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# Benzenetriol and derivatives

## Potential activity against citrus canker

# Abstract

Citrus canker is a plant disease which plagues citrus production worldwide, caused by the bacterium *Xanthomonas citri* sp. *citri*. Anti-microbial sprays, traditionally containing copper compounds, are used to limit the spread of the bacteria. Due to the adverse environmental effect of copper use, alternatives have been sought. Previous research into organic compounds derived from benzene-1,2,4-triol (BTO) revealed that BTO itself also had anti-bacterial activity. BTO is a reactive compound, forming dimers when exposed to air. Thus it was not known if it was BTO or the dimerization derivatives which contributed this activity. The results of this project show that it is BTO itself which provides high antibacterial activity, with the dimer showing lesser activity. Furthermore, the activity of BTO decreases over time in solution, while the activity of dimers does not. The effect of BTO and dimers on membrane permeability, metabolism and intracellular reactive oxygen species generation was investigated. No such effect was observed, with a possible exception of one dimer potentially reducing intracellular ATP concentration. The complicated background fluorescence of the compounds in media made it difficult to interpret experiments involving fluorescence reporters.

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# Introduction

Citrus canker is an infectious plant disease caused by three bacterial species of the genus *Xanthomonas* (Schaad et al., 2006). Of these, the most serious and widespread is the Asiatic form of citrus canker, caused by *Xanthomonas citri* sp. *citri*, (Xac) (Laia et al., 2009). The bacteria form necrotic lesions on the twigs, fruits and foliage of citrus plants. When the lesions get wet, the bacteria ooze out of them, from where they are spread by rain splashes and wind. The bacteria enter the leaves through wounds or directly through stomata (Gottwald, 2014). Heavy infection leads to death of foliage and twigs, as well as to fruit blemishes and premature fruit drop (Graham et al., 2004).

Citrus canker has spread from its origins in Asia to Oceania, Africa and the Americas. The disease reduces crop yield with its damage to the plants. Additional losses are caused by limitations imposed on export from the affected areas, as well as the additional cost of applying anti-microbial spray multiple times per year to maintain yield (Gottwald et al., 2001). To control or attempt to eradicate the disease, culling programs where infected or potentially infected trees are terminated have been implemented, notably in Florida, USA, and São Paulo, Brazil. As the bacteria spread by wind, more susceptible orchards can be protected by windbreak systems (Gottwald, 2014).

Among the most important strategies to prevent and combat infection is spraying with chemical anti-bacterial agents, such as copper compounds (Gottwald, 2014). Copper compounds have been used as anti-microbials in agriculture for over a century. High concentrations of copper compounds, and their accumulation in soil can be toxic towards the plant themselves, as well as adversely affect soil biota and the local ecosystem (Lamichhane et al., 2018). There have also been studies that implicate agricultural copper use in the development of Alzheimer's disease in humans (Coelho et al., 2020).

Due to the problems resulting from copper usage, researchers have investigated alternative compounds to combat citrus canker. Recent studies have demonstrated that various alkylated derivatives of hydroxybenzoic acids demonstrate antibacterial activity against Xac. Alkylated ester derivatives of isomers of dihydroxybenzoic acid and of gallic acid (3,4,5-trihydroxybenzoic acid) for example, have demonstrated good activity against Xac (Nazaré et al., 2018; Silva et al., 2013). Both the linear alkyl chain and the hydroxyl groups are believed to play a role in the activity of the compounds. To expand on this understanding, alkyl ether derivatives of 1,2,4-benzenetriol were tested. Unlike the benzoic acids, the aliphatic chain was linked to the aromatic group with an ether bond instead of an ester bond. Some of the resulting compounds did show antibacterial activity against Xac, and furthermore showed little toxicity to human cells (Cavalca et al., 2021).

The precursor for the Alkyloxy-benzenediols tested was benzene-1,2,4-triol, hereafter referred to as BTO (Cavalca et al., 2021). BTO can be produced from 5-hydroxymethylfurfural, a platform chemical which can be easily produced from cellulose biomass derived from agricultural waste. This represents a renewable source of the compound, compared to traditional petrochemical sources of benzene-derived compounds. BTO is an unstable compound that dimerises when exposed to water and air (Kumalaputri et al., 2018). Investigation into the activity of BTO derivatives revealed that, unlike hydroxybenzoic acids, BTO was shown to be active against Xac (data not published). Due to the compound instability, the question of whether BTO itself or one of its derivatives contributed to this activity was raised. The primary degradation product of BTO is a 5-5 carbon-linked dimer, hereafter referred to as Dimer 1. Dimer 1 can be further oxidised by air into the hydroxyquinone-containing dimer 2. Finally, an alternative dimer structure, Dimer 3, can be formed by heating BTO under acidic conditions (Randolph et al., 2018).

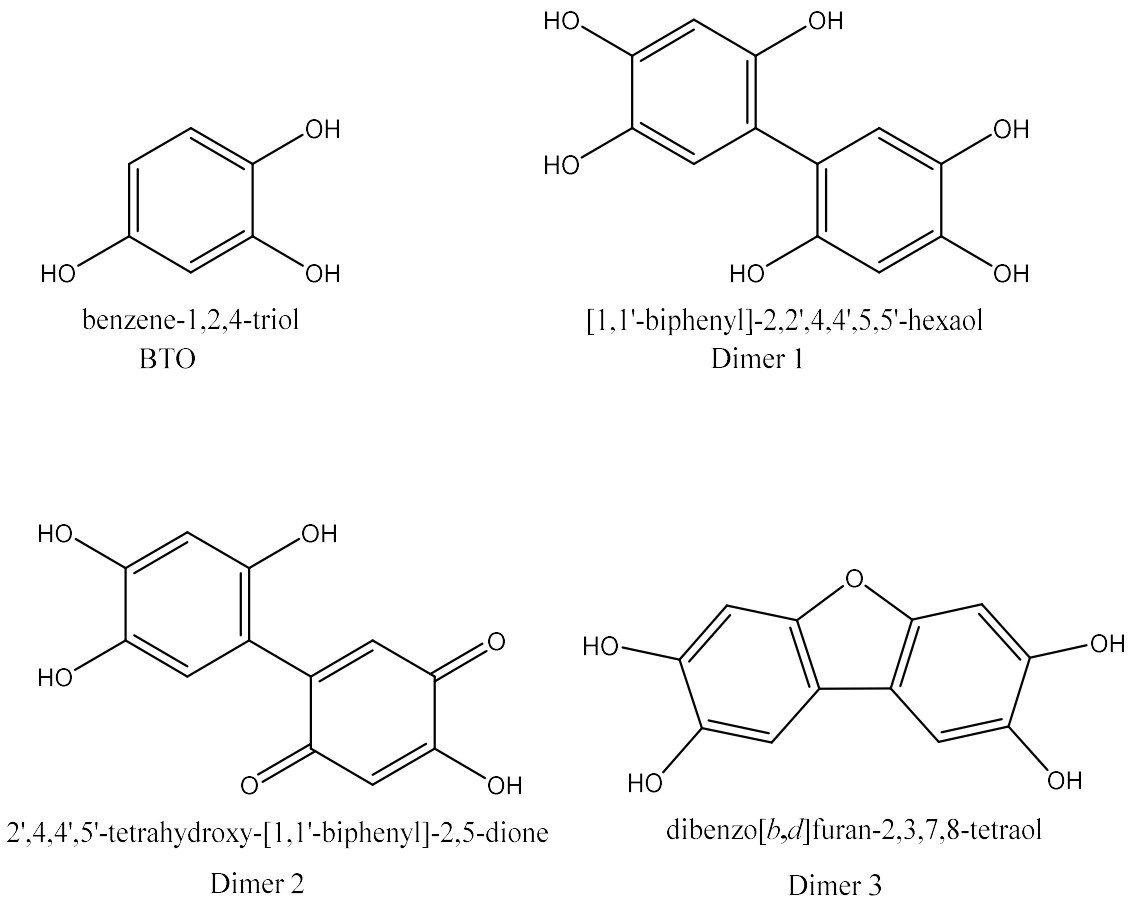


*Figure 1- Structure of the four different compounds tested, BTO and its three different dimer derivatives.*

