with ZnPt and Bronopol (row 3), made it possible to hamper extensive growth within the first inoculations. The biocidal effect is more effective against *S. aureus* than *P. aeruginosa*. Combining ZnPt and Bronopol with the amine (row 4) did not increase the biocidal effect (the increased growth of *P. aeruginosa* compared to incubations without the amine might be due to variation in the test).

The further addition of different combinations of isothiazolinones (BIT, CMIT/MIT and MIT) to a combination of the amine, ZnPt and Bronopol had a limited effect on both *P. aeruginosa* and *S. aureus*, compared to the use of the amine and Bronopol (row 5-8).
The addition of isothiazolinoes (BIT and CMIT/MIT) to a mix of amine, ZnPt and Bronopol did not increase biocidal activity when using the PVA based mat wall paint as test medium (row 12 & 13 compared to row 4 &7).

The results showed that TM seemed to decrease the effect of the biocides more than the PVA based mat wall paint.

The use of DDAC and the amine showed some promise with low or no growth (0-1) until inoculation 11 and 13 for *P. aeruginosa* and *S. aureus*, respectively (row 9).

Each combination was evaluated according to the summed score from the number of viable bacteria detected after each inoculation. To evaluate the collective biocide effect on *P. aeruginosa* and *S. aureus*, the score of each test was combined and evaluated (Tabel 25). The concentration of isothiazolinones and the total concentration of biocides were compared with the bactericidal effect of the different concentrations.

Table 23. Combination and effect of different biocides on *Pseudomonas aeruginosa*. Bacterial culture was added sequentially up to 16 times during the test period (marked in italics). Bacterial growth is ranked from none(0) to completely overgrown(3).Results are color-coded according to biocidal effect evaluated from their summed score: Red, >20; Yellow, 15-20; Green, <15. Test medium: TM = simplified test formulation; MP = PVA based mat wall paint.

												100µl	*100µl*	*1000µl*	*3000 µl is added in each sample*												*Score SUM*
												1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Row No.	Sample no.	Test medium	Bacteria	Amine	ZnPt	Bronopol	CMIT/MIT	MIT	DDAC	BIT																		
1	TM	TM					4.9	33		163		2	0	0	1	0	0	1	1	2	1	0	1	2	3			
2	PATM	TM	P.A.				4.9	33		163		0	0	2	3	3	3	3	3	3	3	3	3	3	3			35
3	15	TM	P.A.		100	50						1	0	0	0	1	1	1	2	1	0	1	0	2	2,5	3	3	18.5
4	1	TM	P.A.	100	100	50						1	2	1	1	1	2	1	1		2	1	0	1	3	3	3	23
5	5	TM	P.A.	100	100	50				300		1	0	0	1	0	0	0	1	0	3	1	2	2	2	3	3	19
6	3	TM	P.A.	100	100	50	15					1	0	0	0	0	0	1	0		1	1	0	3	3	3	2	15
7	7	TM	P.A.	100	100	50	15			300		1	0	0	0	0	3	0	1	0	0	1	1	2	3	3	3	18
8	13	TM	P.A.	100			15	50		300		1	1	0	0	0	1	1	1	0	1	0	2	2	2	3	3	18
9	17	TM	P.A.	100					1000			0	0	0	0	0	0	0	0	1	0	3	0	2	2	3	2	13
10	MP	MP					5.7			75		1	0	0	1	0	0	1	1	0	1	0	0	2	3			
11	PA7	MP	P.A.				5.7			75		1	0	1	2	3	2	3	3	3	3	3	3	3	3			33
12	9	MP	P.A.	100	100	50						0	0	1	0	0	0	0	0	0	1	1	1	1	2	3	2	12
13	11	MP	P.A.	100	100	50	15			300		1	0	1	0	0	0	0	0	1	0	1	1	2	2.5	3	3	15.5

Table 24. Combination and effect of different biocides on *Staphylococcus aureus*. Bacterial culture was added sequentially up to 16 times during the test period (marked in italics). Bacterial growth is ranked from none(0) to completely overgrown(3). Results are color-coded according to biocidal effect evaluated from their summed score: Red, >20; Yellow, 15-20; Green, <15. Test medium: TM = simplified test formulation; MP = PVA based mat wall paint.

											100µl	*100µl*	*1000µl*	*3000 µl is added in each sample*												*Score SUM*
											1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Row no.		Test medium	Bacteria	Amine	ZnPt	Bronopol	CMIT/MIT	MIT	DDAC	BIT																	
1	TM	TM					4.9	33		163	2	0	0	1	0	0	1	1	2	1	0	1	2	3			
2	SATM	TM	S.A.				4.9	33		163	0	0	1	2	3	3	3	3	3	3	3	3	3	3			**33**
3	16	TM	S.A.		100	50					0	0	0	0	0	0	0	1	1	0	1	2	3	2	3	3	**16**
4	2	TM	S.A.	100	100	50					1	1	0	1	0	1	0	0	2	0	1	3	2	3	3	3	**21**
5	6	TM	S.A.	100	100	50				300	1	0	1	0	0	1	0	0	0	1	1	1	2	2	2	2	**14**
6	4	TM	S.A.	100	100	50	15				1	0	0	0	0	1	0	0		0	1	0	2	2	2	3	**12**
7	8	TM	S.A.	100	100	50	15			300	0	0	0	0	0	1	0	0	0	1	0	2	2	3	3	3	**15**
8	14	TM	S.A.	100			15	50		300	1	0	0	0	0	0	0	2	1	1	1	0	2	2	3	2	**15**
9	18	TM	S.A.	100					1000		1	0	0	0	0	0	0	1	1	0	1	1	2	2	2	2	**13**
10	MP	MP					5.7			75	1	0	0	1	0	0	1	1	0	1	0	0	2	3			
11	SA7	MP	S.A.				5.7			75	1	1	1	0	2	2	3	3	3	3	3	3	3	3			**31**
12	10	MP	S.A.	100	100	50					1	1	0	0	0	1	0	1	1	0	1	0	1	1.5	3	3	**14.5**
13	12	MP	S.A.	100	100	50	15			300	1	0	1	0	0	0	0	0	1	2	1	1	2	1	2	2	**14**

Tabel 25. Combination and effect of different biocides on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Results are color-coded according to biocidal effect evaluated from their summed score: Red, >40; Yellow, 30-40; Green, <30. Test medium: TM = simplified test formulation; MP = PVA based mat wall paint.

Row No.	Sample no.	Test medium	Amine	ZnPt	Bronopol	CMIT/MIT	MIT	DDAC	BIT	Score P.A.	Score S.A.	Score Bacteria	Isothiazolinone Concentration (ppm)	Total Biocide Concentration, (ppm)
1	TM	TM				4.9	33		163				200.9	200.9
2	PATM	TM				4.9	33		163	35	33	68	200.9	200.9
3	15	TM		100	50					18.5	16	34.5	0	150
4	1	TM	100	100	50					23	21	44	0	250
5	5	TM	100	100	50				300	19	14	33	300	550
6	3	TM	100	100	50	15				15	12	**27**	15	265
7	7	TM	100	100	50	15			300	18	15	33	315	565
8	13	TM	100			15	50		300	18	15	33	365	465
9	17	TM	100					1000		13	13	**26**	0	1100
10	MP	MP				5.7			75				80.7	80.7
11	PA7	MP				5.7			75	33	31	64	80.7	80.7
12	9	MP	100	100	50					12	14.5	26.5	0	250
13	11	MP	100	100	50	15			300	15.5	14	29.5	315	565

The best results on the test paint (TM) were seen using the following biocide combinations:
- Amine, ZnPt, Bronopol, CMIT/MIT (row 6)
- DDAC, Amine (row 9)

Using PVA based mat wall paint the best results were seen with:

- Amine, ZnPt, Bronopol (row 12)
- Amine, ZnPt, Bronopol, CMIT/MIT, BIT (row 13)

Two of the biocide combinations did not include isothiazolinones, and therefore they do comply with the goal of the project to reduce or completely avoid the need for preservation using isothiazolinones. The concentration of biocides can be difficult to compare directly, but very different concentrations were used to obtain preservation. The DDAC concentrations of 1000 ppm were very high compared to the concentrations of the other biocides (row 9) that were used. Further experiments were needed to evaluate the use of DDAC at even lower concentrations. The biocide concentration used in row 13 was more than twice of the concentration used in row 12.

Therefore, it was necessary to further optimize the combination and concentration of biocides.

3.6 Biocide tests in the PVA based mat wall paint system

3.6.1 Combination of biocides

An extensive set-up was constructed to test the combination of different biocides - including the ones tested in the previous section. The biocidal effects of the different combinations were tested with 5 subsequent inoculations with a mix of *P. aeruginosa* DSM1253 and *S.aureus* DSM799. The number of viable bacteria was analysed prior to each new inoculation.

The number of viable bacteria was evaluated and scored according to the number of viable bacteria present, detected as the number of bacterial colonies (CFU) on agar plates: 0, no CFU; 1, 1-10 CFU; 2, 10-100 CFU; 3; >100; 4, too numerous to count (TNTC).

Challenge tests like these are subject to some variation and interpretation of results, and scores should therefore be used as indications and not as absolute truths. The results are shown in Table 26.

Table 26. Overview of the biocidal effect from different combination of biocides for the first testing. MP = PVA based mat wall paint. The color coding: Red, high level of viable bacteria; Yellow, medium level of viable bacteria; Green, low level of viable bacteria.

Row no.	MP	Bacteria	ZnPt	Amine	DDAC	Bronopol	BIT	MIT	CMIT/MIT	LiCO3	pH	Total biocid	Total isoT.	Before Inoculation	1.	2.	3.	4.	5.	
1	1	X	192.0	99.0								291.0		0	0	0	1	0	1	**0.4**
2	2	X	96.0	99.0								195.0		0	0	1	1	1	4	1.4
3	3	X	48.0	99.0								147.0		0	4	4	4	4	4	4
4	4	X	192.0	99.0		99.0						390.0		1	0	1	0	0	2	**0.6**
5	5	X	96.0	99.0		99.0						294.0		1	0	1	0	0	4	1
6	6	X	48.0	99.0		99.0						246.0		0	1	0	0	4	4	1.8
7	7	X	192.0		300.0							492.0		0	0	1	1	1	1	0.8
8	8	X	96.0		300.0							396.0		0	0	0	0	0	1	**0.2**
9	9	X	48.0		300.0							348.0		1	4	4	4	4	4	4
10	***16***	***X***		***99.0***			***290.65***	***79.75***	***8.35***			***477.75***	***378.75***	***0***	***0***	***1***	***0***	***1***	***1***	***0.6***
11	17	X		49.5			145.33	39.88	4.17			238.87	189.37	0	2	1	0	2	2	1.4
12	18	X		24.75			72.66	19.94	2.09			119.44	94.69	0	2	0	1	4	2	1.8
13		Uncon. + Bac X												1	4	4	4	4	4	4
14		Uncon. - bac												1	3	4	4	4	4	3.8

For the alternative biocide-solutions, the combination of ZnPt and the amine is a isothiazolinone-free combination that shows a good biocidal effect at 291 ppm biocide (row 1-3). Reducing the concentration of ZnPt from 192 ppm to 96 ppm, increased the bacterial growth all through the incubation period. Adding Bronopol to this combination (row 4-6, ZnPt, Amine, Bronopol), decreased the bacterial growth at 96 ppm ZnPt, but did not inhibit complete bacteria overgrowth (score 4) after 5th inoculation. Therefore, ZnPt should be added in concentrations of 196 ppm when combined with the amine. The addition of Bronopol had a limited additional effect on the tested organisms.
When combining ZnPt with DDAC, the concentration of ZnPt could be reduced to 96 ppm combined with 300 ppm DDAC. An effective concentration of 300 pm DDAC reduced the con-

centration with more than 70% compared to the incubations with 1000 ppm DDAC (row 7-9). Further reduction of DDAC to 100 and 200 ppm, resulted in insufficient preservation, and that is why the concentration cannot be reduced more than 300 ppm DDAC (Table 27, row 16-17).

