(12) **PATENT**

(11) **342374** (13) **B1**

(19) NO

**NORWAY** (51) Int Cl.

***A01N 35/06 (2006.01)***
***A61K 31/122 (2006.01)***

**Norwegian Industrial Property Office**

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| (21) | Application nr. | 20161136 | (86) | International Filing Date and Application Number |
| (22) | Date of Filing | 2016.07.08 | (85) | Date of Entry into National Phase |
| (24) | Date of Effect | 2016.07.08 | (30) | Priority |
| (41) | Publicly Available | 2018.01.09 | | |
| (45) | Granted | 2018.05.14 | | |

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(54) Title **Compounds and compositions for biofilm prevention**

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(57) Abstract

The invention discloses use of compounds and compositions for effective biofilm prevention, wherein the compounds are certain cyclic ketones or derivatives thereof.

**Compounds and compositions for biofilm prevention.**

BACKGROUND OF THE INVENTION

Biofilm is defined as microbially derived sessile communities characterized by cells that are attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. Biofilms are almost universal in both natural and manmade environments, and they can develop on innumerable surface types including polymers, glass, stainless steel, water pipes, implants, wounds and teeth. In a biofilm, it has been shown that bacteria are 20 to 1000 times less sensitive to biocides, like e.g. disinfectants and antibiotics compared to planktonic bacteria. For this reason, once microorganisms are established in biofilm, they are very difficult to eradicate.

In the marine environment, biofilms constitute a costly problem both for the maritime industry, for aquaculture and for the petroleum industry, to mention a few. For the maritime industry, biofouling (the colonization of submerged surfaces by unwanted organisms such as bacteria, barnacles and algae) has detrimental effects on shipping and leisure vessels, as well as aquaculture and other marine installations. Biofilm formation by bacteria and other microorganisms serves as the foundation for biofouling. Once a biofilm forms, it is easier for other marine organisms such as barnacles to attach. Fouling on hulls of ships increases the frictional drag and can reduce speed in excess of 10%. A vessel with a fouled hull can burn as much as 40% more fuel which has an impact on fuel costs and on additional greenhouse gas production (estimated to be 20 million tons per annum). In some instances the hull structure and propulsion systems can become damaged. Fouled hulls are also implicated in the spread of 'alien species' around the world, potentially threatening the balance of sensitive ecosystems.

There is an increased focus in the oil and gas industry on the control of microorganisms. Water injection is by far the preferred method of increasing oil recovery efficiency. It also introduces nutrients such as sulphates which stimulate microbial growth in reservoirs and in production facilities. Increases in the production of hot water rich in nutrients lead to three major problems:

- Corrosion, which increases the risk of equipment failure and leads to higher operating costs. A major part of this is due to microorganisms.

- The production of the poisonous gas, hydrogen sulphide ($H_2S$), in reservoirs – also called reservoir souring. This will result in increased corrosion and increases in the use of production chemicals (scavengers). It also increases the risks to personnel on board.
- Biofouling, which reduces the efficiency of production equipment such as heat exchangers.

Biofilms cause complications in several industrial sectors. These vary from the oil and gas industry, animal feed production plants, human and animal medical equipment industry to the marine and maritime production industry among several others. For instance, in the aquaculture, where biofilms cause problems as biofilm grow on the fish nets and cause increased costs for cleaning the nets. Also, in the fish processing industry, the biofilm can harbor pathogenic bacteria like *Listeria monocytogenes* which is a major food safety threat. It can cause severe foodborne disease (listeriosis) with high hospitalization rates and mortality rates in excess of 30 % in humans, making safe food handling paramount to ensure public health. The bacterium is ubiquitous in the environment and is regularly found to contaminate food processing plants, increasing the risk of cross-contaminated products. In particular, the safety of consuming ready-to-eat products like vacuum-packed smoked fish has drawn scrutiny from the public and the scientific communities, as these products have been associated with listeriosis outbreaks. Unlike many other foodborne bacteria, *Listeria* tolerates salty environments and can even multiply at temperatures as low as 2-4 °C. Despite comprehensive surveillance of *Listeria* in the facilities and products, as well as rigorous cleaning and disinfection routines, *Listeria* remains a battle. Results from a recent research project financed by the Norwegian Seafood Research Fund (FHF) showed that *Listeria* is very difficult to eradicate, as more than 30% of samples from production facilities still were positive for *Listeria* after disinfection. Furthermore, the data indicated that the participating plants might harbor "house strains", i.e. strains that are able to persist in the facilities for an extended period of time.

As seen from the examples above, there are numerous areas where the development of bacterial biofilm causes large problems in aquaculture and food production environments. The patent no. JP2007091706 discloses an inhibitor to eliminate biofilm. Different compounds from plant extracts like cedarwood oil and palmarosa oil are suggested, including menthone. However, biofilm is found virtually everywhere and is potentially a problem in practically all terrestrial and marine environments. The available products on the market for the treatment of

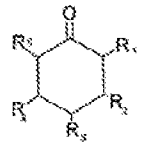
bacterial biofilm, such as biocides, aim at killing bacteria and removing already established biofilms. Some of these measures might work for certain bacteria while leaving others less affected and even promote biofilm formation. Bacteria organized in biofilm are protected and robust, thus the treatment is challenging. Currently, mechanical removal, biocides and desiccation are widely used antibacterial and anti-biofilm measures. A great advantage of the claimed compounds is that they prevent or decrease the build-up of biofilm without killing the bacteria. They will, therefore, delay or prevent the formation of bacterial biofilm as well as ease their eradication. They will act in combination with and increase the effect of e.g. anti-fouling agents and biocides. Further, the claimed compounds can be applied to or incorporated into steel, polymer, and other substances as well as mixed in solutions and paints among others to inhibit and prevent biofilm formation.

## DESCRIPTION OF THE INVENTION

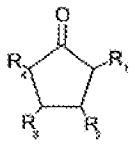
The invention relates to a solution, matrix, powder, gel or coating comprising one or more cyclic ketones or derivatives thereof at a concentration of preferably 0.05 - 500 mg/ml or 0.1-300 mg/ml or at least used in a concentration of 1-100 mg/ml to prevent biofilm formation.

## DETAILED DESCRIPTION OF THE INVENTION

The invention relates to biofilm prevention technology comprising use of typical molecules having the general structures 1 or 2 alone or in combination using two or more molecules.



General structure 1



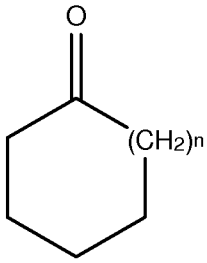
General structure 2

$R^1$, $R^2$, $R^3$, $R^4$ and $R^5$ are independently selected from the group consisting of hydrogen, oxygen, hydroxy, methoxy, ethoxy.