In this study, the effectiveness of BTO, and of its dimerization derivatives, as an antibacterial was tested, to elucidate which compound was the primary contributor to the antibacterial activity of BTO. The effect of the instability of BTO on its antibacterial activity was investigated, as well as the role of the derivatives in it. Finally, efforts were dedicated to elucidate the mode of action of the compounds, by investigating their effects on membrane permeability, metabolism and reactive oxygen species generation.

# Methods

## Growth of Xanthomonas citri subsp. citri

*Xanthomonas citri* was grown in NYG medium (5 g/L peptone, 3 g/L yeast extract and 20 g/L glycerol) at 30°C. Liquid cultures were set up overnight and diluted to $OD_{600}$ of 0.1 and grown for 4 hours, to reach mid-exponential phase with $OD_{600}$ of 0.3-0.4.

As minimal medium, used in the reactive oxygen species assay, Xam1 medium was used (2.46 g/L glycerol, 0.247 g/L $MgSO_4$, 1.0 g/L $(NH_4)_2SO_4$, 10.5 g/L $K_2HPO_4$, 4.5 g/L $KH_2PO_4$, 0.5 g/L trisodium citrate and 0.3 g/L Casamino acids. 1 g/L bovine serum albumin was added filtered after autoclaving).

## Compounds

BTO was acquired from Fluorochem. An initial mix of dimers 1&2 was obtained from Randolph et al, for pilot studies. Dimers were synthesized concurrently to the project, according to Randolph et al., 2018, with modifications. Synthesis was done by Alexandru Trofin, under the supervision of Dr. Peter

Deuss and Lúcia Bonci Cavalca, in the Green Chemical Reaction Engineering department of the ENTEG, which collaborates in the citrus canker project. Purity of compounds was analyzed using 400-MR NMR Spectrometer from Agilent Technologies using DMSO-d6 as solvent. Relative integration values of byproducts and derivatives to that of the main compound was used to estimate their concentration. Compounds were identified from described peaks (Randolph et al., 2018).

## Minimum inhibitory & bactericidal concentration (MIC & MBC) testing

MIC and MBC were determined by microdilution of compounds, incubated over 24 hours in 96 well plates (O'Neill and Chopra, 2004). Concentrations were in the range from 100 to 0.78 µg/mL. Cultures were grown to a mid-exponential phase with $OD_{600}$ of 0.4. Wells were inoculated for a final calculated value of $5\times10^5$ cells per well. Growth in the plates was evaluated by $OD_{600}$ measurements in plate readers (BioTek SynergyMX or Powerwave) to evaluate MIC values. Cultures from the 96-well plates were stamped on agar plates using sterile plastic replicators and growth after 24 hours was used to evaluate MBC. Samples were compared to control grown in 1% v/v DMSO media as a solvent control, a positive control treated with 20 µg/mL kanamycin, and an untreated negative control.

## BTO stability assay

BTO was diluted in NYG media and pre-incubated at 30°C for 1 week or 24 hours prior to inoculation. Stability was determined by measuring MIC and MBC values as described previously.

## Live/dead microscopy

Cultures were grown to $OD_{600}$ of 0.3-0.4 and then incubated with the compound to be tested, for 15 minutes and 1 hour. Compounds were tested at MIC and MBC concentrations. After incubation the cells were treated with LIVE/DEAD BacLight Bacterial Viability Kit™ to assess membrane integrity. The kit contains two fluorescent dyes; SYTO9, a green dye which can cross an intact membrane, and propidium iodide, a red dye which can only enter cells with permeabilised membrane. This way permeabilised cells will be dyed red, while non-permeabilised will be dyed green. The sample was compared to populations treated with DMSO, a positive control treated with 15 minutes of 65°C heat shock and a negative control incubated without any treatment.

## REMA

The resazurin assay was performed to evaluate if the compound had any impact on cellular respiration. Cells were treated and incubated with 100 µg/mL resazurin over 4 hours. Resazurin is reduced by NADH or NADPH into the fluorescent compound resorufin, giving indication of NAD(P)H level and hence the status of cellular respiration. A culture in mid-exponential phase, with $OD_{600}$ in the 0.3-0.4 range, was diluted in medium to a final $OD_{600}$ value approximately 0.1 in the wells. Compounds were tested at MIC concentration. Two antibiotics controls were used: Rifampicin (0.625 µg/mL) and penicillin G (800 µg/mL). DMSO was used as solvent control at 0.45% v/v concentration. Fluorescence at 585 nm was measured in SynergyMX plate reader, every 15 minutes, with excitation wavelength of 530 nm. Samples were incubated in the plate reader at 30°C. Fluorescence values were normalised to fluorescence values of uninoculated samples.

## Intracellular ATP assay

This method was based on previous methodology used by the group as described in (Chakraborty et al., 2021). Cultures were grown to $OD_{600}$ of 0.2 and then diluted 1:1 and incubated over a period of two hours with the tested compound. Samples were taken after 0, 15, 30, 60 and 120 minutes and snap-frozen in liquid nitrogen. Samples were thawed afterwards and mixed with BacTiter-Glo™ reagent, which lyses the cells and produces luminescence proportional to the amount of ATP in the sample. Luminescence was measured in a Tecan F200 PRO. A standardisation curve was measured alongside

the measurement, with ATP concentrations from $10^{-4}$ µM to 1 µM, with tenfold difference between each point. A standardised volume was used for both samples and standardisation curve set-up. Samples were compared to control grown in 1% v/v DMSO media as a solvent control, a positive control treated with 40 µg/mL Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), and an untreated negative control.

Initial testing was done without snap-freezing. Measurements were made at 0-hours and after incubation. A standardisation curve was measured along each measurement, with ATP concentrations from $10^{-5}$ µM to 1 µM, with tenfold difference between each point. Controls were the same as previously described.

The results were analysed with ANOVA and a post-hoc T-test.

### Reactive oxygen species assay

This method was based on previous methodology used by the group as described in (Chakraborty et al., 2021). Cultures were grown in Xam1 minimal medium, until an OD of 0.8, and diluted 1:1 to be in mid-exponential phase during the experiment. Following dilution, they were incubated for 3 hours in Xam1 medium with 10 µM of 2',7'-Dichlorodihydrofluorescein diacetate (H2DCFDA). 900 µL of culture were spun down, washed with medium and resuspended in medium with compound. Cultures were grown in a 96 well plate and measured in SynergyMX plate reader. Excitation frequency was 488 nm and emission frequency 530 nm. Fluorescence values were normalised to the values of wells inoculated with bacteria that had not been incubated with H2DCFDA. Samples were compared to positive controls treated with 10 mM and 20 mM hydrogen peroxide, and negative controls which were treated with 0.45% DMSO or not treated.