DDAC seems to be an effective biocide, but its cationic nature is problematic in the present paint formulations since they employ anionic components. The mix of anionic and cationic components increases the risk of aggregation and subsequent production of paint that is unfit for use.

Row 10 represents a mixture of isothiazolinones and an amine. Reducing the concentration of isothiazolinones by 50% and 25% decreased the preservative effect of the biocide-system dramatically and did therefore not represent a viable solution.

The documented effectiveness and broad spectrum of target-organisms of isothiazolinones make them very hard to replace. The activities of the project have therefore explored possibilities for more long-term goals where the use of added isothiazolinones is completely omitted, and investigated products where isothaizolinones are used with focus on minimizing the concentration of MIT. Further tests were therefore performed to evaluate the use of different isothiazolinones, in combination with other types of biocides. Results are shown in Table 27.

The example using high concentration of MIT, CMIT/MIT, BIT and amine had a good preservative effect, but resulted in a total biocide concentration of 477.75 ppm whereof 378.75 ppm was isothiazolinones (row 18). Further reduction of the concentration of the preservative lowered the preservative effect to an unsatisfactory level (rows 19-20).

Preservation without MIT was tested with a combination of BIT with both the amine and ZnPt, which resulted in the good preservation when using concentrations of BIT to 275.48 ppm and 137.74 ppm (row 13-14). Even further reduction of the concentration decreased the effectiveness of the preservation (row 15). Addition of CMIT/MIT to the combination of BIT, ZnPt and the amine had limited additional effect (rows 13-14) except at the test with the lowest concentration of BIT, where the addition of CMIT/MIT had some effect (row 15). The effect with very low concentrations of CMIT/MIT could be a result of the test variation. However, in previous tests combinations with CMIT/MIT have been very efficient (Tabel 25).

Preservation with BIT as the only isothiazolinone showed the best effect when combined with ZnPt (rows 3-4). The combination of BIT and the amine performed better than equivalent concentrations of ZnPt and the amine, which was insufficient for product preservation (row 1-2). Lowered concentrations of the amine and ZnPt (shown in Table 26, row 2) actually gave an even higher score than in the previous tests, perhaps because the paint was contaminated even before inoculation with *S. aureus* and *P. aeruginosa*. The use of 196 ppm ZnPt in combination with the amine is therefore necessary for sufficient preservation.

Using BIT instead of an amine proved to be more effective, while maintaining the same total biocide concentrations (rows 3 and 4). Substituting an amine with 100 ppm BIT might be more efficient, but will also require classification according to EUH208.

Table 27. Overview of biocidal effect from different combinations of biocides for the second tests. MP = PVA based mat wall paint. B.I.: Before Inoculation. The color coding: Red, high level of viable bacteria; Yellow, medium level of viable bacteria; Green, low level of viable bacteria.

MP	Bacteria	NaPt	$LiCO_3$	CMIT/MIT	MIT	BIT	ZnPt	Amine	DDAC	Bronopol	Total biocid	Total isoT.	B.I.	1.	2.	3.	4.	5.1	5.7	
1	X						96.00	99.0			195.00	0.00	1	4	4	4	4	4	4	4.0
2	X						48.00	99.0			147.00	0.00	3	4	4	4	4	4	4	4.0
3	X					100.00	96.00				196.00	100.00	1	0	0	3	0	1	0	**0.7**
4	X					100.00	48.00				148.00	100.00	0	1	0	3	2	2	4	2.0
7	X					190.00		110.00			300.00	190.00	1	1	1	2	0	4	1	**1.5**
8	X	20				95.00		55.0			170.00	95.00	2	2	1	2	1	4	2	2.0
9	X					95.00	20.16	55.0			170.16	95.00	2	0	0	3	4	4	1	2.0
10	X			8.35		270.75	99.75	99.0			477.85	279.10	0	2	0	3	1	1	0	**1.2**
11	X			4.17		135.38	49.88	49.5			238.92	139.55	1	1	1	2	0	3	0	**1.2**
12	X			2.09		67.69	24.94	24.75			119.46	69.77	1	2	0	3	0	4	0	**1.5**
13	X					275.48	104.48	99.0			478.95	275.48	1	0	0	1	1	2	2	**1.0**
14	X					137.74	52.24	49.5			239.48	137.74	1	0	0	4	0	3	0	**1.2**
15	X					68.87	26.12	24.75			119.74	68.87	0	0	1	1	2	4	4	2.0
16	X						96.00		200.00		296.00	0.00	1	4	4	4	4	4	4	4.0
17	X						96.00		100.00		196.00	0.00	1	4	4	4	4	4	4	4.0
18	X			8.35	79.75	290.65		99.0			477.75	378.75	1	0	1	0	0	4	0	**0.8**
19	X			4.17	39.88	145.33		49.50			238.87	189.37	1	0	0	1	4	4	2	1.8
20	X			2.09	19.94	72.66		24.75			119.44	94.69	1	1	0	2	4	4	4	2.5
Uncon. + Bac													2	4	4	4	4	--	4	4.0
Uncon. - bac	X												3	4	4	4	4	--	4	4.0

Apart from the single experiment that showed an effect when BIT was combined with ZnPt, very high concentrations of isothiazolinones were necessary for sufficient product presser-vation. Reducing the concentration of isothiazolinone, markedly reduced the preservative effect. The best comprise between effect and biocide/isothiazolinone concentration was ob-tained when using combinations of CMIT/MIT (2.09 ppm), BIT (67.69 ppm), ZnPt (24.94 ppm) and amine (24.75 ppm) (row 12) or BIT (100 ppm) and ZnPt (96.00 ppm) (row 3). The follow-ing combinations were selected for further evaluation with regard to their effect on health and environment: The model system combination of CMIT/MIT, MIT, BIT and amine (row 18), the combination of CMIT/MIT, BIT, ZnPt and amine and BIT, ZnPt and amine, a MIT free preser-vation system (row 13).

3.6.2 Effect on pH and Lithium on biocidal activity

Substances that are not classified as biocides, but that could potentially enhance the effect of biocides, were tested. Different combinations of biocides were formulated in a PVA based mat wall paint matrix in combination with lithium ions and in formulations with elevated pH (Table 28).

Comparison of sublethal concentrations of the amine and ZnPt at different pH levels, showed no effect (rows 13-15) and all had scores above >1.5.

Combining lithium ions with ZnPt and the amine did not show effects in concentrations down to 96 ppm ZnPt and 99 ppm amine (row 1-2 and row 10-11). The incubation with the lowest concentration of the amine and ZnPt showed a surprising effect of the biocide concentration. That effect could be a question of test variation since the effect of lithium was less pronounced at higher concentrations of the amine and ZnPt.

Table 28. Overview of the biocidal effect from different combinations of biocides combined with lithium or elevated pH. Rows 1-3 are similar to those shown in Table 26. MP = PVA based mat wall paint. The color coding: Red, high level of viable bacteria; Yellow, medium level of viable bacteria; Green, low level of viable bacteria.

MP	Bacteria	ZnPt	Amine	DDAC	Brono-pol	BIT	MIT	CMIT/MIT	LiCO3	pH	Total biocid	Total isoT.	Before inocula-tion	1.	2.	3.	4.	5.	
1	X	192.0	99.0	0.0							291.0		0	0	0	1	0	1	**0.4**
2	X	96.0	99.0	0.0							195.0		0	0	1	1	1	4	1.4
3	X	48.0	99.0	0.0							147.0		0	4	4	4	4	4	4
10	X	192.0	99.0	0.0					500.0		291.0		0	1	0	1	0	1	**0.6**
11	X	96.0	99.0	0.0					500.0		195.0		0	0	3	1	1	4	1.8
12	X	48.0	99.0	0.0					500.0		147.0		1	0	0	0	0	3	**0.6**
13	X	48.0	99.0	0.0						9.0	147.0		4	1	0	0	4	3	1.6
14	X	48.0	99.0	0.0						10.0	147.0		1	3	3	0	4	2	2.4
15	X	48.0	99.0	0.0						11.0	147.0		1	4	1	1	1	2	1.8
	Uncon. + Bac X												1	4	4	4	4	4	4
	Uncon. - bac												1	3	4	4	4	4	3.8

Further elucidation of the effect of lithium was investigated with a combination of BIT and ZnPt. Again, some effect of lithium was shown only in incubations with low concentrations of biocide (Table 29, row 4 compared to row 6).

Table 29. Overview of the biocidal effect from different combination of biocides combined with lithium. MP = PVA based mat wall paint. The color coding: Red, high level of viable bacteria; Yellow, medium level of viable bacteria; Green, low level of viable bacteria.

MP	Bacteria	NaPt	Li	CMIT/MIT	MIT	BIT	ZnPt	Amine	DDAC	Bronopol	Total biocid	Total isoT.	B.I.	1.	2.	3.	4.	5.1	5.7	
3	X		0.00	0.00		100.00	96.00	0.00	0.00	0.00	196.00	100.00	1	0	0	3	0	1	0	**0.7**
4	X		0.00	0.00		100.00	48.00	0.00	0.00	0.00	148.00	100.00	0	1	0	3	2	2	4	2.0
5	X		500.00	0.00		100.00	96.00	0.00	0.00	0.00	196.00	100.00	1	2	1	2	0	2	0	**1.2**
6	X		500.00	0.00		100.00	48.00	0.00	0.00	0.00	148.00	100.00	1	1	1	0	4	2	0	**1.3**
Uncon. + Bac													2	4	4	4	4	--	4	4.0
Uncon. - bac	X												3	4	4	4	4	--	4	4.0

Based on the test results shown above, pH was shown not to have sufficient effect, and was therefore not used for further product development. Lithium was shown to have some effect at low concentrations of biocide and was therefore evaluated with regard to its effect on CLP classification.

3.7 Overall evaluation of the combination of different biocides

3.7.1 Evaluation of biocides and non-biocidal compounds

Based on the concentration and type of biocides, the biocides were assessed for use in paints (e.g. CLP-classification and MAL-code) and health and environmental (Pregnant status, hormonal effect, BPR etc.). The biocides were evaluated both as single substances and in combination with each other(See the total evaluation in Appendix 3). The individual evaluation is shown in Tabel 30.

There is already focus on MIT in consumer products and the media due to a significantly increased number of allergy cases on this substance.Therefore, the classification of MIT will be intensified further within the next fewyears to demand a H317-label if the concentration is 15 ppm or higher.

Although the use of MIT is omitted, the use of BIT still requires CLP-classification using the EUH208, that may be unfavourable in the future although the vapour pressure for BIT is mich lower compared to MIT.. Further assessment of the developed biocide combinations without isothiazolinones show that some of these combinations could be promising candidates for biocide solutions in future products. The use of DDAC does not lower the overall concentration of biocides and furthermore presents some challenges regarding formulation of the product. The most likely candidate will be the combination of zinc pyrithione with the amine since this represents a complete absence of isothiazolinones and has a 40% reduction of the total biocide concentration in the end-product. But it is worth noting that zinc pyrithione might have an additional classification in the future with Rep. Tox 1B, and therefore will be subjected to REACH Appendix XVII - Conditions of Restriction, entry 30.

If this should happen, then zinc pyrithione may not be used in products to private consumers in levels that are equal or exceed the limit for the classification irrespective of which product type it falls under in BPR. The generic limit for Repr. 1B is 0.3 %, but Flügger generally avoids chemical compounds that are classified as category 1 for Carc., Mut. or Repr. due to their negative effect on health and environment.