The invention relates to use of a solution, matrix, powder, gel or coating comprising one or more cyclic ketones or derivatives thereof at a concentration of preferably 0.05 - 500 mg/ml or 0.1-300mg/ml or at least used in a concentration of 1-100mg/ml to prevent biofilm formation.

Preferably the solution, matrix, powder, gel or coating according to claim 1 comprises cyclic ketones with 5-7 carbon atoms in the cyclic structure or derivatives thereof at a concentration of between 0.05 and 500 mg/ml to prevent biofilm formation.

More preferable the invention is directed to use of a solution, matrix, powder, gel or coating composition which comprises one or more of the compounds:




wherein n is 0-2;
substituted by one =O group; and
optionally substituted by 1-2 groups selected from -OH, -$C_{1-6}$ alkyl, -O-$C_{1-6}$ alkyl, -CH=O and -C(OH)=O;
or salts, hydrates, solvates or tautomeres thereof;
at a concentration of between 0.05 and 500 mg/ml or mg/g in preventing biofilm formation.

Even more preferable the solution, matrix, powder, gel or coating according to any of the claims comprises compounds selected from the group consisting of:

1,3-cyclohexanedione;
1,4-cyclohexanedione;
5-ethylcyclohexane-1,3 dione; and
5-methyl-1,3-cyclohexanedione.

The solution, matrix, powder, gel or coating disclosed canbe combined with one or more biocides and/or antibacterial agents to prevent biofilm formation.

Preferably the solution, matrix, powder, gel or coating is combined with biocides/or antibacterial agent selected from disinfectants and general biocidal products, preservatives, pest control agents or other biocidal products like antifouling agents.

The solution, matrix, powder, gel or coating above is preferably combined with biocides/or antibacterial agent selected from the group consisting of 4-hydroxy-3-methoxybenzaldehyde, cetylpyridinium chloride, quorum sensing inhibitors, biguanides, iodophors, quaternary ammonium compounds, Boric acid, cationic tensides, alcohol based, chlorine based, peroxy based and acid based compounds, Tetracyclines, Amphenicols, Beta-lactam antibiotics, Sulphonamides and trimetophrim, macrolides, linkosamides and streptogramins, Aminoglycosides, Quinolones and other antibacterial compounds.

The solution, matrix, powder, gel or coating above can be used in a two-step process to prevent biofilm formation.

Typically the solution, matrix, powder, gel or coating composition according to any of the claims of the invention comprises:

a. A solvent or solvent mixture;
b. An industrial paint or varnish;
c. Anti-fouling coatings and/or impregnations for marine use;
d. Anti-fouling coatings for maritime use;
e. A coating bound to the surface of or mixed in polymer material;
f. Solutions or coatings attached/linked to inert surfaces;
g. A coating bound to the surface of or mixed in fiber glass materials.
h. A solution, ointment, salve or dressing used in human or veterinary medicine or for a medical purpose.

The invention is directed to compositions and use of compositions which comprise molecules selected from the general structures above, and particular as proved in the formula of the claims. The molecules are known molecules not previously applied or used as biofilm inhibitors. The invention is a biofilm prevention technology/anti-biofilm technology as the technology does not remove biofilm or kill bacteria. The use of the compounds strictly

prevent the bacteria from establishing and forming biofilm, leaving the bacteria 'free floating'.

The following are examples of uses of the compounds exemplified in the application or substantiated based on literature.

1. One or more of the molecules selected from general structure used in solvent to a concentration ranging from preferably 0.05 to 500 mg/ml, or 0.1-300mg/ml or at least used in a concentration of 1-100mg/ml to prevent biofilm formation.
2. One or more of the molecules selected from general structure mixed in industrial paint and varnishes to a concentration ranging from 0.05 to 500 mg/ml to prevent biofilm formation.
3. One or more of the molecules selected from general structure mixed in anti-fouling coatings and or impregnations for marine use to a concentration ranging from 0.05 to 500 mg/ml to prevent biofilm formation.
4. One or more of the molecules selected from general structure mixed in anti-fouling coatings and or impregnations to be used as an anti-slime technology at a concentration ranging from 0.05 to 500 mg/ml to prevent biofilm formation.
5. One or more of the molecules selected from general structure mixed in anti-fouling coatings for maritime use to a concentration ranging from 0.05 to 500 mg/ml to prevent biofilm formation.
6. One or more of the molecules selected from general structure mixed in or attached to polymer materials to a concentration ranging from 0.5 to 500 mg/ml to prevent biofilm formation.
7. One or more of the molecules selected from general structure chemically and/or physically attached to inert surfaces (such as metal, glass and so on) to a concentration ranging from 0.05 to 500 mg/ml to prevent biofilm formation.
8. One or more of the molecules selected from general structure bound to the surface of or mixed in fibre glass materials to a concentration ranging from 0.5 to 500 mg/ml to prevent biofilm formation.
9. One or more of the molecules selected from general structure in a concentration ranging from 0.05 to 500 mg/ml to prevent biofilm formation intended for use in human or animal medicine. Comprising use on implants, wound dressings, wound treatment products, suture materials, ointments, spray, jelly or any other product used in animal or human medicine.

10. One or more of the molecules selected from general structure in a concentration ranging from 0.05 to 500 mg/ml to be used to potentiate the effect of biocides such as disinfectants, surfactants, antibacterial, electrolyzed water, anti-biofilm specific compounds or antiseptic solutions to prevent biofilm formation. Where the biocides are used in a concentration according to the manufacturer's recommendation or lower.
11. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml in combination with biocides such as disinfectants, surfactants, antibacterial, electrolyzed water, anti-biofilm specific compounds or antiseptic solutions to prevent biofilm formation. Where the biocides are used in a concentration according to the manufacturer's recommendation or lower.
12. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml according to 9 and 10 where the disinfectants are selected from any of the following groups: Humans and animals, Veterinary hygiene, Food and feed area, preservatives for products during storage and drinking water. Comprising: air disinfectants, aldehydes, alcohols, oxidizing agents, silver, phenolics, quaternary ammonium compounds, Biguanide, Thymol based disinfectants, ultraviolet germicidal radiation, Sodium bicarbonate and lactic acid among others.
13. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml according to 9 and 10 where the surfactant is selected from any of the following groups comprising: Anionic, cationic, zwitterionic, non-ionic, ionic surfactants and biosurfactants among others.
14. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml according to 9 and 10 where the antiseptics are selected from any of the following groups comprising: Alcohols, quaternary ammonium compounds, boric acid, brilliant green, chlorhexidine gluconate, hydrogen peroxide, iodine, Manuka honey, Mercurochrome, Octenidine dihydrochloride, Phenol, Sodium Chloride, polyhexanide, Sodium hypochloride, Calcium hypochloride, Sodium bicarbonate or Balsam of Peru among others.
15. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml according to 9 and 10 where the antimicrobials are selected from any of the following groups comprising: Tetracyclines, Aminophenichols, β- lactam antibiotics (penicillins and others), Sulphonamides and thrimetophrim, Macrolides, lincosamides and Streptogramines, Aminoglycosides,

Quinolones, Vancomycin, Teicoplanine, Colistin, Fucidinic acid, Metronidazol, Nitrofurantoin, Metenamine, Linezolid and Daptomycine among others.

16. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml according to 9 and 10 comprising: Preservatives such as preservatives for products during storage, film preservatives, wood preservatives, fibre, leather, rubber and polymerized materials preservatives, construction material preservatives, preservatives for liquid- cooling and processing systems, slimicides or working or cutting metal, glass and other materials.
17. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml according to 9 and 10 comprising biocides in antifouling products.
18. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml together with and comprising groups of adjuvants and additives such as emulsifying agents, alkylating agents, oxidizing agent, wetting agents, humectants, surface active agent, chelating agents and buffering agents among others.
19. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml together with agents to enhance or mask smell and taste selected from any of the following groups as examples consisting of natural or artificial: lime, lemon, orange, pineapple, grapefruit anisaldehyde, vanillin and its derivatives, red chilipepper, anethole, dihydroanethole, eugenol, ethyl maltol and mixtures thereof, benzaldehyde and its derivatives , cinnamon, clove, bay, allspice, anise, wintergreen, spearmint, peppermint, cherry and mixtures thereof.
20. One or more of the molecules selected from the general structure in a concentration ranging from 0.05 to 500mg/ml together with agents to enhance or mask colour with the following examples natural or artificial: Brilliant blue FC , Indigotine, Fast green FCF, Erythrocine, Allura red, Vanillic acid, Ethyl- vanillin ,O- vanillin, turmeric, saffron, anisaldehyde, Tartrazine, Sunset yellow or titanium oxider.

In points 1-20 above “general structure” refers to the formula of claims 1 and 8.

The compounds are unique as anti-biofilm agents in the fact that they have a non-toxic reduction of the establishment of biofilm and can be incorporated in different compounds (polymer, fiber glass, textiles etc.) and solutions (paint, varnish, anti-fouling etc.). They can

be used solely in solution (such as paint, coatings) as well as in combination with biocides, antiseptics and antibiotics to further enhance the effect of these. When used together with other active ingredients they can be used in combination with or as part of a two-step process. In the latter case they can be used after the initial treatment with a biocide, antiseptic or antibiotic as a prevention of further biofilm build-up. All but one (5-methyl-1,3-cyclohexanedione 98% at 1mg/ml) of the biofilm formation results show a statistically significant decrease in biofilm formation. This is shown using Confidence interval (CI) in the statistical calculation (CI not including 100% are considered statistically significant at $p<0.05$).

LIST OF FIGURES/DRAWINGS

Fig. 1: 1,3-cyclohexanedione. Gram positive and Gram negative bacteria.
The graph shows a decrease in biofilm formation of 74 and 70% with 1,3-cyclohexanedione in a concentration of 1 mg/ml on biofilm produced by Gram positive and Gram negative bacteria. Further, a decrease of 98% was seen at a concentration of 10 mg/ml in Gram positive bacteria and 87% in Gram negative bacteria. The asterisks' shows that the reduction is statistically significant ($p<0.05$)

Fig.2: 1,4-cyclohexanedione. Gram positive and Gram negative bacteria. The graph shows a 53% decrease in biofilm produced by Gram positive bacteria with 10 mg/ml 1,4-cyclohexanedione. Similarly, a decrease of 91% was seen in biofilm formation by Gram negative bacteria.
The asterisks' shows that the reduction is statistically significant ($p<0.05$)

Fig.3: cyclopentanone (reference compound). Gram negative bacteria. The graph shows a decrease of 77% in biofilm formation by Gram negative bacteria using cyclopentanone at a concentration of 10 mg/ml.
The asterisks shows that the reduction is statistically significant ($p<0.05$)

Fig. 4: 5-ethylcyclohexane-1,3 Dione. Gram positive bacteria. The graph shows decrease of 55 % in biofilm production using 5-ethylcyclohexane-1,3 Dione at 1 mg/ml, 74 % at 2 mg/ml and 98% at 10 mg/ml.
The asterisks' shows that the reduction is statistically significant ($p<0.05$)..

Fig.5: 5-methyl-1,3 cyclohexanedione. Gram positive bacteria. The graph shows a decrease of 32 % in biofilm formation using 5-methyl-1,3 cyclohexanedione at 1mg/ml, 85% at 2 mg/ml and 98% at 10mg/ml.
The asterisks' shows that the reduction is statistically significant ($p<0.05$)

Fig. 6: 1,3-cyclohexanedione. *Listeria monocytogenes*. The graph shows a decrease of 27%, 40%, 53% and 70% using 1,3-cyclohexanedione at 0,625 mg/ml, 2,5 mg/ml, 5 mg/ml and 10 mg/ml respectively.
The asterisks' shows that the reduction is statistically significant ($p<0.05$)

Fig.7: 1,4-cyclohexanedione. Sulphate reducing bacteria (SRB). Arrow A in the picture points to the slide with SRB bacterial biofilm included as a control. Arrow B points to a slide that has been immersed in medium containing 1,4-cyclohexanedione at a concentration of 10mg/ml.

Fig.8: cyclopentanone (Reference compound). Sulpha reducing bacteria (SRB). Arrow A in the picture points to the slide with SRB bacterial biofilm included as a control. Arrow B points to a slide that has been immersed in medium containing cyclopentanone at concentration of 10mg/ml.

Fig.9: 1,3-cyclohexanedione. Resistance to increasing temperature. The graph show biofilm formation after heating the solutions containing 1,3-cyclohexanedione compound to 80 °C, 90 °C and 100 °C. The graph shows a decrease of 38%, 40% and 28% respectively. The unheated control shows a decrease of 46%.

Fig 10: 1,3-cyclohexanedione. Stainless steel. The figure shows the average of log values of the parallel experiments using 1,3-cyclohexanedione in two concentrations. A decrease was seen with 1,3-cyclohexanedione in a concentration of 1 mg/ml. At 10 mg/ml, we could not detect any colony forming units (CFU`s).