## Results

### NMR spectra of BTO stock and dimer mix

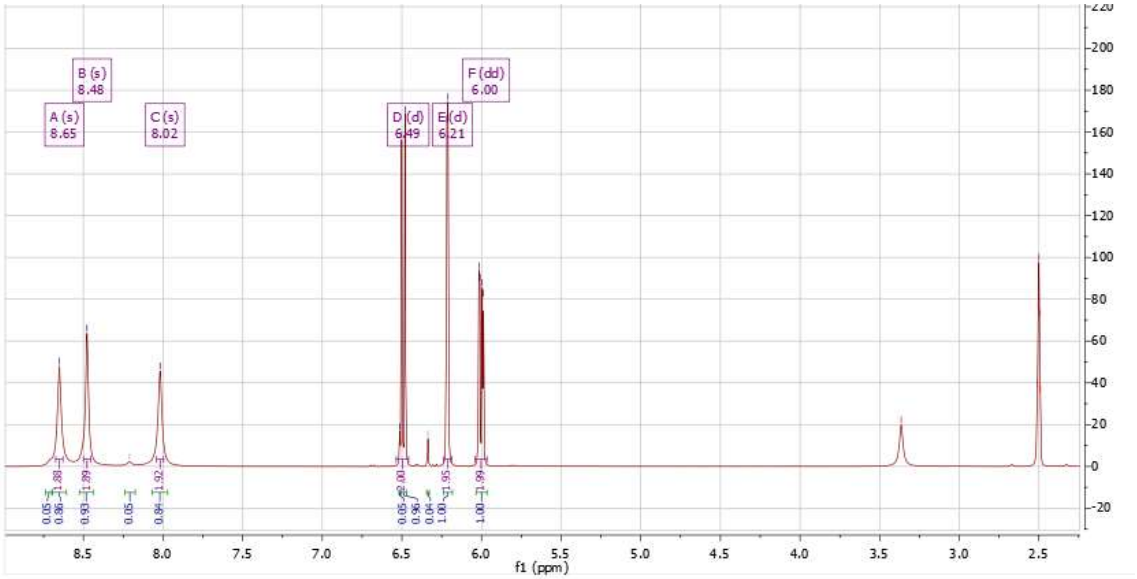


*Figure 2 - NMR spectra of BTO stock sample. BTO peaks are observed at 8.65, 8.48, 8.02, 6.49, 6.21 and 6.00. Peaks corresponding to Dimer 1 are 8.71, 8.20, 6.51 and 6.34.*

Analysis of BTO stock sample showed peaks corresponding to dimer 1 showed up with relative integration value of 0.05 to BTO. Adjusted for the number of each hydrogen atom in the molecule this

gives 2.4% of Dimer 1 in the BTO stock. None of the peaks corresponding to Dimer 2 and Dimer 3 were observed.

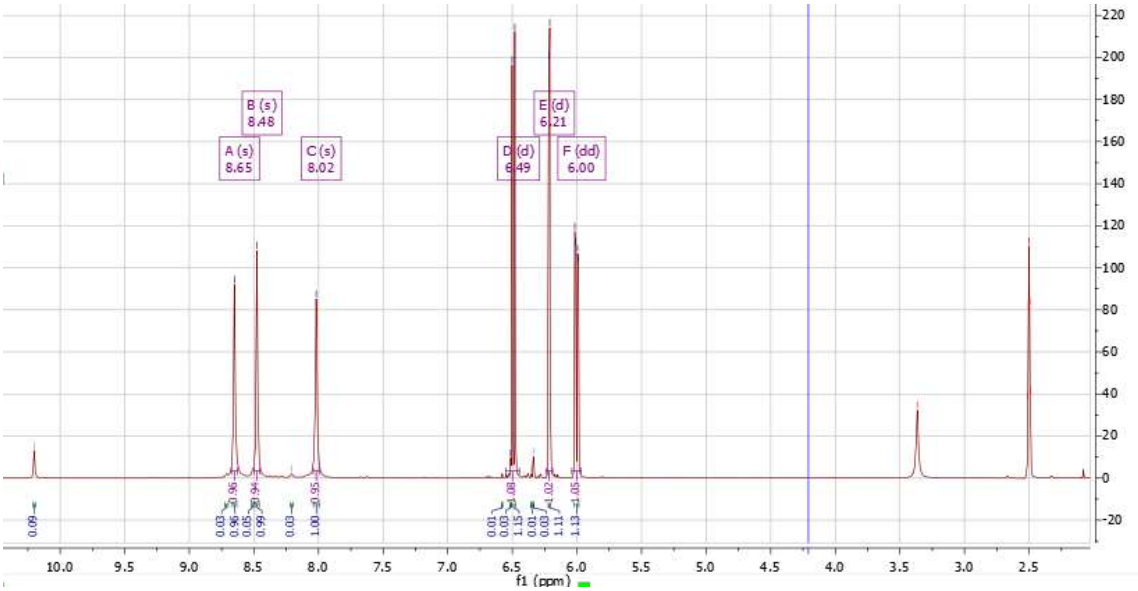


*Figure 3 - NMR spectra of BTO stock after being kept 2 weeks in a fridge dissolved in DMSO. BTO peaks are observed at 8.65, 8.48, 8.02, 6.49, 6.21 and 6.00. Dimer 1 peaks are observed at 8.71, 8.50, 8.21, 6.51 and 6.34.*

Stock sample was tested again after being kept 2 weeks dissolved in DMSO in a fridge, to evaluate if the compound kept stable enough under those conditions to be used for experimentation. Relative integration values for Dimer 1 were 0.03. Adjusted for the number of each hydrogen atom in the molecule this gives 1.48% of Dimer 1. This is lower than the previous value. This may be due to possible degradation of Dimer 1 or due to measurement variance. Only one potential Dimer 2 peak was observed, with a small relative integration value. Overall, this suggests that BTO is stable enough under these conditions to be used for experimentation.