Tabel 30. Evaluation of the biocides tested with regard to toxicology and classification.

Abb. for chemical compound	CAS No.	REACH (REACH. reg. No.)	Classification	MAL No.	TEB	VOC	Pregnant status (Arbejdsmiljøhuset)	Biocid reg.	Hormone disruptor effects
CMIT/MIT (3:1)	55965-84-9[I]	No	Acute Tox. 3, Skin Corr. 1B, Skin Sens. 1, Eye Dam. 1, Aquatic Acute 1, Aquatic Chronic 1 // H301, H311, H314, H317, H318, H331, H400, H410 , (M-acute = 10), (M-chronic = 1)	0	>= 0.003% => -3, >= 1% => -6	No	OK	OK (Approved in PT6 and on art. 95)	No
BIT	2634-33-5[II]	No	Acute Tox. 4, Skin Irrit. 2, Skin Sens. 1, Eye Dam. 1 Aquatic Acute 1 Aquatic Chronic 3 // H302, H315, H317, H318, H400, H412, (M-acute = 1)	0	>= 1% => -3	No	OK	OK (under review PT6 and on art. 95)	No
MIT	2682-20-4[III]	No	Acute Tox. 3, Skin Corr. 1B, Skin Sens. 1A, Eye Dam. 1, STOT SE 3 Aquatic Acute 1 Aquatic Chronic 2 // H301, H311, H314, H317, H318, H335, H400, H411, (M-acute = 1)	0	>= 0.03% => -3, >= 1% => -6	No	OK	OK (under review PT6 and on art. 95)	No
Bronopol	52-51-7[IV]	01-2119980938-15	Self-react. D Acute Tox. 3 Acute Tox. 4, Skin Irrit. 2, Eye Dam. 1, STOT SE 3 Aquatic Acute 1 Aquatic Chronic 2 // H242, H301, H312, H315, H318, H331, H335, H400, H411 (M-acute = 10)	0 (under App. 2A*)	> 0.2% => -6 (under app. 3A*)	ja	* AF: < 0.1%, MB: < 0.4%, MIA: >= 0.4% (pH < 9.0)	OK (under review PT6 and on art. 95)	No
ZnPt	13463-41-7[V]	01-2119511196-46	Acute Tox. 3, Eye Dam. 1, Acute Tox. 3 Aquatic Acute 1 Aquatic Chronic 1 // H301, H318, H331, H400, H410 (M-acute = 100) (M-chronic = 10)	0	>= 1% => -3	No	OK	OK (under review PT6 and on art. 95)	No
DDAC	7173-51-5	01-2119945987-15	Acute Tox. 3, Skin Corr. 1B, Eye Dam. 1 Aquatic Acute 1 Aquatic Chronic 2 // H301, H314, H318, H400, H411 (M-acute = 10)	0	>=1% => -3	No	OK	OK (under review PT6 and on art. 95)	No
Amine	-	-	Acute Tox. 3, Skin Corr. 1B, Eye Dam. 1, STOT RE 2 Aquatic Acute 1 Aquatic Chronic 1 // H301, H314, H318, H373, H400, H410, (M-acute = 10), (M-chronic = 1)	0	>= 0.2% => -6	No	OK	OK (under review PT6 and on art. 95)	No

[I] >= 15 ppm => H317, 1,5-15 ppm EUH208, [II] >= 500 ppm => H317, 50-500 ppm => EUH208, [III] >= 1000 ppm => H317, >= 100 ppm => EUH208, Flügger has chosen to follow CEPE's recommondation to use EUH208 when MIT >= 15 ppm. It is expected to be lowered >= 15 ppm for H317 in 2019, [IV] Reach reg. has H302, H312, H315, H318, H335, H400, H411. Not formaldehyde releaser (iflg. Lanxess), [V] In 2019 it is expected that an addition classifaction will come with Rep.tox 1B and M-faktor akut = 1000, * Executive Order no. 301 of the 13th of May 1993 on the determination of code numbers, The Danish Working Environment Service

3.7.2 Combinations of different biocides

In bacterial growth media, biocides showed growth inhibition already at very low concentrations, proving that the combination of different biocides has a promising effect. Further evaluation in paint matrices employed tests under rigorous conditions with sequential additions of bacteria to formulations containing different combinations of biocides.

The most promising combinations were evaluated and are shown in Table 31. The assessment and the resulting label after adding the different biocidal packages to the standard recipes differed depending on the biocides and the concentration.

Table 31. Biocide combinations selected for health and environmental assessment.

Combination	CMIT/MIT	MIT	BIT	ZnPt	Amine	DDAC	Bronopol	
1	x	x	x		x			Model system
2	x		x	x	x			Upscale test
3			x	x	x			Free for MIT and CMIT/MIT
4				x	x		x	IT-free
5				x		x		IT-free Formulation issues
6				x	x			IT-free

By combining the use of BIT with an amine and ZnPt, the problematic MIT could be omitted, and the concentration of isothiazolinones could hereby be decreased (Combination 3).

Bronopol contributes negatively to the pregnancy assessment of the final product, which pregnant painters may use. However, as all the contributions in a recipe are summed up, it is hard to say how much bronopol can be added.

The health and environmental assessments are often based on the sum of all contributions in a paint when combining multiple biocides. To obtain comparable outcomes, the biocidal combinations were added to a standard PVA-based mat wall paint recipe (with no added biocidal product).

This recipe includes some raw materials (binders etc. that also contain isothiazolinones, so the contributions from these are also included). In Appendix 3, the added total concentration of the individual biocidal product is shown with regard to the biocidal commodity combinations described on the left side of the table (with wt.%).

All combinations from the standard formula shown in Appendix 3 meet the existing requirements of Flügger, except combination 5 using DDAC. The combination has a too high second summed value for ECO label, which must not exceed 600 ppm. But it is worth noting that the reason why combination 5 exceeds the biocidal concentration limit is due to the contribution of biocides from the raw materials and not the tested biocide combination. Since neither DDAC nor zinc pyrithione is classified under the EUH208 regulation, the combination would still be relevant but not in relation to ECO-label due to the added biocides from the raw material supplier.

All combinations have the CLP classification EUH208 (BIT and CMIT/MIT) due to the biocide added to the binders by the supplier. This is the same phrase that is on Flügger's aqueous indoor products and thus acceptable. In the same way, VOC requirements and pregnancy status requirements are fulfilled in all combinations. Combination 4-6 would not be labelled with the EUH208 regulation if it was possible to use unpreserved raw materials or raw materials that are not preserved with isothiazolinones such as BIT, MIT or CMIT/MIT.

With regard to the MAL-code, all combinations give the code 00-1. Combination 1 has the highest contribution to -3 on 0.9 (If the sum reaches 1, then the product will be labelled with code 00-3 instead).

Rigorous testing of the different biocide combinations in water-based paint has shown the potential of these combinations in realistic situations. The next step is the formulation and subsequent approval of a commercial product. It is worth noting that the outcome of the toxicological assessment may alter if another paint recipe was used for the assessment than the mat PVA paint.

3.7.3 Combination of biocides with non-biocidal compounds

The use of non-biocidal substance from the cosmetics industry and the paint from coating industry in combination with different biocides did not have any significant effect. Neither did the use of elevated pH up to pH 11. At pH above 11.5, products have to be labelled as corrosive.

The addition of lithium did show some promising effect at low concentration. The use of lithium with regard to CLP-classification was evaluated. Despite a potential effect, the price of lithium is at the moment too high, and a product using 500 ppm of lithium is therefore not an optimal solution.

4. Reduced water potential as alternative means of conservation

Solutes, such as salts and sugars have been used as product preservation in the food and feed industry for many years.
In this part of the project, the effects on bacterial viability and growth in liquid medium and water-based paints were tested by reducing the concentration of water, or using solutes such as potassium chloride and urea, . Although the addition of solutes to some extent could inhibit growth, the required concentrations to do so were so high that the performance of the paint would be severely hampered.
The potential of high-solid paints (HSP), where water is excluded, was furthermore tested with no effect on water activity and only limited effect on bacterial viability compared to the other solutions developed and tested in the project.

4.1 Introduction

Water is an essential part of all living organisms and thus a prerequisite for life. The deficiency of water can therefore be lethal to both macro- and microorganisms. The availability of water can be described by the term "water activity" (a_w), which can be calculated as:

$$a_W = \frac{p_w}{p_W^0} = \frac{RH}{100}$$

Where p_W and p_W^0 are the vapour pressures of water in the system and of pure liquid water at the same temperature respectively. Distilled water has a value of one. RH is defined as the percent relative humidity of the air layer in equilibrium with the sample (Sereno *et al*, 2001). The presence of water is of utmost importance for the viability of the microbial cells, and decreased water activity limits microbial growth and as a final consequence, microbial viability, leading to cell-death.

Different organisms have different ways of coping with water stress, like producing compatible solutes inside their cells to counter decreased water activity of the surrounding medium (osmoregulation). The limits for growth , i.e. at which water activity organisms can proliferate , differs from types and species of microorganisms. In general, Gram-negative bacteria seem to be more susceptible to water stress (decreased water activity) than Gram-positive bacteria. Most bacteria are limited below a_w = 0.85 whereas yeasts and molds can tolerate water activities of 0.7-0.6. Table 32 shows the tolerance-level of different bacteria.

Table 32. Minimum water activity for growth of various bacteria using sodium chloride as solute (Sperber, 1983).

Organisms	Minimum water activity for growth
Moraxella/Acinetobacter sp.	*0.99*
Clostridium perfringens	*0.970*

Pseudomonas fluorescens	*0.957*
Escherichia coli	*0.950*
Bacillus cereus	*0.920*
Bacillus subtilis	*0.900*
Staphylococcus aureus	*0.860*

Historically, water activity as a controlling factor for microbial growth has been used in food and feed to prevent microbial spoilage. The conservation of foods has been achieved by e.g. salting of meat to hamper microbial spoilage. Today, the mechanisms behind this are recognized as to be decreasing water activity: water activity can be decreased by the addition of both organic and inorganic soluble compounds (solutes), e.g. salts such as sodium chloride or potassium chloride and organic molecules such as glucose or urea. By affecting the water activity, it is possible to affect the microbial growth and thus reduce the need for biocides. There is also evidence that water-stressed organisms are more susceptible to other modes of conservation like high or low pH, temperature, or chemical preservatives.

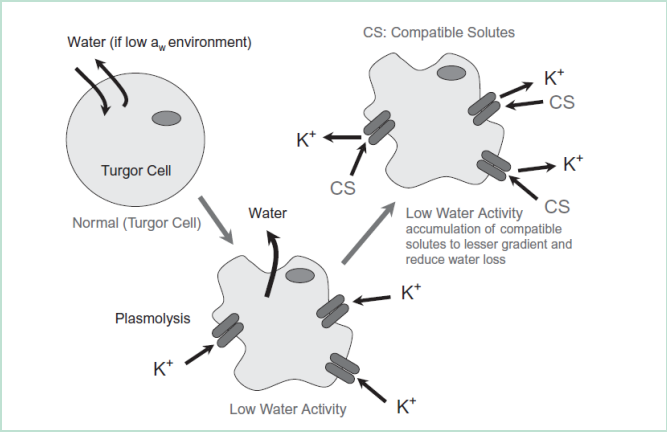


Figure 13. Microbial response to low water activity (Rahman, 2009)

The addition of solutes affects the availability and activity of water, which in turn influence the hydrostatic pressure inside the microbial cells (Figure 13). In addition to the effect on the hydrostatic pressure, some solutes do not only affect the water activity,but will also affect internal structure and processes of the microbial cells. Especially proteins, which are large macromolecular structures and are very dependent on optimal water activity to ensure proper folding and activity. Correct protein folding is dependent on hydrogen bonds to stabilize structure. Solutes can be divided into three categories according to their effect on both water molecules and hydrogen bonds of biological macromolecules (biopolymers) such as proteins and nucleic acids (DNA, RNA):
(1) *Chaotrophic* solutes, which destabilize hydrogen bonds
(2) Solutes with no effect on hydrogen bond stability
(3) *Kosmotropic* solutes with stabilizing effect on hydrogen bond stability and consequently macromolecular structure (Record *et al*, 2013).