DEFINITIONS
In the present context, the compounds or molecules are typically 1,3-cyclohexanedione ($C_6H_8O_2$), 1,4-cyclohexanedione ($C_6H_{12}O_2$), 5-ethylcyclohexane-1,3 Dione ($C_8H_{12}O_2$) and 5-methyl-1,3 cyclohexanedione 98% ($CH_3C_6H_7(=O)_2$) used as anti-biofilm agent in solutions,

or incorporated into or onto materials. Examples, but not limited thereto are paint, antifouling coating, polymer, glass or metal surfaces.

In the present context, a biofilm is an extracellular matrix community of sessile, stable attached microorganisms, such as bacteria, embedded in a self-produced matrix consisting of various components, including extracellular polymeric substances. Biofilm formation consists of three steps; attachment, growth and detachment in order to recolonize another surface. Extracellular matrix is continuously formed during the first two steps.

In the present context, a biofilm is considered to have been established from the moment when one or more microorganisms is/are irreversibly attached to a surface. Examples, but not limited thereto of a surface, is a wound and wound area.

The term “surface” is intended to relate to any surface which may be partially or fully covered by a biofilm. Examples, but not limited to, of surfaces are metal, polymer, fibre glass, human skin, epithelial cells, muscle tissue and surgical suture material or any coated or impregnated area.

The term “effective amount” as used herein refers to an amount effect, at dosages and for periods of time necessary to achieve a desired result.

In the present context the term “effective amount” refers to an amount of a compound or compounds that is sufficient to effect treatment when administered to a subject in need of such treatment.

EXPERIMENTAL

The following examples are illustrations within the scope of the claims.

EXAMPLE 1

**Biofilm formation experiments**

All experiments were performed by using sterile 96-wells polystyrene microtiter plates (Nunc, Nuncleon, Roskilde, Denmark) under conditions promoting biofilm formation by the different bacterial genera. Inhibio molecule preparations 1,3-cyclohexanedione, 1,4-cyclohexanedione, cyclopentanone (Reference compound), 5-ethylcyclohexane-1,3 Dione and 5-methyl-1,3 cyclohexanedione 98% were solved directly in TSB 1+1 or LB broth without NaCl (LB $^{wo}$/NaCl) to obtain the test concentrations, i.e. from 1 mg/ml to 10 mg/ml.

*Gram positive bacteria*

Three *Staphylococcus aureus* strains of animal origin were used in this study. All strains were stored at -80 $^{0}$C in BHI (Difco, BD, Franklin Lakes, NJ, USA) supplemented with 15 % glycerine (Merck KGaA, Darmstadt, Germany) and were recovered on blood agar at 37.0 ± 1.0 $^{0}$C. The bacterial cultures were then transferred into TSB and were incubated statically overnight at 37.0 ± 1.0 $^{0}$C to obtain an overnight working culture. A total of 2 µL of this suspension was transferred to each well in 96 wells polystyrene microtiter plates (Nunc, Nuncleon, Roskilde, Denmark) containing 198 µl TSB 1+1 with dissolved Inhibio compounds in the following concentrations: 0 mg/ml, 1 mg/ml and 10 mg/ml for 1,3-cyclohexanedione; 0 mg/ml and 10 mg/ml for 1,4-cyclohexanedione; and 0 mg/ml, 1 mg/ml, 2 mg/ml and 10 mg/ml for 5-ethylcyclohexane-1,3-dione and 5-Methyl-1,3-cyclohexanedione in the total amount of broth. Three parallels of each strain were used and the microtiter plates were incubated statically for one day, at 37 ± 1.0 $^{0}$C. After incubation, $OD_{595}$ was measured in a microplate photometer (Multiscan EX; Thermo Fisher Scientific Inc, Waltham, MA, USA) before the plates were gently washed three times with 290 µl tap water. The plates were dried in room temperature before addition of 220 µL 1 % crystal violet (Sigma-Aldrich, St. Louis, MO, USA). After 30 minutes incubation in room temperature, the plates were washed three times with tap water before the addition of 220 µL ethanol:acetone (70:30 w:w) to dissolve the bound dye. The plates were incubated for 10 minutes in room temperature before $OD_{595}$ was measured after the bound dye was dissolved using ethanol: acetone. For each strain, the result was calculated by subtracting the median $OD_{595}$ of the three parallels of the control (test broth only) from the median $OD_{595}$ of the three parallels of sample. Further, the average result of all three Gram positive strains included in the study were calculated. Three independent experiments were performed and the average was evaluated.

*Gram negative bacteria*

One *Salmonella* ser.Typhimurium (*S*.Typhimurium), two *Salmonella* ser. Agona (*S*.Agona) and two *Escherichia coli* (*E.coli)* isolates were used in these studies. The studies performed using 1,3-cyclohexanedione and 1,4-cyclohexanedione were done on *Salmonella* isolates only. The salmonella isolates were isolated from Norwegian feed factories except one *S.* Typhimurium strain which was a culture collection strain (ATCC 14028). The *E.coli* isolates were of animal origin. All strains were stored at -80 $^{0}$C in BHI supplemented with 15 % glycerine and were recovered on blood agar at 37.0 ± 1.0 $^{0}$C. The bacterial cultures were then transferred into LB broth and incubated statically overnight at 37.0 ± 1.0 $^{0}$C to obtain an overnight working culture. A total of 30µL of this suspension was transferred to each well in 96 wells polystyrene microtiter plates (Nunc, Nuncleon, Roskilde, Denmark) in triplets. The wells were containing 100µL LB $^{wo}$/NaCl (bacto-tryptone 10 g/l, yeast extract 5 g/l) with 1,3 cyclohexanedione dissolved in concentration 0mg/ml, 1mg/ml and 10 mg/ml in the total amount of broth and 1,4-cyclohexanedione and cyclopentanone (Reference compound) at 0mg/ml and 10mg/ml. The microtiter plates were incubated statically for two days, at 20 ± 1.0 $^{0}$C. After incubation, $OD_{595}$ was measured before the plates were gently washed one time with 150 µl tap water. The plates were dried in room temperature before addition of 140 µL 1 % crystal violet (Sigma-Aldrich). After 30 minutes incubation in room temperature, the plates were washed three times with tap water before addition of 140µL ethanol:acetone (70:30 w:w) and incubated for another 10 minutes in room temperature. $OD_{595}$ was measured in a microplate photometer (Multiscan EX) after the bound dye was dissolved using ethanol:acetone. For each strain, the result was calculated by subtracting the median $OD_{595}$ of the three parallels of the control (test broth only) from the median $OD_{595}$ of the three parallels of sample. Further, the averages of the Gram negative strains were calculated. Three independent experiments were performed and the average was evaluated.