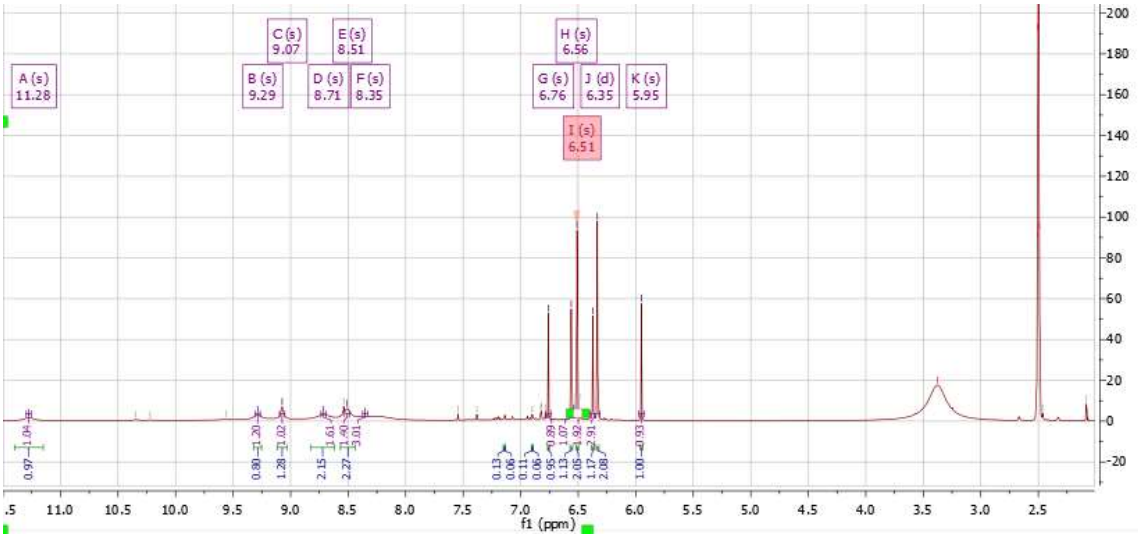


*Figure 4 - NMR spectra of 50:50 dimer mix. Peaks corresponding to Dimer 1 are observed at 8.71, 8.51, 6.51 and 6.35. Peaks corresponding to dimer 2 are observed at 11.28, 9.29, 9.07, 8.35, 6.76, 6.56, 6.35 and 5.95.*

An NMR spectrum was taken of a mix of Dimers 1&2. Concentration of dimers were equal. A larger number of smaller peaks was observed compared to the BTO stock, suggesting a higher level of impurities compared to the stock. No peaks corresponding to BTO were observed.

## Initial pilot studies

Initial pilot experiments on BTO and a mixture of dimers 1 & 2 revealed BTO to have antibacterial activity at low concentrations. Minimal bactericidal concentration was 12.5 µg/mL. At that concentration, incubation for 1.5 hours was enough for no growth to be visible on the stamped agar plate.

The mixture of dimers 1&2 demonstrated antibacterial activity at 50 µg/mL. Due to the chemical similarity of the dimers they are difficult to separate. Synthesis of the dimers was investigated in parallel to the investigation into antibacterial activity. Later experiments used dimers successfully synthesised at higher concentrations. See *Figure 5* for stamped plates.

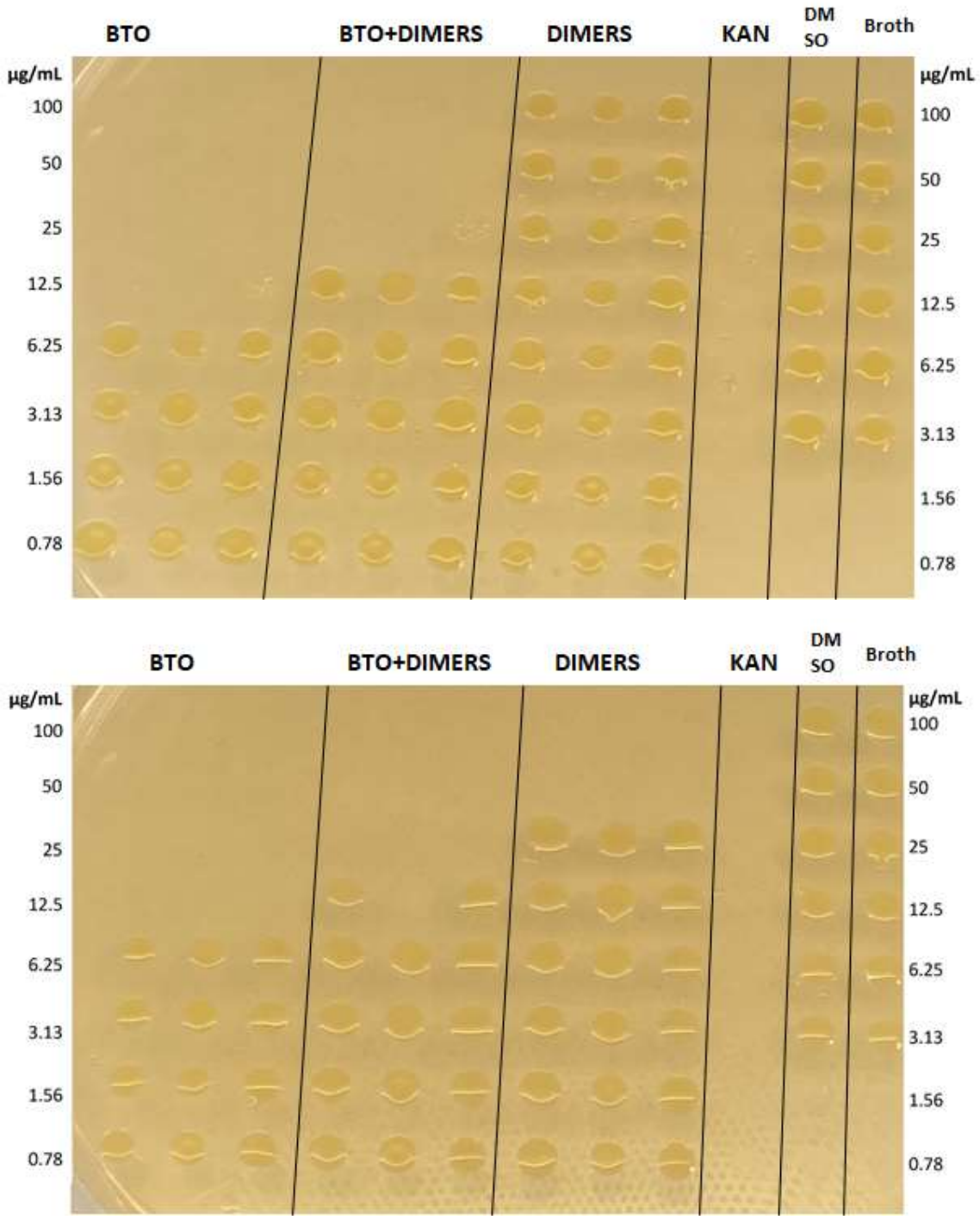


*Figure 5-Pilot study results. Plates stamped with cultures after incubation with compounds for 1.5 hours and 24 hours. The compounds tested are BTO (columns 1-3), a 50:50 mix of dimers 1&2 (columns 7-9) and an even mix of both solutions (4-6). Controls are 20 µg/mL kanamycin (column 10), 1% DMSO (column 11) and NYG broth without additions (column 12).*

## MIC evaluation and growth curve under BTO treatment

Observation of growth in media with dilution of BTO gave a minimum inhibitory concentration of 6.25 µg/mL for *X. citri* subsp. *citri*. This compares favourably to heptyl-2,4-dihydroxybenzoate with MIC of 13.2 µg/mL (Nazaré et al., 2018) and n-heptyl gallate with a MIC value of 31.2 µg/mL (Silva et al., 2013).

Treatment with small quantities of BTO, as well as DMSO, resulted in slower growth over time. Growth seems negatively correlated with higher BTO concentration, but the difference between cultures is small. Upon reaching the concentration of 6.25 µg/mL growth ceased (See *Figure 6*).