The effect of solutes is ranked in a Hofmeister series according to their effect on proteins (Figure 14).

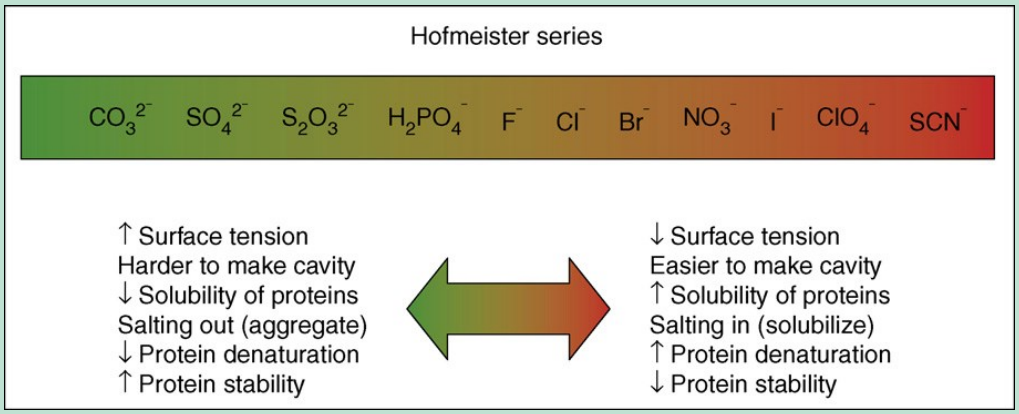


Figure 14. Hofmeister series showing the effect of salts on the stability of Proteins. Solutes can have destabilizing effect (left), no effect (Middle) or stabilizing effect on macromulecular structure and stability. (Zhang & Cremer, 2006 - http://biowiki.ucdavis.edu/)

Degradation can be hampered by lowering the activity of water, but according to the Hofmeister series, some solutes will hamper biological activity better than others. A solute like glycerol depresses water activity, but may enter bacterial cells without causing osmostic stress (Mathlouthi, 2001). Studies indicate that water activity and osmostic stress is only part of the effect, and that the addition of chaotropic agents like LiCl, urea, ethylene glycol and some anions, have destabilizing effects on cellular macromolecules and bacterial growth (Hallsworth *et al*, 2003; Lo Nostro *et al.*, 2005)). It is furthermore indicated that this chaotropic effect can be somewhat alleviated by the addition of kosmotropic agents (Williams & Hallsworth, 2003), a fact that might be noteworthy when working in complex matrices like water-based paints. The choice of solute might therefore affect macromolecular stability, microbial viability and growth according to its ranking on the Hofmeister series.

4.2 Screening of water activity in different formulations

To elucidate the effect of water activity on product contamination, products with different composition with different levels of contamination were examined. These contaminations can have different causes, e.g. microbial load from production site and raw materials, different biological degradability of raw materials, end-user handling, final biocide concentration and stability or water activity. Analysis showed only small differences in the water activity (a_w 0.980-0.993) between these products (Table 33). Although the water activities in some of these products is potentially sufficiently low to inhibit the growth of some microorganisms, it will not be sufficient for inhibiting most bacteria or fungi (Table 33). Therefore, it is necessary to add supplementary solutes to decrease the water activity of the paints to a level which will inhibit microbial growth sufficiently. This decrease in water activity could be obtained by adding different solutes.

Table 33. Analysis of water activity for different formulations of water-based paints. The number of contaminated products (contamination level) is defined as none/low, medium or high based on the number of product reclamations.

Product	aw	Temperature	Contamination level
Paint A	0.986	22.2	None/Low
Paint B	0.984	22.3	None/Low
Paint C	0.987	22.3	None/Low
Paint D	0.989	22.6	Medium
Paint E	0.993	22.7	None/Low
Paint F	0.980	22.8	None/Low

Product	aw	Temperature	Contamination level
Reference	0.839	22.1	-

4.3 Decreasing water activity by adding solutes

4.3.1 Analysis of water activity

Initial experiments focused on studying different solutes and their effect on water activity. Experiments were carried out in both water and water-based paint.
The stability of the paint was studied by visual characterization, and did not show significant signs of instability after mixing and letting the mixture set for 5-10 minutes, but more extensive tests would be needed for evaluation of long-term effects on product stability.

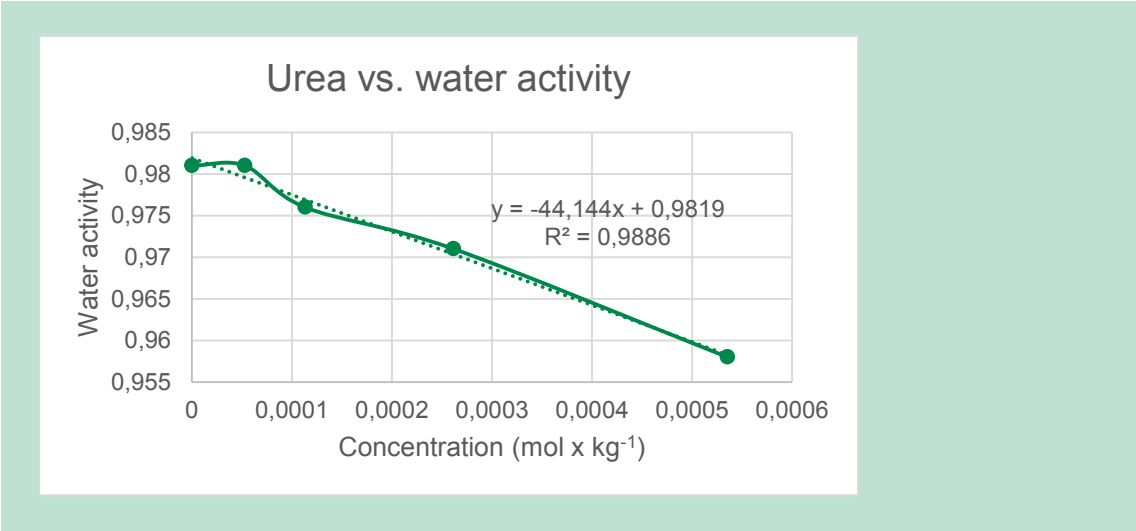


Figure 15. Effect of urea concentration on water activity in the PVA-based mat wall paint.

The initial water activity of the studied paint was 0.982, so below that of distilled water. Extrapolation of the results (Figure 15) show that a final concentration 1.86 M of urea is needed for water activity below 0.90 which corresponds to the addition of about 112 g urea/ kg paint. The effect of adding the salt KCl as solute to PVA-based mat wall paint with 50 wt% water is shown in Figure 16.

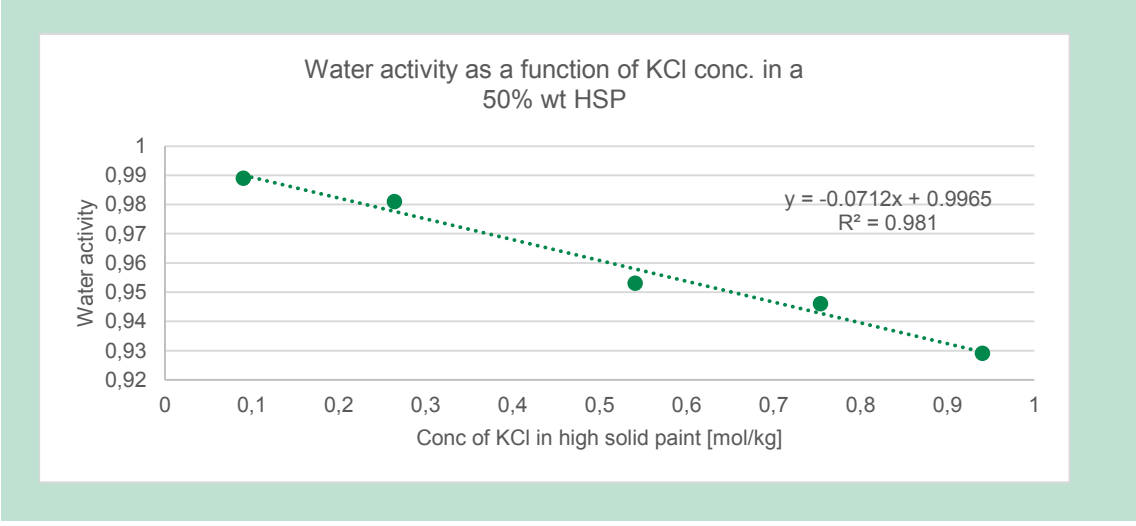


Figure 16. Water activity as function of increasing KCl concentration in high solid paint.

The concentration of KCl to obtain water activity of 0.9 is 1.4 mol x kg^{-1} corresponding to the addition of 104.7 g of KCl/kg paint.

The concentrations of both KCl and urea needed for sufficiently decreasing water activity, is very high and in these concentrations will affect paint performance and quality.

4.3.2 Effects of water activity on bacterial growth

Looking at the Hofmeister series, different solutes could be identified as chaotrophic or with no effect. Experiments with urea and $CaCl_2$ (chaotropic agents) and KCl (a solute with no kosmotropic or chaotropic effect)showed their effect on water activity. The effect of $CaCl_2$ is most pronounced, possibly because the dissociated form consists of three ions (One Ca-ion and two Cl-ions) (Figure 17).

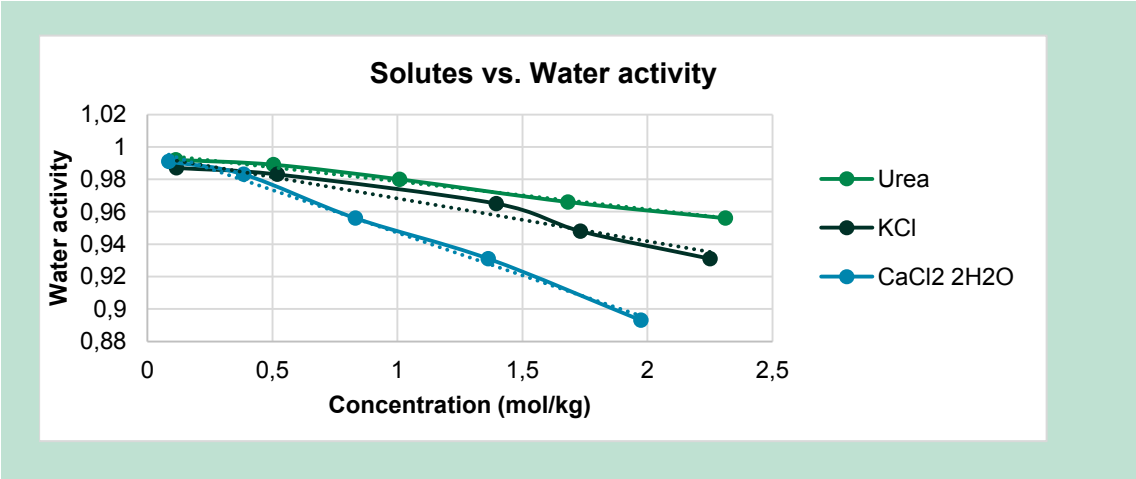


Figure 17. Effect of solute concentrations (in distilled water) on water activity.