*Results:*

The results are expressed as a decrease in biofilm formation calculated in percentage of the control (without the compound). A decrease of 74 and 70% in biofilm production was found using a concentration of 1 mg/ml 1, 3-cyclohexanedione on biofilm produced by Gram positive and Gram negative bacteria. Further, a decrease of 98% was seen at a concentration of 10 mg/ml 1,3-cyclohexanedione in Gram positive bacteria and 87% in Gram negative bacteria. For 1,4-cyclohexanedione, the results show a 53% decrease in biofilm produced by

Gram positive bacteria at 10mg/ml. Similarly, a decrease of 91% was seen in biofilm formation by Gram negative bacteria. Using cyclopentanone (Reference compound), a decrease of 77% in biofilm formation by Gram negative bacteria was detected at a concentration of 10mg/ml. Further, a decrease of 55 % of biofilm formation was found using 5-ethylcyclohexane- 1,3 Dione in 1mg/ml, 74% in 2 mg/ml and 98% in 10mg/ml in Gram positive bacteria. Lastly, a decrease of 32% was found using 5-methyl-1,3 cyclohexanedione 98% at 1 mg/ml, 85% decrease at 2 mg/ml and 98% at 10 mg/ml. All results were statistically significant at confidence interval 95% except 5-methyl-1,3 cyclohexanedione 98% at 1 mg/ml. See figures 1, 2, 3, 4 and 5.

*Listeria*

Six strains of *Listeria monocytogenes* were used in this study isolated from food research . The strains were recovered in 200µl TSB broth in a microtiter plate and incubated at 37°C for 24 hours. A total of 5µl of the bacterial suspension was transferred to a 96 wells polystyrene microtiter plate (Thermo scientific Nucleon Delta surface) together with 200µl medium (LB $^{wo}$/ NaCl) with 1,3-cyclohexanedione (dissolved to 0,625mg/ml, 2,5mg/ml, 5mg/ml and 10mg/ml) to each well. Each strain was added in duplex and incubated at 35 °C for 24 hours. After incubation, $OD_{595}$ was measured before the plates were gently washed one time with 200 µl tap water. This was repeated once. The plates were dried in room temperature before addition of 200 µL 0, 1% crystal violet (Sigma-Aldrich). After 30 minutes incubation in room temperature, the plates were washed two times with 200µL tap water and once with 240 µL. This was followed by the addition of 200µL ethanol:acetone (70:30 w:w) and incubated for another 10 minutes in room temperature. $OD_{595}$ was measured in a microplate photometer (Multiscan EX) after the bound dye was dissolved using ethanol:acetone. For each strain, the result was calculated by subtracting the average $OD_{595}$ of the two parallels of the control (test broth only) from the average $OD_{595}$ of the two parallels of sample. Three independent experiments were performed. The average of the 6 strains as well as the average between the experiments was calculated.

*Results:*

The results are shown as a decrease in biofilm formation in percentage of the control (no compound present). A decrease of 27%, 40%, 53%, 70% was found using 1,3-cyclohexanedione at 0.625 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml respectively. All results were statistically significant at confidence interval 95%. See figure 6.

EXAMPLE 2

**The effect of 1,4-cyclohexanedione and cyclopentanone (Reference compound) on biofilm produced by SRB (Sulphate reducing bacteria) in fluids.**

One SRB isolate (The culture collection strain: ATCC 29579 *Desulfovibrio vulgaris subspecies vulgaris*) was tested. 1, 4- cyclohexanedione and cyclopentanone (Reference compound) were diluted in ATCC 1242 to a concentration of 10mg/ml (1%) and added to each their 50ml falcon centrifugal tube. A third tube, with 10ml of ATCC only was included as a control. 50 µl SRB starting culture was added to each of the three tubes together with a carbon steel coupon. Biofilm was formed on the coupon by incubating at 20 °C for 12 days. After incubation, the coupons were washed in 40 ml sterile saline to remove loosely adhered cells. A sample from this fluid was injected into SRB medium and blackening of the medium was visualized, showing that there were still free-floating bacteria present.

*Results:*

The characteristic black biofilm was seen on the coupon that is not treated. In contrast, on the coupon treated with 1,4-cyclohexanedione or cyclopentanone (Reference compound) at a concentration of 10 mg/ml only scarce amount of biofilm could be visualized. See figures 7 and 8.

EXAMPLE 3

**The effect of increased temperature on 1,3-cyclohexanedione**

The experiment was performed using 1,3-cyclohexanedione in concentration of 1mg/ml. One strain of *Salmonella* was used in this study. All strains were stored at -80 °C in BHI (Difco, BD, Franklin Lakes, NJ, USA) supplemented 220 µl etanol:aceton 70:30 with 15 % glycerine (Merck KGaA, Darmstadt, Germany) and were recovered on blood agar at 37.0 ± 1.0 °C. The bacterial culture was transferred into LB broth and was incubated statically overnight at 37.0 ± 1.0 °C to obtain an overnight working culture. 1,3-cyclohexanedione was diluted in LB $^{wo}$/NaCl at a concentration of 1 mg/ml and divided into 4 tubes. The tubes were heated, for two minutes, to 80 °C, 90 °C, 100 °C and the last tube was not heated and included as a

control. 100 µl from each tube was added to a sterile 96-wells polystyrene microtiter plates (Nunc, Nuncleon, Roskilde, Denmark) together with 30µl bacterial culture or 30µl LB $^{wo}$/ NaCl in the case of the blank controls. The plates were incubated for 72 hours at 20°C. The plate was emptied and washed twice using 200µl tap water in each well each time. This was followed by the addition of 140 µl 1 % Crystal violet to each well and, after 30 minutes, the plate was again emptied and washed 3 times using 200µl tap water. 140 µl etanol:acetone 70:30 is added and $OD_{595}$ is measured after 10 minutes. For each strain, the result was calculated by subtracting the median $OD_{595}$ of the three parallels of the control (test broth only) from the median $OD_{595}$ of the three parallels of sample. At least two experiments were performed and the average was estimated.

Results:

The results show a decrease of 38%, 40% and 28% in biofilm formation after heating the solutions containing 1,3-cyclohexanedione compound to 80 °C, 90 °C and 100 °C. The unheated control showed a decrease of 46%. Considering normal variations, this shows that the Inhibio compound was still effective at higher temperatures. See figure 9.