Stamping of culture on agar gave repetition of the results of the pilot study with MBC of 12.5 µg/mL. Cultures growing at 6.25 µg/mL, the MIC value, tended to be faint and colourless after incubation for 24 hours. They would gain colour and the normal appearance of Xac colonies after further growth.

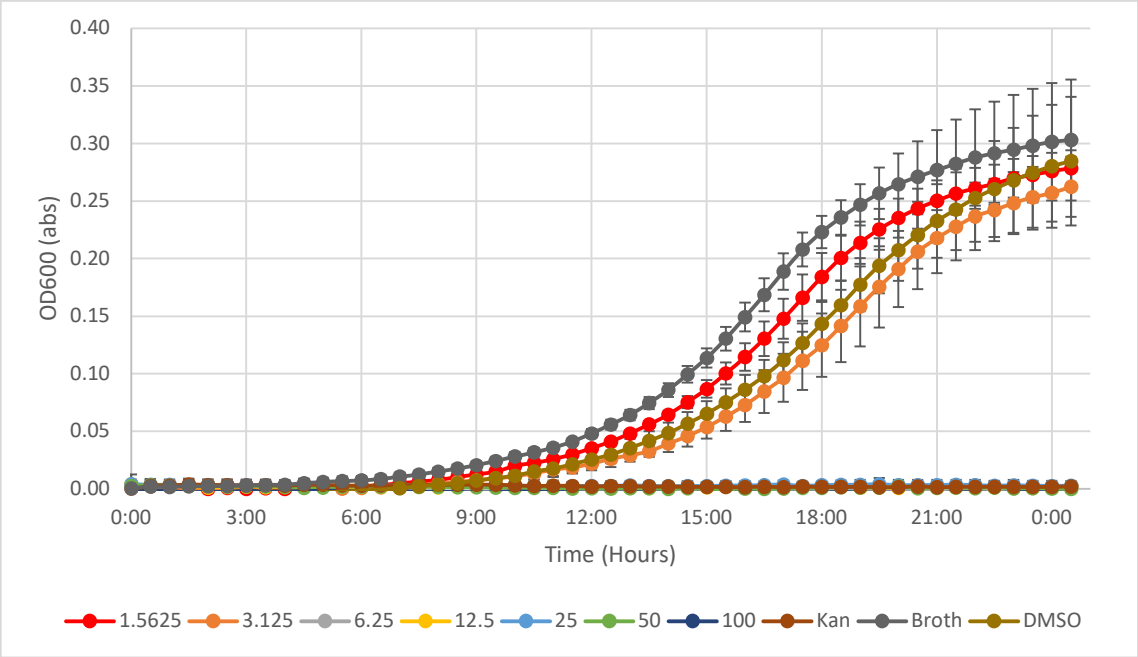


*Figure 6 -Growth of Xac over 24 hours in liquid medium with varying concentrations (µg/mL) of BTO. Comparisons are to positive kanamycin-treated control (Kan), negative non-treated control (Broth) and a solvent control (DMSO). Cultures treated with 3.125 µg/mL of BTO or less were able to grow.*

## Stability of BTO

Cells were treated with pre-incubated BTO to evaluate the effects of the degradation of BTO on its effectiveness as an antimicrobial. Samples were pre-incubated in medium at 30 degrees for 1 week and 24 hours.

MIC values were double for both 24 hours and 1 week pre-incubation, while MBC values were double for 1 week (*Table 1*). This demonstrates that the antibacterial activity of BTO decreases with time and degradation of compound.

*Table 1-Comparison of MIC and MBC values for BTO after pre-incubation.*

| **0 hour** | | **24 hours** | | **1 week** | |
|---|---|---|---|---|---|
| MIC | MBC | MIC | MBC | MIC | MBC |
| 6.25 µg/mL | 12.5 µg/mL | 12.5 µg/mL | 12.5 µg/mL | 12.5 µg/mL | 25 µg/mL |

## Live/dead staining

Evaluation of membrane permeabilisation by the compounds was done by live/dead staining. Cultures were treated with BTO at MIC and MBC concentrations (See *Figure 7*) and dimers at MBC concentration (See *Figure 8*), for 15 minutes or 1 hour. Treated cultures had similar numbers of permeabilised cells

compared to untreated samples (See *Figure 9*). This was consistent for all compounds, whether treated for 15 minutes or 1 hour (See *Table 2*).

*Table 2 - Total cells counted for live/dead assay. Cells with non-permeabilised membrane fluorescence green and cells with permeabilised membrane red. The number of cells which were not dyed, and their percentage compared to total dyed cells is also included.*

| Treatment | Concentration (µg/mL) | Incubation time (min) | Green % | Red % | Total | Undyed % |
|---|---|---|---|---|---|---|
| BTO | 6.25 | 15 | 95.58 | 4.42 | 633 | 0.47 |
| | 6.25 | 60 | 97.87 | 2.13 | 1035 | 0.29 |
| | 12.5 | 15 | 96.87 | 3.13 | 1054 | 0.57 |
| | 12.5 | 60 | 96.35 | 3.65 | 1150 | 1.83 |
| Dimer 1 | 25 | 15 | 92.69 | 7.31 | 1300 | 0.31 |
| | 25 | 60 | 90.11 | 9.89 | 1153 | 0.52 |
| Dimer 2 | 100 | 15 | 95.15 | 4.85 | 1670 | 0.18 |
| | 100 | 60 | 94.21 | 5.79 | 1105 | 0.54 |
| Dimer 3 | 50 | 15 | 93.61 | 6.39 | 1033 | 1.45 |
| | 50 | 60 | 91.62 | 8.38 | 990 | 4.04 |
| Controls | | | | | | |
| DMSO | | Solvent Control | 93.08 | 6.92 | 6562 | 0.67 |
| 30°C | | Negative Control | 93.96 | 6.04 | 8059 | 1.15 |
| 65°C | | Positive Control | 0.04 | 99.96 | 8208 | 0.51 |

## Viability assay at higher initial culture density

To check if any bacteria remained viable in the culture treated in the live/dead assay, samples were spread onto agar plates and incubated overnight. As the inoculum used for microscopy had a 40-fold concentration of bacteria compared to the MIC experiments, which may lead to increased survival of the bacteria. Dilutions were made from cultures starting at $OD_{600}$ = 0.4, which were treated with all four compounds at both MIC and MBC value. Incubation was done for 15 minutes and 60 minutes. Dilutions were $10^{-1}$ and $10^{-4}$. In all cases a thin film of bacteria was formed (not shown).