The solutes' effect on growth of *Pseudomonas aeruginosa* was furthermore studied to determine the inhibitory concentrations of the urea, KCl and $CaCl_2$, and to elucidate the effect of both water activity and chaotropic effect.

It was found that urea and KCl inhibit growth of *P. aeruginosa*, whereas $CaCl_2$ stimulates growth (Figure 18 and Figure 19). The latter might be due to the fact that calcium helps to stabilize cell walls in many microorganisms (Madigan, Chap 5, 12th Ed). Although only a very limited number of solutes were tested here, the results indicate that predicition of the biological effect of solutes cannot be based solely on its predicted kosmotropic/chaotropic effect – at least not in the concentrations tested here.

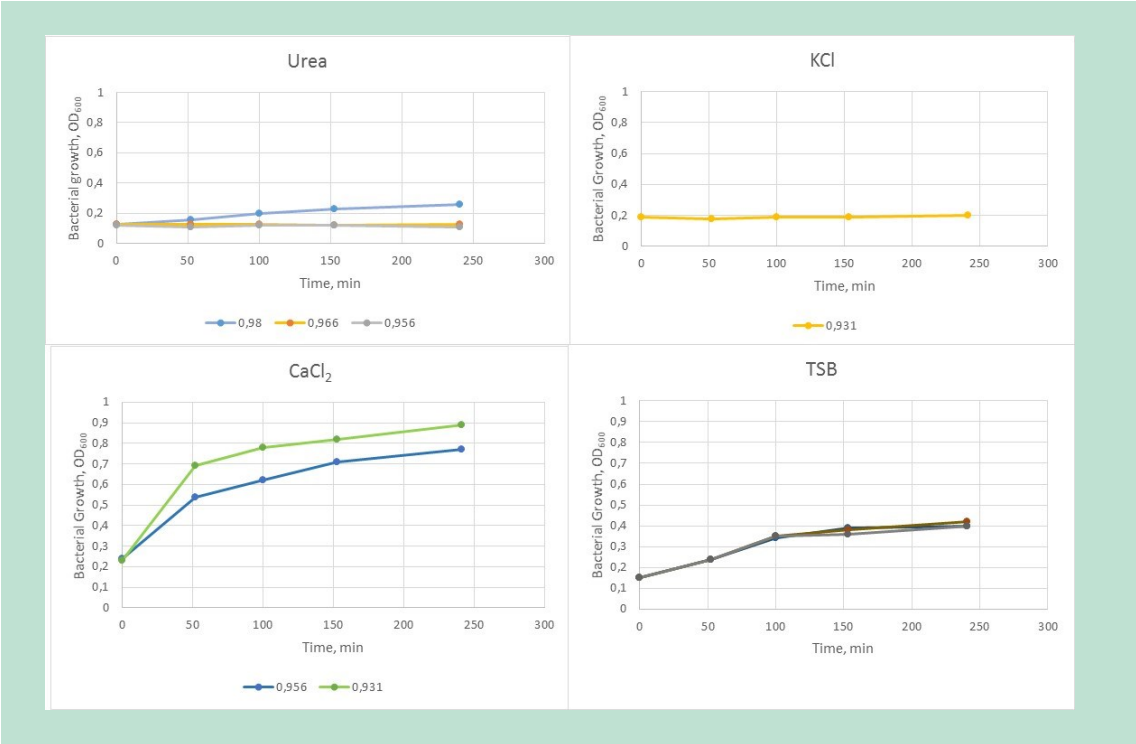


Figure 18. Growth of *P. aeruginosa* in dilute growth media amended with tryptic soy broth (1:10) and urea, potassium chloride (KCl) or Calcium dichloride ($CaCl_2$) to obtain different water activities. Growth in Tryptic soy broth (1:10) was employed as control.

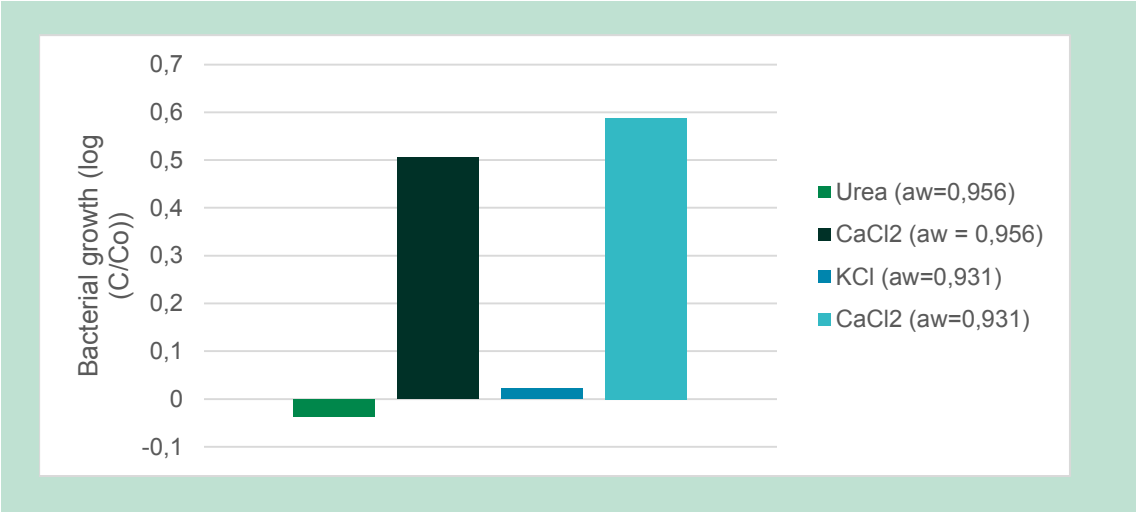


Figure 19. Growth of *P. aeruginosa* in dilute growth media amended with tryptic soy broth (1:10) and urea, potassium chloride (KCl) or Calcium dichloride (CaCl2). Growth is compared to solutions with similar water activity. Growth calculations are based on initial bacterial concentrations and final bacterial concentrations during a growth-period of 4 hours.

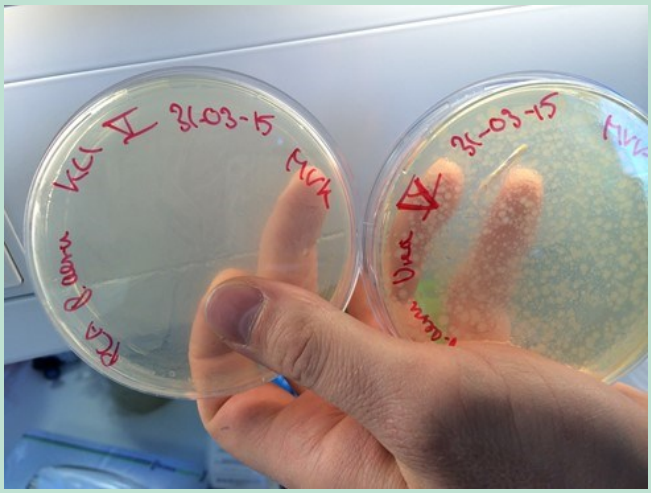


Figure 20. Streaking of agar plates after incubations with urea and potassium chloride. Bacterial colonies are observed on both plates.

Streaking of the bacterial cultures on agar plates (PCA) shows that although growth was hampered by the addition of solution, the bacterial cultures were still viable (Figure 20). Hence, it can be deduced that high concentrations of solutes could inhibit growth, but not kill all cells.

Furthermore, the results shown in Figure 17 indicate that very high concentrations of solutes are necessary to lower water activity in a regular paint with a water concentration of 50 wt%.

4.3.3 Combination of solutes and biocides

Although the concentration of solutes needed for complete inhibition of bacterial growth is high, an increased effect might be obtained by combining solutes with the use of biocides in order to stress bacterial cells on different levels.

To study the combined effect of biocides and solutes, cultures of actively growing *Pseudomonas aeruginosa* (5 x 10^7 CFU/mL) were incubated with different concentrations of either KCl or urea at equal a_w values ($\pm$0.001) combined with 0, 2.5 or 5 ppm BIT (Table 34). The combined effect of solutes and BIT was studied in bacterial growth medium (Tryptic Soy Broth) using a Varioskan Lux multi-well plate-reader (Thermo Scientific).

Bacterial growth was analysed as increase in optical density of the bacterial cultures (OD_{600}).

Table 34. Concentration and water activity of incubations with urea or potasium chloride. Incubations contained either 0, 2.5 or 5 ppm BIT. a_w values were calculated from linear regressions of the data shown in Figure 17.

	Solution 1	Solution 2
Urea		
Concentration (mol/L)	0.010	0.028
Water activity (a_w)	0.994	0.991
KCl		
Concentration (mol/L)	0.0052	0.017
Water activity (a_w)	0.993	0.990

After comparing the bacterial growth at a_w=1 to that of a_w=0.993 and a_w=0.990, no effect could be observed on combining KCl with low concentrations of BIT (Figure 21). Although there was

some variation of the data, the effect of using urea was also limited when comparing growth without urea (a_w=1) to that where urea was added (a_w=0.994 and a_w=0.991).

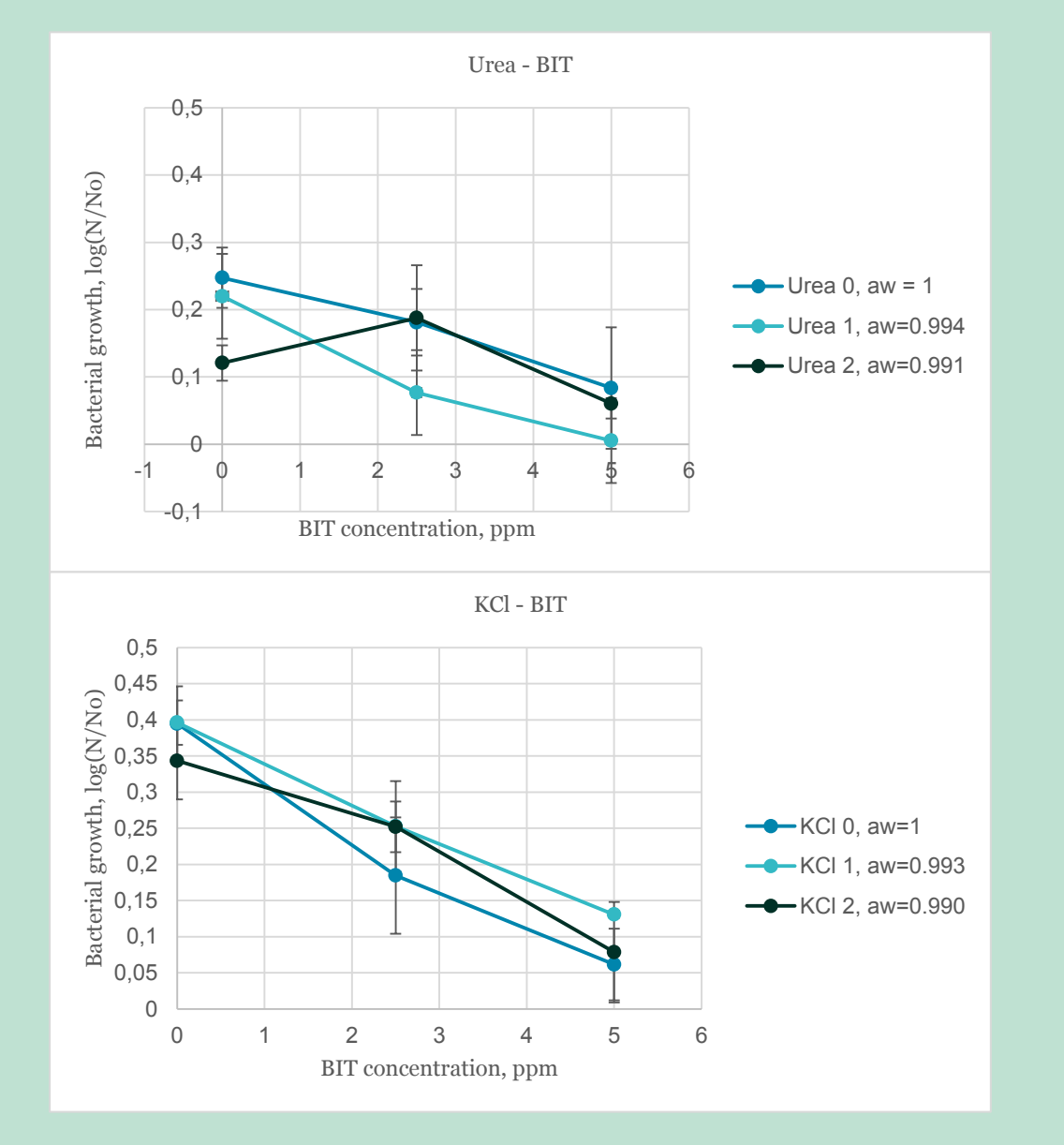


Figure 21. Bacterial inhibition as a function of added BIT combined with either urea or KCl. Zero refers to no added urea or KCl, while 1 and 2 refers to solution 1 and 2 in Table 34.