EXAMPLE 4

**The effect of 1,3-cyclohexanedione on biofilm formed on stainless steel coupons by Gram negative bacteria**

One strain of *S.* Agona (Fig 10) and one strain of *E.coli* (Fig.10) were used in this study. The study was performed using 1,3-cyclohexanedione in a concentration of 1 and 10 mg/ml. The strain was stored at -80 °C in BHI (Difco, BD, Franklin Lakes, NJ, USA) supplemented with 15 % glycerine (Merck KGaA, Darmstadt, Germany) and was recovered on blood agar at 37.0 ± 1.0 °C. The bacterial culture was then transferred into LB broth and was incubated statically overnight at 37.0 ± 1.0 °C to obtain an overnight working culture. The Inhibio compound was dissolved in LB $^{wo}$/NaCl to a concentration of 10mg/ml and then further diluted to a concentration of 1mg/ml. 10 ml of each solution was added to a 50ml falcon tube and, in a third tube, only LB broth $^{wo}$/NaCl was added as a control. To each tube, 200 µl bacterial culture and an autoclaved stainless steel coupon was added and the tubes were incubated at 20 °C for 72 hours.

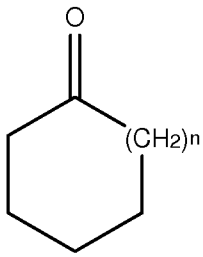
Following incubation, each coupon is dipped three times in three different tubes containing physiological saline and further transferred to a tube containing 5ml cold physiological saline as well as 20 sterile silica glass beads. Each coupon is further scraped with a sterile cell scraper before the coupon is removed and the solution is vortexed at 2000 rpm for one minute. A 10-fold dilution is made in a Nunc microtiterplate (kept on ice) with 180 µl physiological saline and 20 µl of the previous dilution for each well. 100 µl are spread on a blood agar plate and incubated on 37 °C for 24 hours. After incubation, the bacterial colonies are counted. If more than 200 colonies on a plate it is considered overgrown. At least two experiments were performed.

Results:

The Colony forming units (CFU) on the plate is calculated into CFU in biofilm by multiplying with a factor for each dilution. Further, the log value is calculated of the average of each dilution. A significant decrease is seen with 1,3-cyclohexanedione in a concentration of 1 mg/ml. At 10 mg/ml, there were no bacteria found in the biofilm. See figure 10.

**CLAIMS**

1. Use of a solution, matrix, powder, gel or coating composition comprising one or more of the compounds:




wherein n is 0-2;
substituted by one =O group; and
optionally substituted by 1-2 groups selected from -OH, -$C_{1-6}$ alkyl, -O-$C_{1-6}$ alkyl, -CH=O and -C(OH)=O;
or salts, hydrates, solvates or tautomeres thereof;
at a concentration of between 0.05 and 500 mg/ml or mg/g in preventing biofilm formation.

2. Use according to claim 1, of a composition according to claim 1, wherein the compounds are selected from the group consisting of:
   1,3-cyclohexanedione;
   1,4-cyclohexanedione;
   5-ethylcyclohexane-1,3-dione; and
   5-methyl-1,3-cyclohexanedione.

3. Use according to any of the preceding claims, in combination with one or more biocides and/or antibacterial agents.

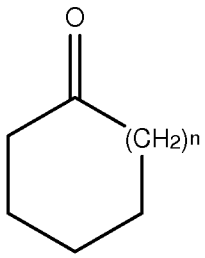
4. Use according to claim 3, wherein the biocides/or antibacterial agent is selected from disinfectants and general biocidal products, preservatives, pest control agents or other biocidal products like antifouling agents.

5. Use according to claim 3 or 4 wherein the biocides/or antibacterial agent is selected from the group consisting of 4-hydroxy-3-methoxybenzaldehyde, cetylpyridinium chloride, quorum sensing inhibitors, biguanides, iodophors, quaternary ammonium compounds, boric acid, cationic tensides, alcohol based, chlorine based, peroxy based and acid based compounds, tetracyclines, Amphenicols, Beta-lactam antibiotics, Sulphonamides and trimetophrim, macrolides, linkosamides and streptogramins, Aminoglycosides, Quinolones and other antibacterial compounds.

6. Use according to any of the claims 3-5, wherein the combination of one or more biocides and/or antibacterial agents are for use in a two-step process to prevent biofilm formation.

7. Use of a composition according to any of the preceding claims, applicable for either of the following uses as:
    a. A solvent or solvent mixture;
    b. An industrial paint or varnish;
    c. Anti-fouling coatings and/or impregnations for marine use;
    d. Anti-fouling coatings for maritime use;
    e. A coating bound to the surface of or mixed in polymer material;
    f. Solutions or coatings attached/linked to inert surfaces;
    g. A coating bound to the surface of or mixed in fiber glass materials.

8. A composition comprising one or more of the compounds:




wherein n is 0-2;

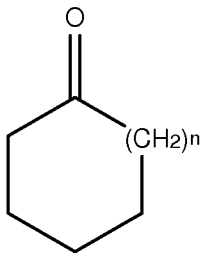
substituted by one =O group; and

optionally substituted by 1-2 groups selected from -OH, -$C_{1-6}$ alkyl, -O-$C_{1-6}$ alkyl, -CH=O and -C(OH)=O;
or salts, hydrates, solvates or tautomeres thereof;
at a concentration of between 0.05 and 500 mg/ml or mg/ for use in medicine in preventing biofilm formation.

9. A composition according to claim 8 for use in a solution, ointment or dressing in human or veterinary medicine or for a medical purpose.

**Patentkrav:**

1. Anvendelse av en løsnings-, matriks-, pulver-, gel- eller beleggsammensetning omfattende en eller flere av forbindelsene:




hvor n er 0-2;
substituert med en =O gruppe; og
eventuelt substituert med 1-2 grupper valgt fra -OH, -$C_{1-6}$ alkyl, -O-$C_{1-6}$ alkyl, -CH=O og -C(OH)=O;
eller salter, hydrater, solvater eller tautomere derav;
ved en konsentrasjon på mellom 0.05 og 500 mg/ml eller mg/g for å hindre biofilmdannelse.

2. Anvendelse ifølge krav 1, av en sammensetning ifølge krav 1, hvor forbindelsene er valgt fra gruppen bestående av:
   1,3-sykloheksanedione;
   1,4-sykloheksanedione;
   5-etylsykloheksane-1,3-dione; and
   5-metyl-1,3-sykloheksanedione.

3. Anvendelse ifølge et hvilket som helst av de foregående krav, i kombinasjon med en eller flere biocider og/eller antibakterielle midler.