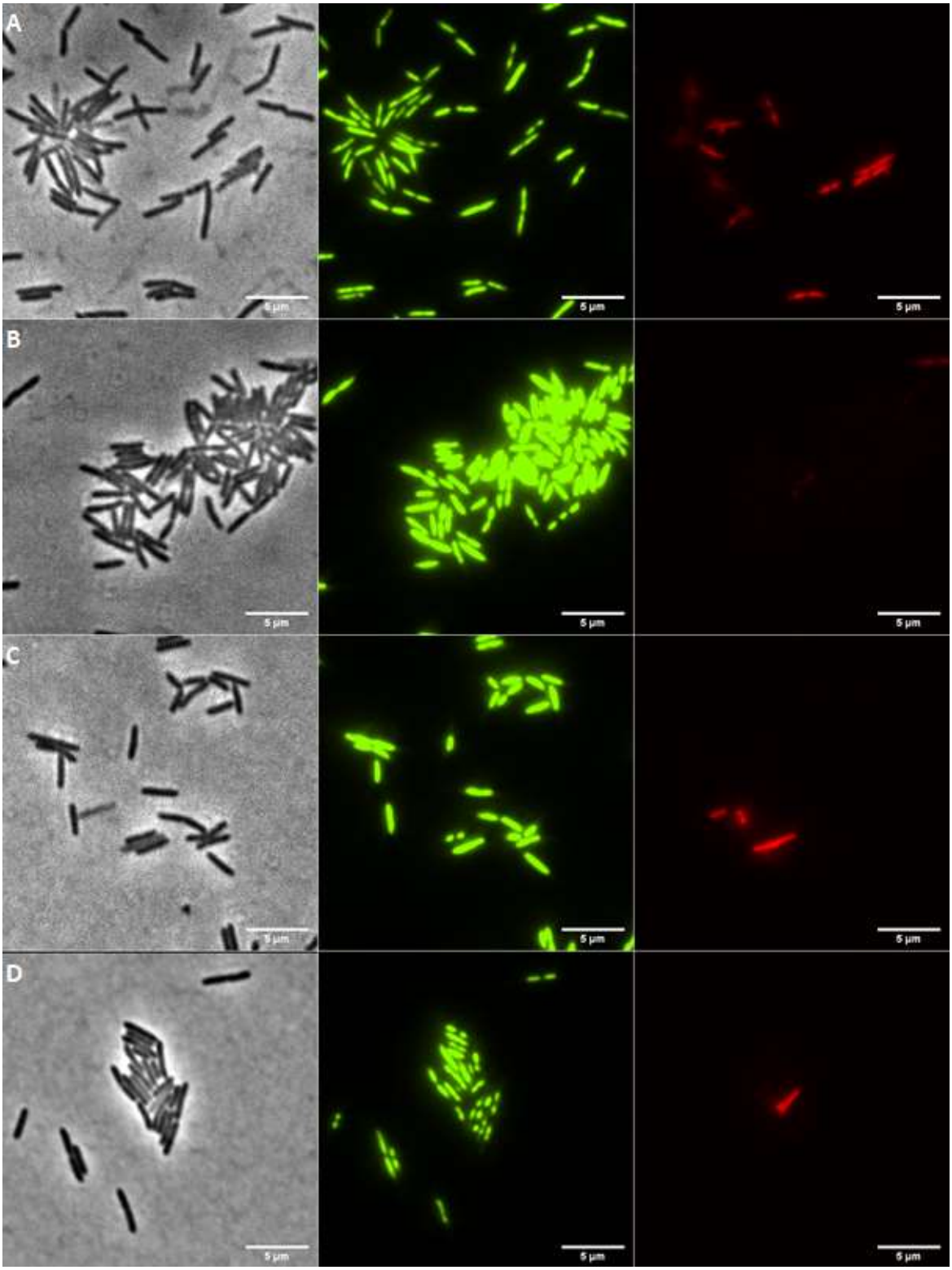


*Figure 7 - Comparison of live/dead dyed cells after treatments for 15 minutes with 6.25 µg/mL (A) and 12.5 µg/mL (B), and for 60 minutes with 6.25 µg/mL (C) and 12.5 µg/mL (D) of BTO. From left to right there is contrast picture, cells with non-permeabilised membrane, which are dyed with SYTO9 and cells with permeabilised membrane, dyed with propidium iodide.*

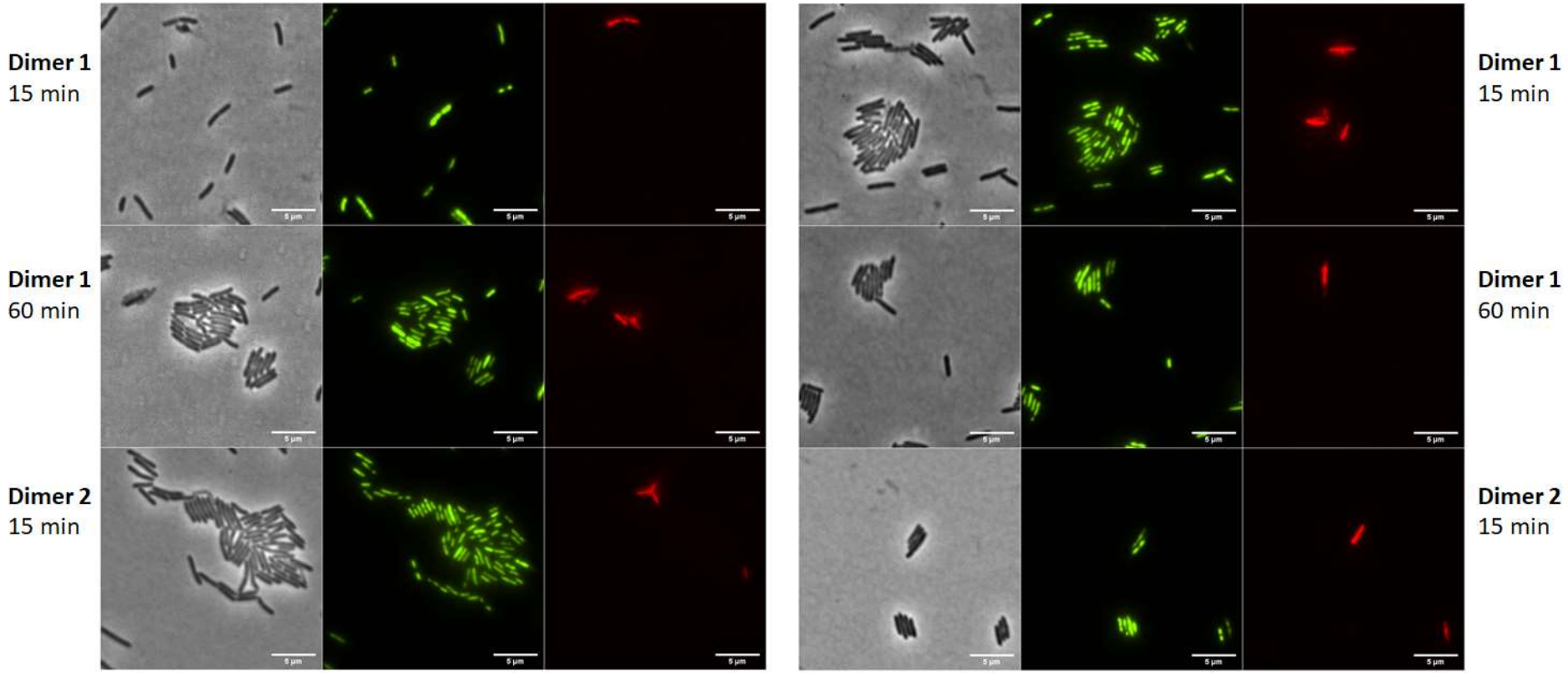


*Figure 8 - Comparison of live/dead dyed cells after treatments with Dimers 1-3, for 15 and 60 minutes. From left to right there is contrast picture, cells with non-permeabilised membrane, which are dyed with SYTO9 and cells with permeabilised membrane, dyed with propidium iodide.*