Further experiments on testing the use of urea as a possible booster of biocidal activity are described in section 3.4.1.

4.4 Reduced water-activity by use of polymers

Some polymers are known to bind water. The binding of water does not necessarily make it unavailable to microorganisms, but products like Osmocide™ (Sederma) designed for use in,e.g. cosmetics based on acrylate polymers (and some surface active compounds) claim

biocidal effect due to decreased water activity. To test the water binding of polyacrylates on water activity, tests were performed with two polyacrylate-based thickeners used in formulation of paint. The thickeners were adjusted to the desired pH 10, and water activity was measured. The results are shown in Table 35 and Table 36.

Table 35. Water activity in Thickener I

pH	Water activity
3	0.986
6.5	0.983
~10	0.981

Table 36. Water activity in Thickener J

pH	Water activity
2.5	0.985
4.5	0.981
~10	0.982

Even though the texture of the thickener changed from liquid to elastic, the water activity only changed very little. The binding of the water therefore did not change the concentration of the available water.

4.5 High-solids paint (HSP)

4.5.1 Water activity in high-solids paint

As an alternative to the addition of solutes or use of polymers, the water activity can potentially be decreased by simply reducing the concentration of water in the product hereby producing a so-called high-solids paint (HSP). The goal is again to reduce the water accessible to microorganisms and hereby inhibit their growth.

High-solid paints are paints with a high concentration of dry matter, where the main part of the solvent (in this case water) is added by the consumer prior to use. The low amount of water in the product increase the density as more solid matter is present. Due to more solid in the paint less product shall be applied compared to conventional paints. As the concentration of water is decreased in the product, this might also affect the product's water activity and thus its susceptibility to microbial degradation.

A formulation for a high-solids paint was developed, and the water activity of the different components was measured (Figure 22). Most of the components are powdery (solids) substances with a water activity below 0.4. Few components could not be obtained as solids, but also these had a low water activity below 0.82.

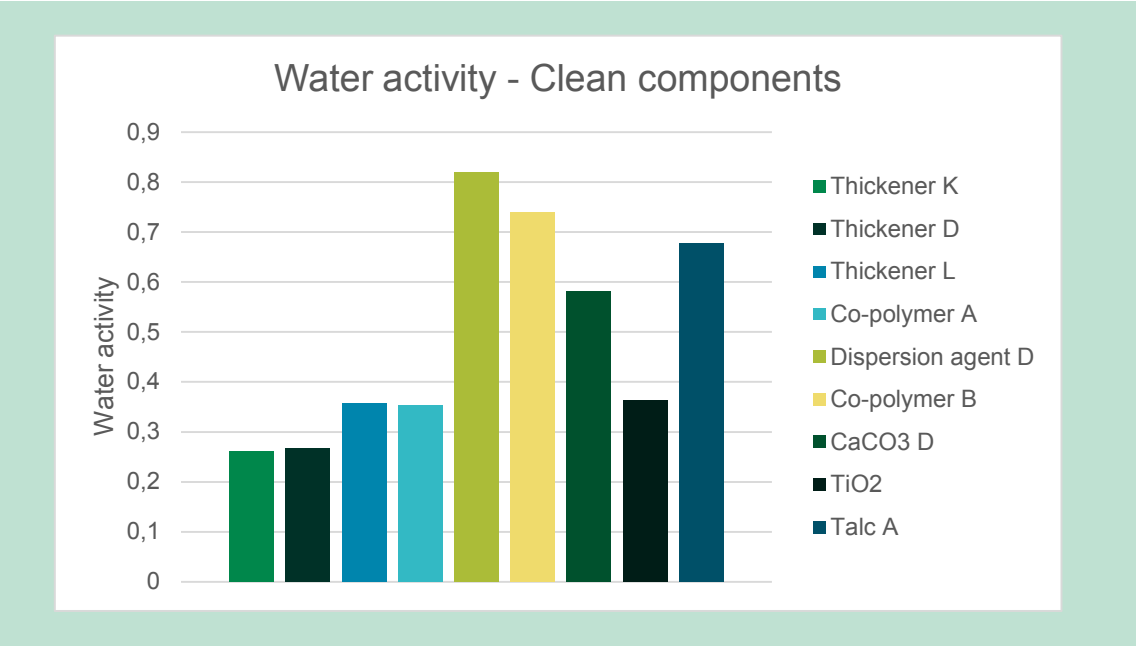


Figure 22. Water activity of different components for high-solid paint

Mixing of all the components resulted in a component mix with a water activity of 0.53 before adding water. To study the water activity in relation to the concentration of added water, different volumes of water was added to the component mix. A standard water-based paint has approximately 50 wt% water compared to powder paint which has none.

With the addition of water, the water activity of the high solids powder was still very high, even at water concentrations as low as 10-20%, where the mix still resembled a powder (Figure 23). Figure 24 shows the consistency of the corresponding samples with 10-100 wt% water in the high-solids powder. A paint with 30 wt% water will have lost its powdery consistency, but will have a water activity of 0.99- Even at 20 wt%, the water activity is 0.99.
A high-solid paint with a low concentration of water will thus not in itself have a sufficiently low water activity to inhibit microbial growth.

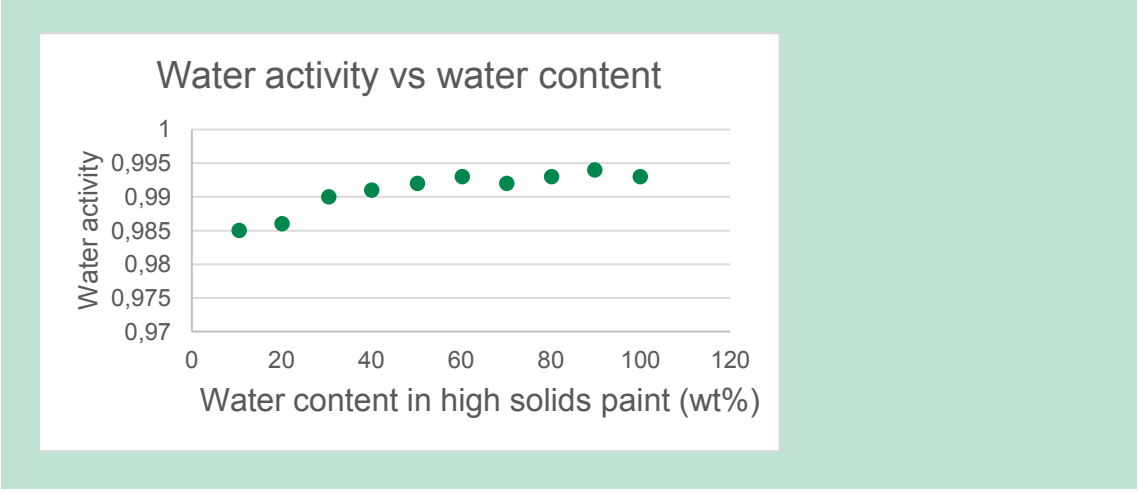


Figure 23. Water activity as a function of water content in a mix of components for a high-solid paint.

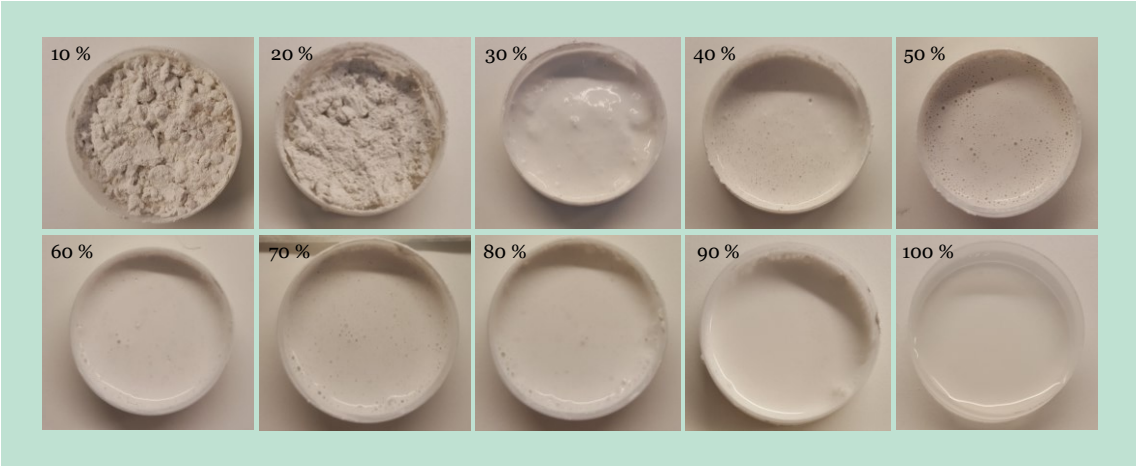


Figure 24. Pictures of the high solids components with 10-100 wt% water

4.5.2 Bacterial growth in high-solids paints

Bacterial growth was compared in the high solids paint with four different water concentrations (20, 30, 40 and 50 % water), to study the effect of water activity on bacterial growth. The high solids paint (HSP) was autoclaved prior to mixing to ensure no bacterial contamination. The high solids paint was then mixed with sterilized water and liquid bacterial culture *Pseudomonas aeruginosa*. At the lowest water concentration of 20 %, the bacterial culture could not be homogenously dispersed into the HSP due to the highly viscous texture of the mix, which resulted in inaccurate cell-counts at this water concentration.

The bacterial concentration at time zero was 1.5 x 10^4 CFU/ml for high solids paint with 20 % water and 2 x 10^6 CFU/ml for the three other paints (Table 37). The test showed that the bacterial concentration decreased after 7 days proportionally to the lowered water concentrations. After 14 days, the concentration of bacteria had increased in HSP with 50 % water, whereas no viable bacteria could be detected in HSPs with 20-40% water.

Table 37. Bacterial growth as a function of time in high solids paint with water content from 20-50%. The number of colonies on agar plates from the analysis of HSP 50 % water was too numerous to count, and the concentration of viable bacteria could therefore not be accurately quantified.