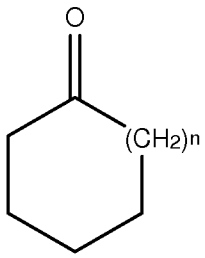
4. Anvendelse ifølge krav 3, hvor biocider og/eller antibakterielle midler er valgt fra desinfeksjonsmidler og generelle biocidholdige produkter, konserveringsmidler, skadedyrskontrollmidler eller andre biocidholdige produkter som begroingshindrende midler.

5. Anvendelse ifølge krav 3 eller 4 der biocider og/eller antibakterielle midler er valgt fra gruppen bestående av 4-hydroksy-3-metoksybenzaldehyd, cetylpyridiniumklorid, quorum sensing inhibitorer, biguanider, jodoforer, kvartære ammoniumforbindelser, borsyre, kationiske tensider, alkoholbaserte-, klorbaserte-, peroksybaserte- og syrebaserte-forbindelser, tetracykliner, amfenikoler, beta-laktam antibiotika, sulfonamider og trimetophrim, makrolider, linkosamider og streptograminer, aminoglykosider, kinoloner og andre antibakterielle forbindelser.

6. Anvendelse ifølge et hvilket som helst av kravene 3-5, hvor kombinasjon med en eller flere biocider og/eller antibakterielle midler er for anvendelse i en to-trinns prosess for å hindre biofilmdannelse.

7. Anvendelse ifølge et hvilket som helst av de foregående krav, anvendelig for en av følgende bruksområder som:
    a. Et løsningsmiddel eller en løsningsmiddelblanding;
    b. En industriell maling eller lakk;
    c. Begroingshindrende belegg og/eller impregneringer for marine bruk;
    d. Begroingshindrende belegg for maritim bruk;
    e. Et belegg bundet til overflaten av eller blandet i polymermateriale;
    f. Løsninger eller belegg festet/koblet til inerte overflater;
    g. Et belegg bundet til overflaten av eller blandet i fiberglassmaterialer.

8. Sammensetning omfattende en eller flere av forbindelsene:




hvor n er 0-2;

substituert med en =O gruppe; og

eventuelt substituert med 1-2 grupper valgt fra -OH, -$C_{1-6}$ alkyl, -O-$C_{1-6}$ alkyl, -CH=O og -C(OH)=O;

eller salter, hydrater, solvater eller tautomere derav;

ved en konsentrasjon på mellom 0.05 og 500 mg/ml eller mg/g for anvendelse i medisin for å hindre biofilmdannelse.

9. Sammensetning ifølge krav 8 for anvendelse i en løsning, salve eller bandasje i human- eller veterinærmedisin eller til medisinsk formål.