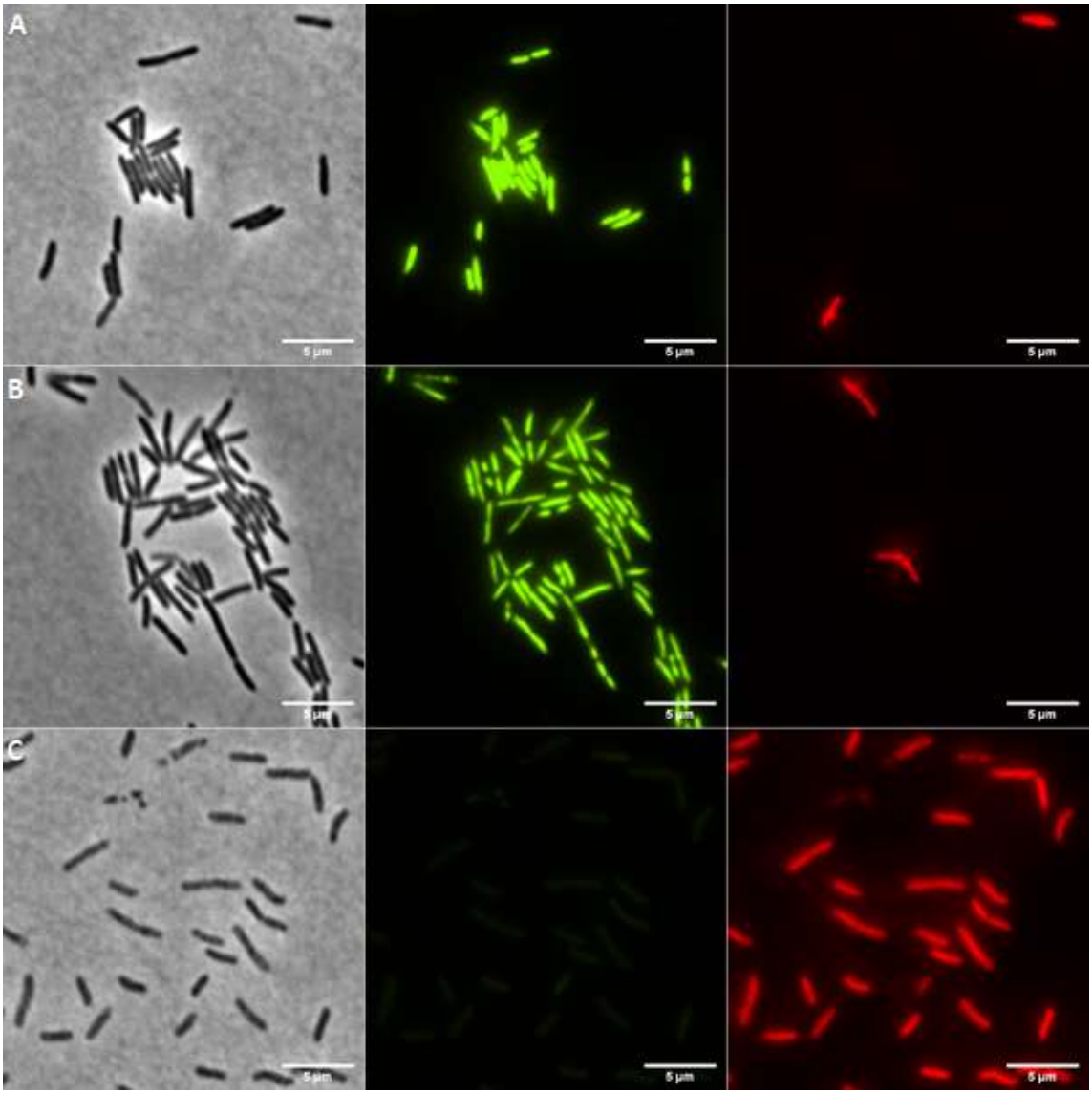


*Figure 9 - Comparison of controls, cells treated with DMSO (A), untreated cells (B) and cells treated with heat shock (C). From left to right there is contrast picture, cells with non-permeabilised membrane, which are dyed with SYTO9 and cells with permeabilised membrane, dyed with propidium iodide.Resazurin assay of BTO and dimer 3*

BTO and dimer 3 showed, respectively, 40% and 60% of the fluorescence exhibited by untreated and DMSO-treated controls, when normalised to uninoculated controls. Fluorescence was also lower than for antibiotic controls treated with rifampicin and penicillin G (See *Figure 10*). This would indicate that BTO and dimer 3 treatment decrease metabolism significantly compared to both untreated samples and samples treated with antibiotics. However, both BTO and dimer 3 are themselves fluorescent, with samples that do not contain bacteria but do contain medium and compound showing a fluorescence signal, that appears to increase over time and that is not stable (See *Figure 11*). This background fluorescence may lead to the underestimation of the fluorescence signal resulting from the reduction of resazurin, since the addition of the different fluorescent factors is not necessarily linear.

It was noticed during an initial measurement of emission and excitation spectra that, after exposure to light, samples in medium with a high concentration (50 µg/mL) of BTO had lost all the colour of resazurin. After being left for 2 days in medium with the same concentration of Dimer 1, it had lost its

colour as well. This did not happen in samples where compound and resazurin were mixed in water instead of medium. This indicates that BTO and Dimer 1 react with NYG medium and resazurin. The resazurin assay was discontinued due to the problems with background fluorescence before usable samples of dimers 1 and 2 were synthesised.

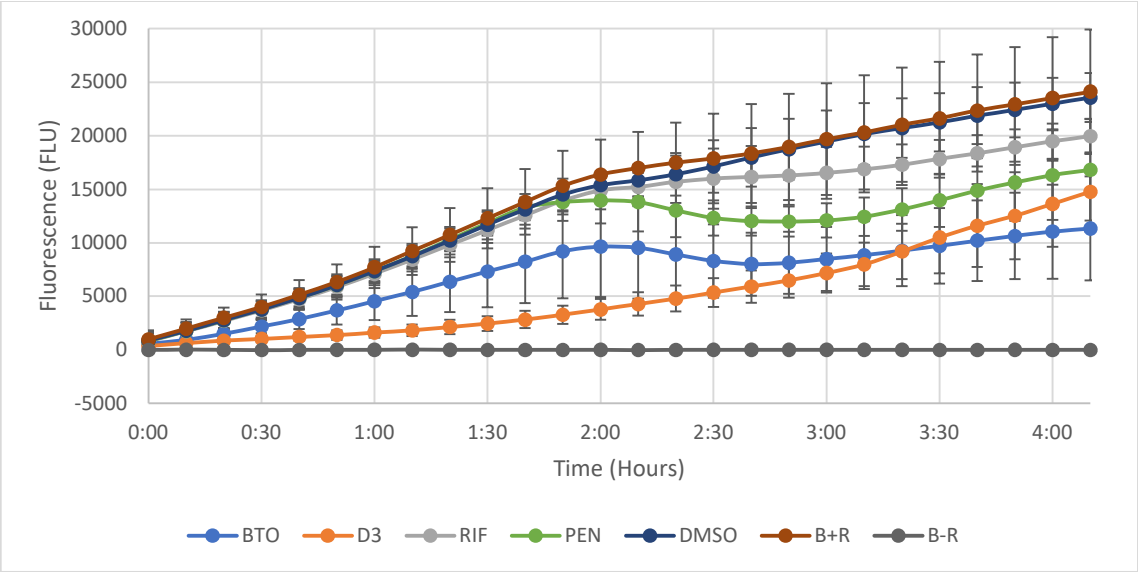


*Figure 10 - Change in fluorescence in resazurin assay over time. Comparison of cultures treated with BTO, dimer 3 (D3), rifampicin (RIF), penicillin G (PEN) and controls treated with DMSO and not at all (B+R). Also, a sample of untreated culture without resazurin was measured (B-R). N=18, 12 (D3), 6 (B-R).*