	Water concentration			
Day	**20%**	**30%**	**40%**	**50%**
0	1.45E+04	2.01E+06	1.97E+06	1.91E+06
7	2.73E+03	1.00E+04	1.10E+05	3.64E+05
14	0.00E+00	0.00E+00	0.00E+00	>1.00E+07

These results indicate that water activity is not the only parameter that determines the basis for microbial growth in the analysed HSP. The components of the HSP will potentially contain other substances with microbial inhibitory effect, which will be diluted at water concentrations of 50%. Liquid components are often preserved by biocides added by the manufacturer to prevent spoilage of these raw materials. By decreasing the concentration of water in the HSP, the relative concentration of these biocides is hereby increased. The bacteriocidal effect observed at water concentrations of 20-40% is therefore not necessarily a result of lowered water activity - as this was very limited - but more a result of a relative increase in the concentration of biocides in the HSP at lower water concentrations. By lowering the concentration of water, the concentration of biocides is thus not lowered in the product. Nonetheless, the use of HSP

could decrease the end-user exposure to biocides as the concentration of biocides will be lowered by the addition of water to the HSP before use.

4.6 Conclusions and future perspectives

Lowering water activity by removal of water or addition of solutes or water-binding polymers was tested:

- Bacterial growth was shown to be inhibited in short-term experiments by addition of the solutes urea and potassium. The concentrations needed were so high that these were considered unrealistic as regards paint functionality.
- Combination of biocides with KCl and urea showed no or limited effect on bacterial growth.
- The use of water-binding polymers did not change the water activity significantly.
- Although removal of water did not change water activity, even at very low concentrations of water, tests in high-solid paints showed bacterial inhibition when the concentration of water was lowered. This could be due to a relative increase in concentration of biocides or other inhibitory compounds present in the raw materials, which is increased by reducing the concentration of water in HSP, and is thus not linked directly to the lowering of water activity.

None of the above-mentioned approaches were considered for use in water-based paints, based on the measurement for water activity.. The development of new products based on HSP-technology will require new production procedures as well as increased handling by the consumers. Based on the results decribed in this chapter, the potential of HSP-based products are limited for the time being compared to the other approaches tested in the project.
On a longer-term, an alternative to formulation of HSP might be the development of powder paints, where no water is added and all components are powder-based. This is a very different approach that,just like high-solids paint, will require adaption by consumers and end-users who are used to traditional water-based paints, which requires a limited pre-treatment before use.
Currently, the suppliers do not sell all of the components for powder paints in bulk, and the large scale development of a powder-based paint is therefore not an option at the moment. Nonetheless, the potential for powder paints is the complete omission of biocides due to very low water activities of the products.

5. Process development to reduce microbial product contamination

The production facilities in Kolding have been evaluated during the project and actions have been identified. The focus was e.g. on optimizing the pipes to decrease dead-ends with high bacteria concentrations and focusing on an extra cleaning process. The optimizing actions are constantly reviewed and taken into consideration for further development at the facility.

The bacteria level is monitored in the process plant and the changes at the facility have already had a positive effect on the claims from consumers. The focus on maintaining an environment which is hostile to bacteria is a priority and will be investigated in a new project.

5.1 Introduction

An essential point when implementing new procedures for inhibiting bacterial growth by use of biocides or new product formulations is ensuring as clean a product as possible.

Microorganisms are ubiquitous in both natural and technical systems – including paint production facilities. The requirements for microbial growth is both moisture and nutrients.

$$N_t = N_0 \times e^{\mu t}$$

Where N_0 the initial number or concentration of microbes at time zero, N_t is the bacterial concentration of microbes after a given time interval. μ is the specific growth rate of the microorganism. The equation above illustrates the microorganisms potential to multiply exponentially, thereby reaching a problematic level within a short period (hours-days), depending on the presence of nutrients and moisture, initial microbial concentration (N_0) and the growth rate of the specific microorganism (μ).

As microbial growth is primarily controlled by the availability of nutrients, cleaning of process facilities to remove residual products which can fuel microbial growth.

In industries where use of biocides have been limited, and where product contamination has great implications, e.g. in food and feed industry, there has been continued development and optimization of production processes to limit the basis for microbial growth and subsequent product contamination to decrease the need for preservation by use of biocides.
An optimization process like this requires evaluation of the individual components in the production chain (raw materials, packaging, process equipment) influencing the contamination level of the final product.

One of the major differences between the industries, where focus on hygiene is a natural part of the process, and the coating industry is that partly dried films often are left in the production system and pasteurisation or sterilisation is impossible due to the final product stability. Total

and proper cleaning is consequently a necessity and involves a need for more insight into options parallel to already known techniques.

The process development at the production facilities in Kolding was a result of an increase in the number of product claims-after a significant reduction in the use and concentrations of MIT, which was used at concentrations up to 200 ppm in some products. Following the reduction of used MIT concentrations in 2014, the significant increase in number of reclamations prompted the new initiatives

5.2 Process evaluation and development at production facilities, Kolding

Production facilities at Flügger, Kolding, are today producing approx. 330 different products. This wide assortment of products requires a highly flexible processing system and has today resulted in a complex system with interconnecting pipes between storage tanks for raw materials and finished products, mixer tanks, filling stations etc. (Figure 25). A large part of this system is today not directly accessible for manual cleaning and thus requires different CIP (cleaning in place) procedures.

Figure 25. Pipe Connection point for conncecting different tanks (left). Discarded production pipe with paint residues (right).

The cleaning of the system and removal of residues which can form the basis for microbial growth is complicated by (1) the viscosity of the products, and (2) cross-binding ability of the paint, which challenges the need for removal of paint residues (Figure 25).

The production facilities in Kolding are under continued reconstruction to decrease product contamination during production.

The optimizations can be divided into (1) solutions which minimized the risk of microbial contamination of the production system and (2) quality control systems that will reduce the risk of contamination and hereby decrease the possibility of microbial growth in the production system.

5.2.1 Reduced bacterial contamination

Contamination risk is now reduced by reducing the contamination from different sources:

- The headspace in storage tanks for binders is modified to reduce the risk of contamination by use of chlordioxide during storage over night.

- Raw materials suspected to have a major impact on the contamination risk are now checked for microbial contamination, to prevent introduction of microbial contaminants. New procedures are furthermore introduced regarding transport and transfer of paints at the production facility including control procedures at product transfer.

Continued tests in the summer of 2016 has pinpointed further points in the production facilities which could be improved to decrease in-house product contamination (data not shown). As a result, new cleaning procedures has been implemented.

5.2.2 Reduced bacterial growth

Paint and product residues can form the basis for bacterial growth if not adequately removed. These residues can thereby form the basis for growth of microorganisms in the production system and be an inherent source of microbial contaminants to produced products.
Upgrading the production facilities in Kolding focused on avoidance of paint residues being left in the production process. So called dead-ends where old pipes have been cut are totally removed have been removed where it was possible.

To allow for more in-depth cleaning and removal of paint residues in the pipes, a new pigging system has been introduced.
New equipment for cleaning is furthermore introduced to remove residues of wet and dry paint from storage and production tanks by high-pressure cleaning. Several of these tanks are now equipped with disinfection systems to ensure removal or inactivation of bacterial biofilms in the parts of the tanks, which cannot be adequately cleaned by high-pressure cleaning.

There has also been set an extra focus on new cleaning procedures, which now include manual cleaning of areas identified as problematic with regards to microbial contamination.
Production facilities have been monitored over a long period and the final conclusion is that cleaning down to blank steel is an absolute necessary procedure to adequately reduce the risk of microbial contamination. The need for cleaning is at the moment evaluated by visual inspection by the operators .

Cleaning of pipes is still a concern, and the final solution that is examined is the addition of the disinfectant Chlorinedioxide, which is added in small amounts to the water (approx. 1 ppm). A slightly stronger solution of chlorinedioxide is used to flush filters, pipes and will be used to run the pigging system. This will supply a small amount of Chlorinedioxide to every spot in the piping system, even parts which cannot be reached today. Also 5 ppm chlorine dioxide is added to avoid contamination on the surface of the liquid paint during storage of the paints in the tanks over night.
A monitoring of filters over some weeks showed an effective degradation of bacteria when flushing with this solution, providing a clean filter when used for paints.

A problem observed in all paint factories is the build up of biofilms in the pipes. Even with a pigging system and the use of traditional biocides biofilms will not be removed. There is therefore a continuous risk of product contamination. To reduce the risk of biofilm build-up contamination a chlordioxide disinfection system is is introduced for disinfection of pipes and production tanks. Chlorine dioxide has previously been shown to be effective against biofilm in e.g. water systems, and hereby counter the unavoidable build up of bacteria. The combined use of pigging system and disinfection inactivates microorganisms and removes organic substrates which could form the basis of microbial growth and biofilm formation.

An important factor is to monitor the level of bacteria throughout the production facility. Various and increased number of controls were introduced in 2016, including monitoring of the production tanks, filters filling machine and various pipes. At each place the sample is tested on agar and the growth is evaluated with regards to contamination. Less contamination is observed, after the implementation of this control programme. An example of the tests performed and actions required is given in Figure 26.

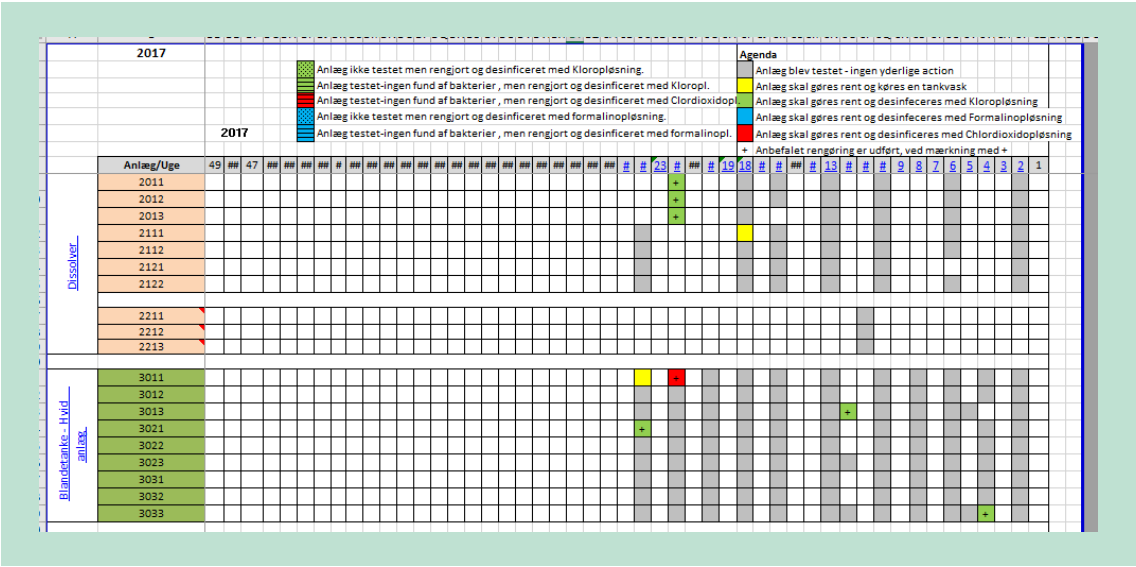


Figure 26. Overview of tests performed at Flügger

The combination of pigging systems for cleaning pipes, intensified control and chlorine dioxide has resulted in a dramatic drop in the contaminations in the production environment.

5.3 Evaluating process development

The effect of these new initiatives, described in the previous sections, led to a decrease in the number of claims to a level which is equal to that in 2013, before the level of MIT was reduced in the paint (Figure 27). With optimized production and processes, it is therefore possible to significantly decrease the necessary biocide-concentration in the products to a level equal to that before the reduction in biocide concentration.