FIGURES/DRAWINGS

FIGURE 1

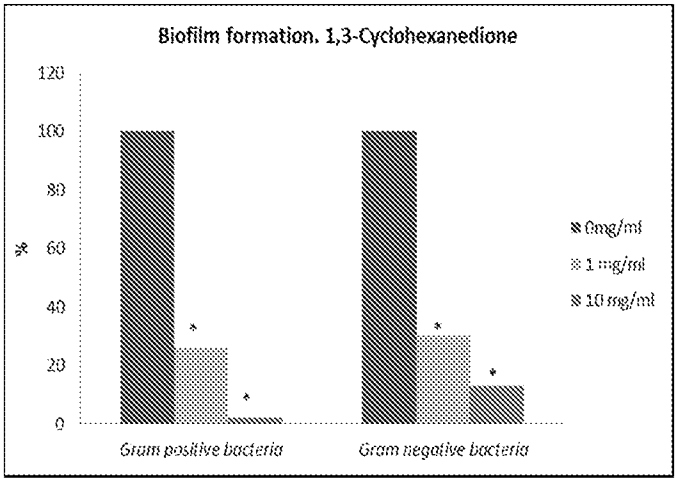



FIGURE 2

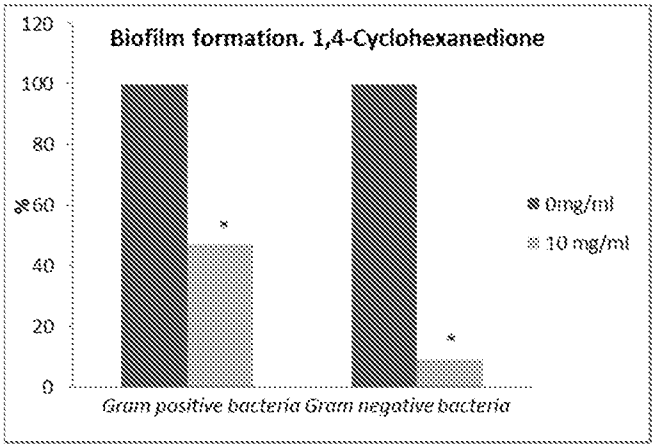



FIGURE 3

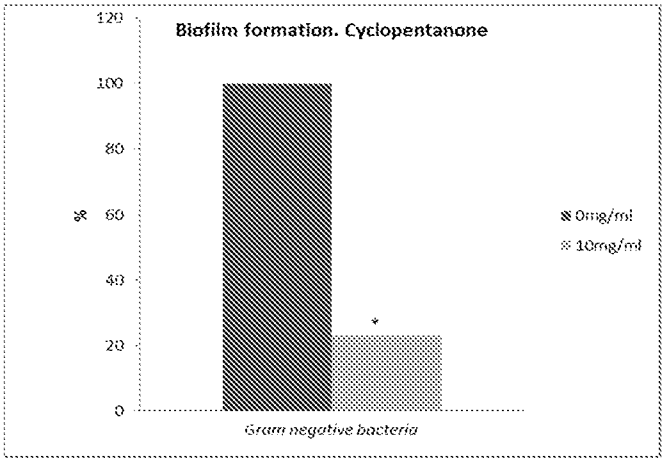


FIGURE 4

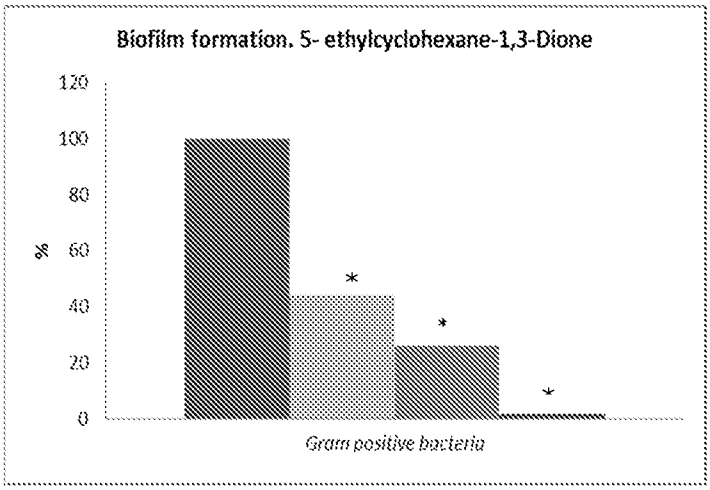



FIGURE 5

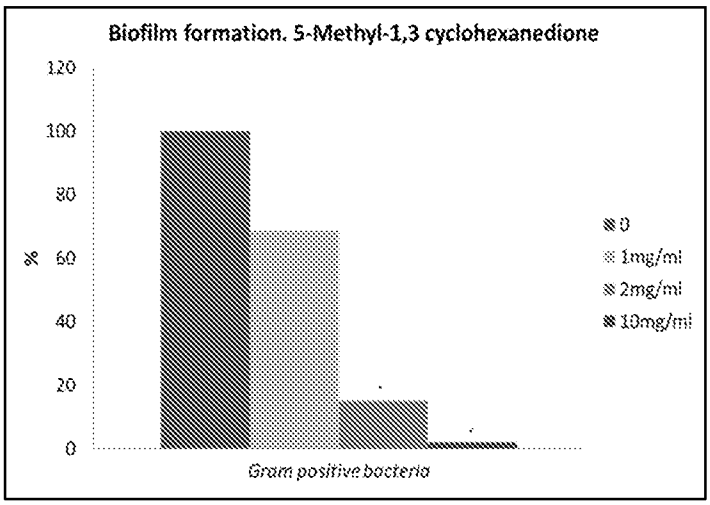



FIGURE 6

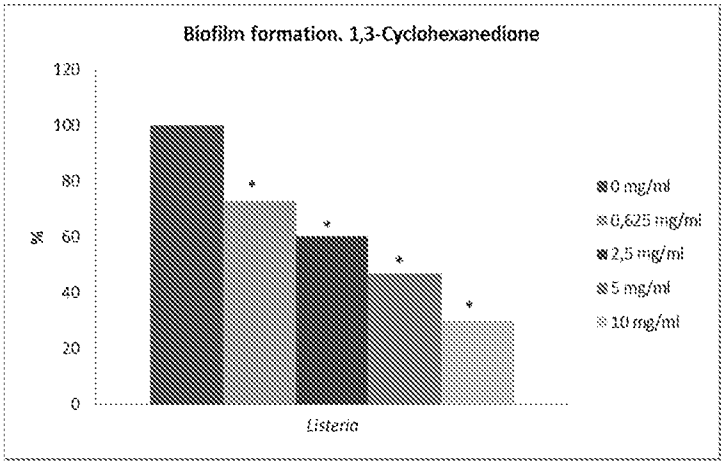


FIGURE 7

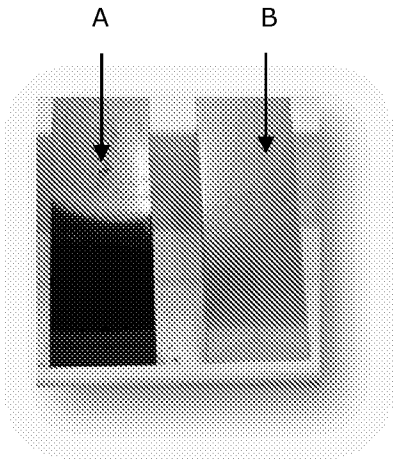



FIGURE 8

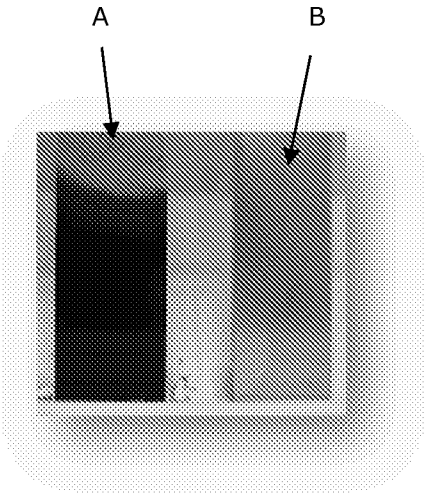



FIGURE 9

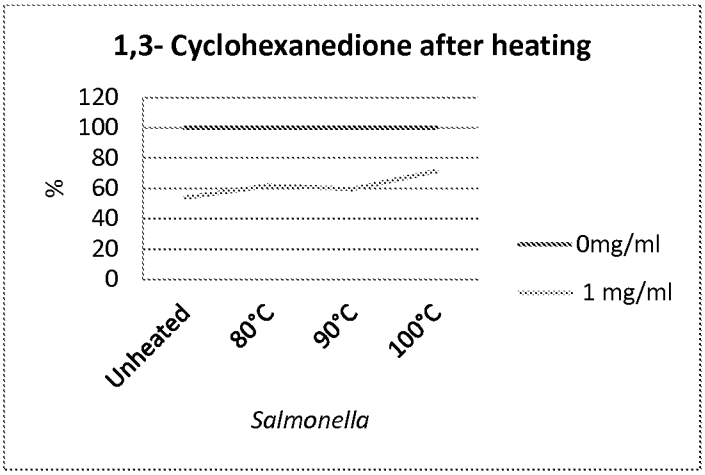
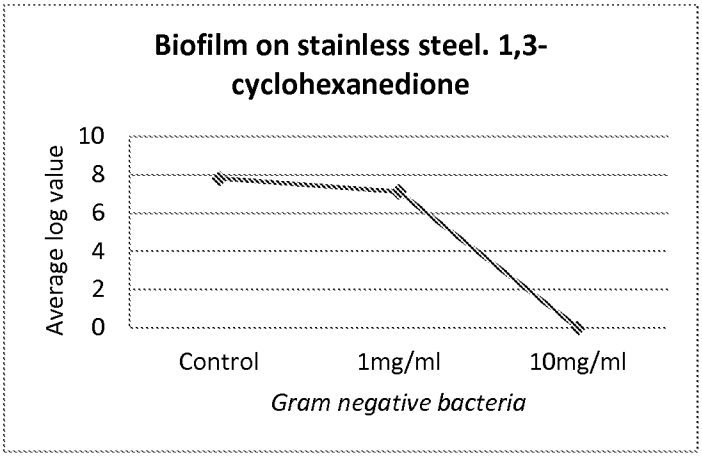

1,3- Cyclohexanedione after heating
120
100
80
60
40
20
0
%
0mg/ml
1 mg/ml
Unheated
80°C
90°C
100°C
Salmonella

FIGURE 10


Biofilm on stainless steel. 1,3-cyclohexanedione
10
8
6
4
2
0
Average log value
Control
1mg/ml
10mg/ml
Gram negative bacteria