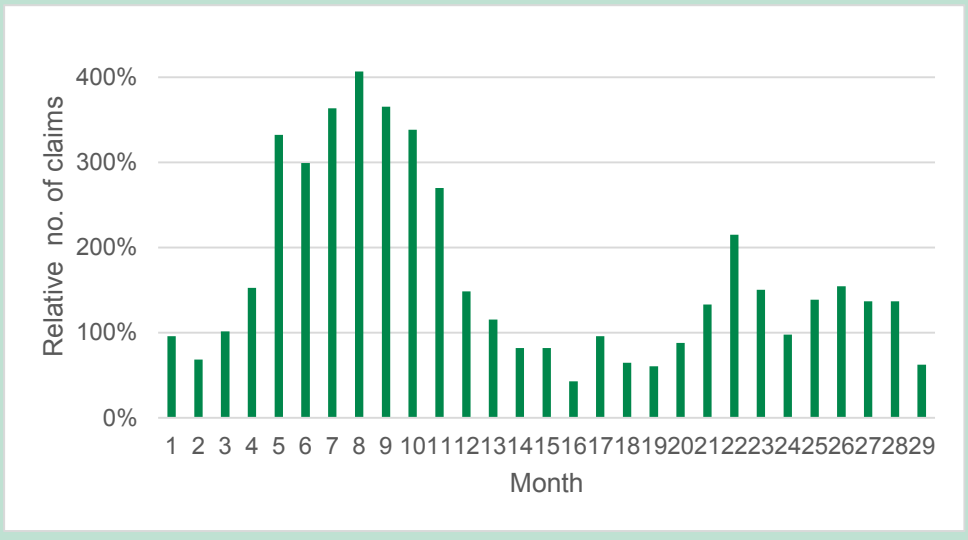


Figure 27. Relative number of claims after optimisation.

The continued reconstruction is believed to significantly decrease the number of claims.
There will be a continued focus on raw materials and component contamination e.g. fillers and pigments (talcum, $CaCO_3$) which today is produced by open mining. Tests showed that prod-

ucts like these soil-derived minerals contain a number of microorganisms which has to be effectively inactivated to obtain uncontaminated products (see section 2.4.1).
Today biocides are added at different processing steps to inactivate the microbial contaminants. As an alternative approach to inactivate contaminating microorganisms with the use of biocides could be by pasteurization through heat exchangers. Implementation of a pasteurization in parts of the production (shredding), has an estimated cost of 4.1 mio. DKK. As previous mentioned this option should be carefully investigated as influence on product stability may be present.

Neither biocides nor pasteurization is expected to inactivate bacterial spores from soil-dwelling microbes like *Bacillus* which has high resilience to both chemical and thermic treatment. An effective biocide system is therefore still a necessity to inactivate vegetative cells formed from bacterial spores - also during production.

5.4 Biocide tolerance of production facility microflora

Biocides are today added during production to inactivate microorganisms in raw materials (incl. water) and from production facilities. Therefore, it is expected biocide tolerante microorganisms which can tolerate the levels of added biocide is among the primary microbial contaminants.

To evaluate the risk of contamination from airborne biocide tolerant microorganisms, we examined the number of air-borne biocide-tolerant microorganisms (bacterial total count) at the production facilities in Bollebygd. Agar plates with different concentrations of biocides were placed at different sites for 3 days and hereafter incubated at 30 °C. Results showed only limited tolerance to biocide with 10 ppm CMIT/MIT (Table 38).

It should be noted that these experiments do not show the presence of biocide-tolerant microorganism in pipes and machinery utilities. Future studies are needed to elucidate to which extent biocide-tolerant organisms is a problem in the production systems.

Table 38. Screening of biocide tolerant microorganisms from the tap at the production facility at Bollebygd, Sweden. TNTC = Too numerious to count

	No biocide	5 ppm CMIT/MIT	10 ppm CMIT/MIT	200 ppm BIT	400 ppm BIT	500 ppm BIT	15 ppm CMIT/MIT 400 ppm BIT	100 ppm Amine 15 ppm CMIT/MIT 400 ppm BIT
PCA	TNTC	Same as blank	Few colonies	0	0	0	0	0
Malt Extract Agar	TNTC	TNTC	0	0	0	0	0	0
Kings B Agar	TNTC	0	0	0	0	0	0	0

Table 39. Screening of biocide tolerant microorganisms from the starting point at the production facility at Bollebygd, sweden. TNTC = Too numerious to count

	No bio-cide	5 ppm CMIT/MIT	10 ppm CMIT/MIT	200 ppm BIT	400 ppm BIT	500 ppm BIT	15 ppm CMIT/MIT 400 ppm BIT	100 ppm Amine 15 ppm CMIT/MIT 400 ppm BIT
PCA	TNTC	TNTC	Few colonies	0	0	0	0	0
Malt Extract Agar	TNTC	TNTC	0	0	0	0	0	0
Kings B Agar	TNTC	0	0	0	0	0	0	0

6. Conclusion & Perspectives

The aim of this project was to reduce the need and use of problematic biocides for in-can preservation of water-based paint. To achieve this, different methods where investigated to create an unfriendly environment for the microorganisms which inhabit and spoil the water-based paint:

- Substitution of components that are prone to support microbial growth (WP1)
- Enhance biocidal effect by combining different types of biocides (WP2)
- Optimize the biocidal effect by adding non-biocidal substances that limit microbial growth (WP3)
- Process optimization to lower the number of microorganisms in the production environment (WP4).

Substitution of components. Growth experiments using *P. aeruginosa*, isolated from contaminated paints, showed that through substitution of different raw materials and components, it was possible to reduce the basis for bacterial growth in the paint. As an example, the concentration of the macronutrient phosphor was reduced in the formulation, reducing bacterial growth without compromising the function of paint.
By lowering the nutritional value of the formulation even further, it was surprising that it showed differences in how identical raw materials from different suppliers affect the nutritional value of paint formulation. To select the best candidates for formulation and low nutritional value, a test platform was set up to screen the optimal components such as fillers (e.g. calcium carbonate and talc). Based on components less prone to fuel bacterial growth, a formulation was developed which was substantially less susceptible to bacterial growth. These first full-concept experiments showed how knowledge of raw materials and components can be transformed into products which are more resilient to microbial contamination even at lowered biocide concentrations. Screening of raw materials and their effect on microbial growth is therefore of utmost importance when designing and formulating new products.

The importance of careful evaluation and selection of raw materials was emphasized in subsequent tests, where some of the components were shown to interact with the added biocides. Several of the tested components were shown to interact with the commonly used biocide, BIT. A considerable effect was seen for example when testing a combination of binders and BIT. These experiments showed that the biocidal activity of BIT is affected by the binders and that its biocidal effect is considerably reduced when used in combination with binders, even at BIT concentrations as high as 300 ppm.
This inactivation poses a potential challenge for the reduction of biocide concentrations since the added amount of biocide is not equal to the actual concentration of active biocide in the product.
The exact mechanism for this inactivation is currently unknown, but the interaction of components and biocides has high potential importance for the reduction of necessary biocide concentrations and therefore requires further elucidation.

Enhanced biocidal effect. In order to ensure that low biocide concentrations become a reality in water-based paint, it is furthermore necessary to ensure optimal effect of the added biocides. To this end, multiple studies were conducted to investigate how biocidal effects could be optimized by combining different biocides and non-biocidal compounds. The studies included tests both with and without isothiazolinones. The amine biocide did show promising effect in combination with ZnPt even without the addition of isothiazolinones (except for the ones present in the raw materials), making it possible to formulate an isothiazolinone-free biocide solution. Although the use of ZnPt is now a possibility, future restrictions on the approved PT-6 list

could reduce the usefulness of ZnPt as a sustainable solution for future preservation. Continued restrictions on the PT-6 list makes the work to find suitable solutions for in-can preservation an ongoing process.

Non-biocidal substances. Although the combination of biocides with other non-biocidal compounds and techniques, such as lithium and elevated pH, did increase biocidal effect, these solutions were not seen as suitable for the production and handling of paint due to the required amount of lithium and pH level. Present products on the market claiming to be biocide free, where an elevated pH is used as technology, occupies a small part of the market and do not cover market requirements for a total offerering of the paint assortment.

The removal of biocides lowering the water activity by removing water, and adding solutes or water-binding polymers was tested. None of the approaches reduced water-activity to a level where they were able to inhibit microbial growth, and were thus not seen as suitable solutions for preserving water-based paints compared to other solutions investigated in this project.

Process optimization. As none of the tested approaches had an effect equal to that of the tested biocide solutions, the continued development and optimization of biocidal solutions is necessary. A part of this is to ensure that a maximum concentration of the added biocide is present for in-can preservation and not consumed during the production process. The optimization of Flügger's production facility in Kolding showed a clear effect of process hygiene: troubleshooting throughout the facility indicated potential critical points for bacterial growth and product contamination. Efforts to alleviate the risk of product contamination included the implementation of disinfection systems, additional cleaning cycles and removal of dead-end pipes. The changes showed a positive effect on Flüggers production and quickly lowered the amount of claims.

Perspectives. The challenge of reducing the biocidal concentration used for in-can preservation will be even more difficult if the biocides are used or inactivated during production before they reach the container. Therefore, a clean production environment will be a prerequisite for future production of paint and coatings if the concentration of biocides is to be reduced. Based on the studies carried out in this project, benefits could be obtained through further process optimization, leaning on years of experience from e.g the food industry.
The studies carried out in this project also revealed surprising interactions between raw materials and biocides which reduced biocidal activity considerably. Further studies are needed to examine how these interactions work, but the presented data show a need for screening the effect of different components with regards to how well they support microbial growth and how they interact with added biocides. The gained knowledge can be used to formulate products that lowerthe nutritional value for microorganisms while still retaining optimal biocide activity.
Although important knowledge has been gained in this project, further knowledge is necessary on the impact of both formulation and production process on microbial growth in order to reduce the need for biocides for preservation of water-based paint effectively.

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Reducing biocide concentrations for preservation of water-based paints

Preservatives in paints are a necessity, but some of the most widely used preservation agents are classified as allergenic, e.g. MIT (Metylisothiazolinone). Danish Technological Institute and Flügger have worked together focusing on reducing the usage of these preservation agents. This resulted in a 3-year collaboration funded by the Environmental Technology Development and Demonstration Program (MUDP) at the Danish Ministry of the Environment. The aim of this project was to optimize those parameters that could be changed in the formulation process and investigate both the possible internal and external challenges regarding the contamination. Tests showed that the choice of raw materials, the combination of preservatives and the level of product hygiene all had a great importance in the usage of preservatives and the degree of contamination in the paint. By focusing on MIT and other isothiazolinones, it was possible to reduce the volume of these allergenic substances.
The project has made it possible to move one step closer towards a world without allergenic preservatives in paints. However, the need to reduce the use of preservatives is still great both regarding the consumers' health, but also due to the enforcement of stricter regulations.

Reduceret biocidkoncentration I vandbaseret maling

Konserveringsmidler i maling er nødvendige, men nogle af de hyppigt anvendte konserveringsmidler er klassificeret som allergifremkaldende, som fx MIT (Metylisotiazolinon). Teknologisk Institut og Flügger har arbejdet fokuseret på at mindske brugen af disse konserveringsmidler. Samarbejdet har forløbet henover tre år med støtte fra Miljøstyrelsen igennem MUDP-programmet. Formålet var at klarlægge samt optimere de parametre, der kunne ændres i formuleringsprocessen og undersøge, hvor udfordringerne i forhold til kontaminering er, udefra kommende såvel som interne. Forsøg viste, at både valget af råvarer, sammen-sætning af konserveringsmidler og niveauet af produktionshygiejne alle havde stor betydning for forbruget af konserveringsmidler samt kontamineringsgraden i malingen. Ved at fokusere på MIT og andre isothiazolinoner, blev det muligt at sænke mængden af de allergifremkaldende stoffer.
Projektet har gjort det muligt at komme et skridt nærmere en verden uden allergifremkaldende konserveringsmidler i maling. Der er dog et fortsat behov for at mindske disse yderligere, både af hensyn til forbrugernes sundhed men også på grund af skærpet lovgivning.

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