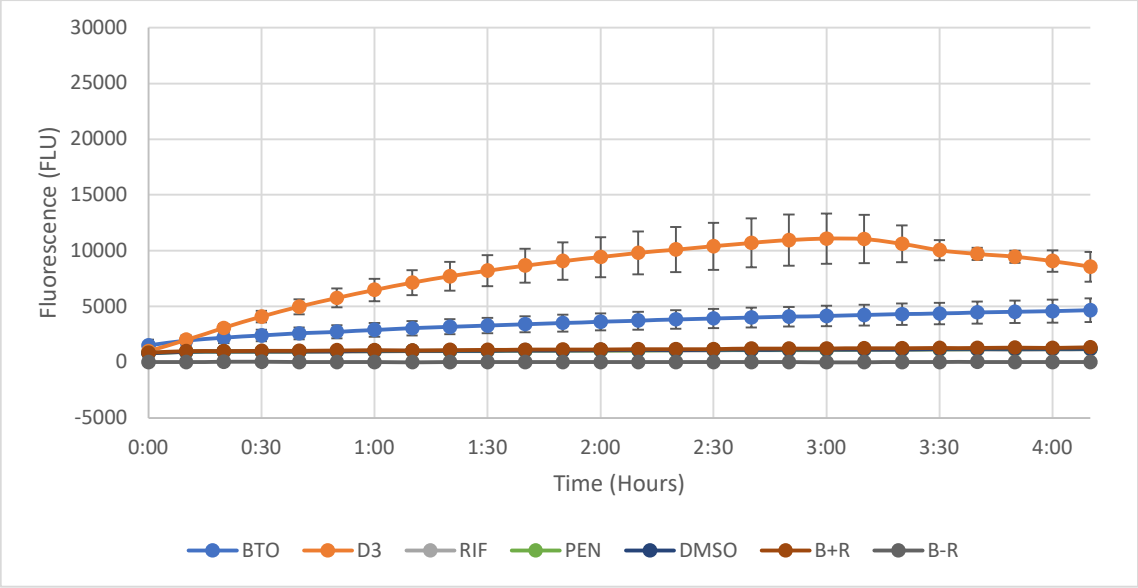


*Figure 11 - Change in fluorescence in uninoculated wells over time during a resazurin assay. Comparison of medium with BTO, dimer 3 (D3), rifampicin (RIF), penicillin G (PEN) and DMSO, in addition to pure media with and without resazurin (B+R and B-R). N=9, 6 (D3), 3 (B-R).*

## Intracellular ATP concentration

Intracellular ATP was measured using luminescence produced by BacTiter-Glo™ reagent in the presence of ATP. Samples were snap-frozen in liquid nitrogen. Treated samples showed delayed increase in ATP concentration compared to untreated and DMSO treated controls. Treated samples

had reached ATP concentrations similar to controls after 2 hours of incubation. The exception was the sample treated with dimer 3, which showed 0.5-fold compared to the untreated sample (See *Figure 12*).

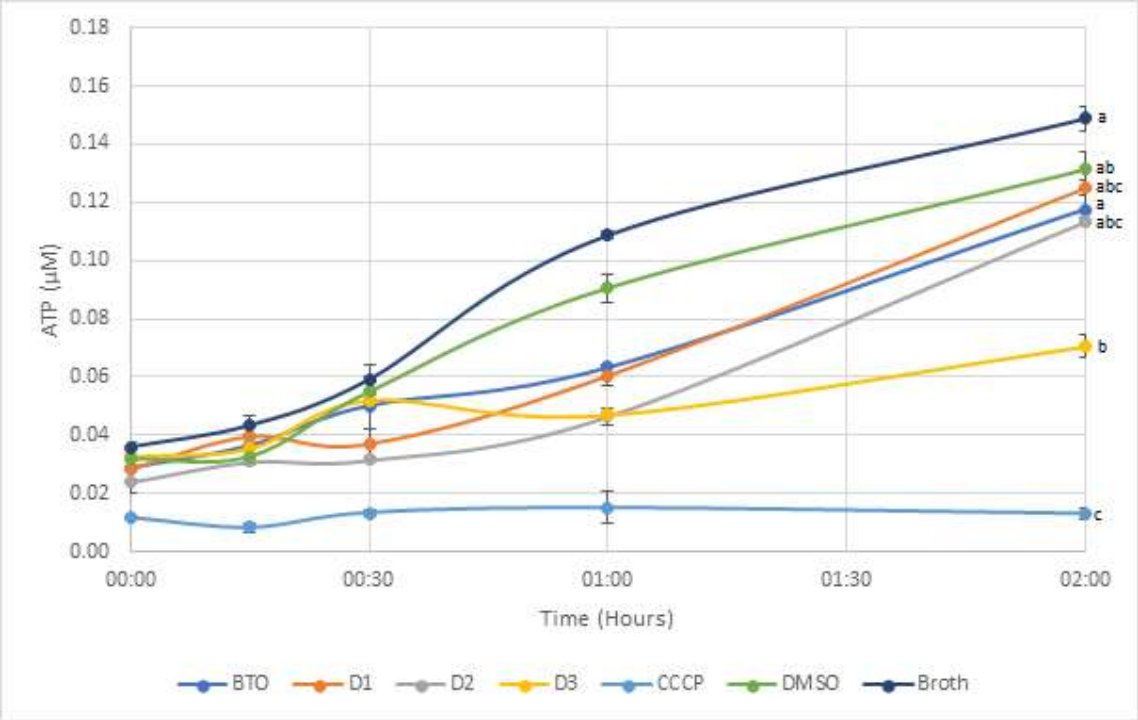


*Figure 12– ATP concentration over two hours of incubation. Values are compared for cultures treated with BTO, Dimer 1 (D1), Dimer 2 (D2), Dimer 3 (D3), CCCP, DMSO and an untreated culture (Broth). Samples were taken and snap-frozen at 0, 15, 30, 60 and 120 minutes. ANOVA was applied to determine if groups were statistically significant. Data sets for the final measurement at 2 hours are grouped based on post-hoc t-tests with Bonferroni correction. Groups marked with the same letter are not statistically different.*

Initial measurements without snap-freezing were made for treatment for 0, 1 and 2 hours. Initial (0 hour) values varied between the measurements, as such each measurement could only be compared to its concurrent 0-hour value. Differently treated cultures at the 0-hour value also showed often significant variance between groups, while after incubation for 1 or 2 hours, only the positive control treated with CCCP showed significantly lower ATP concentration (see *Figure 13*).

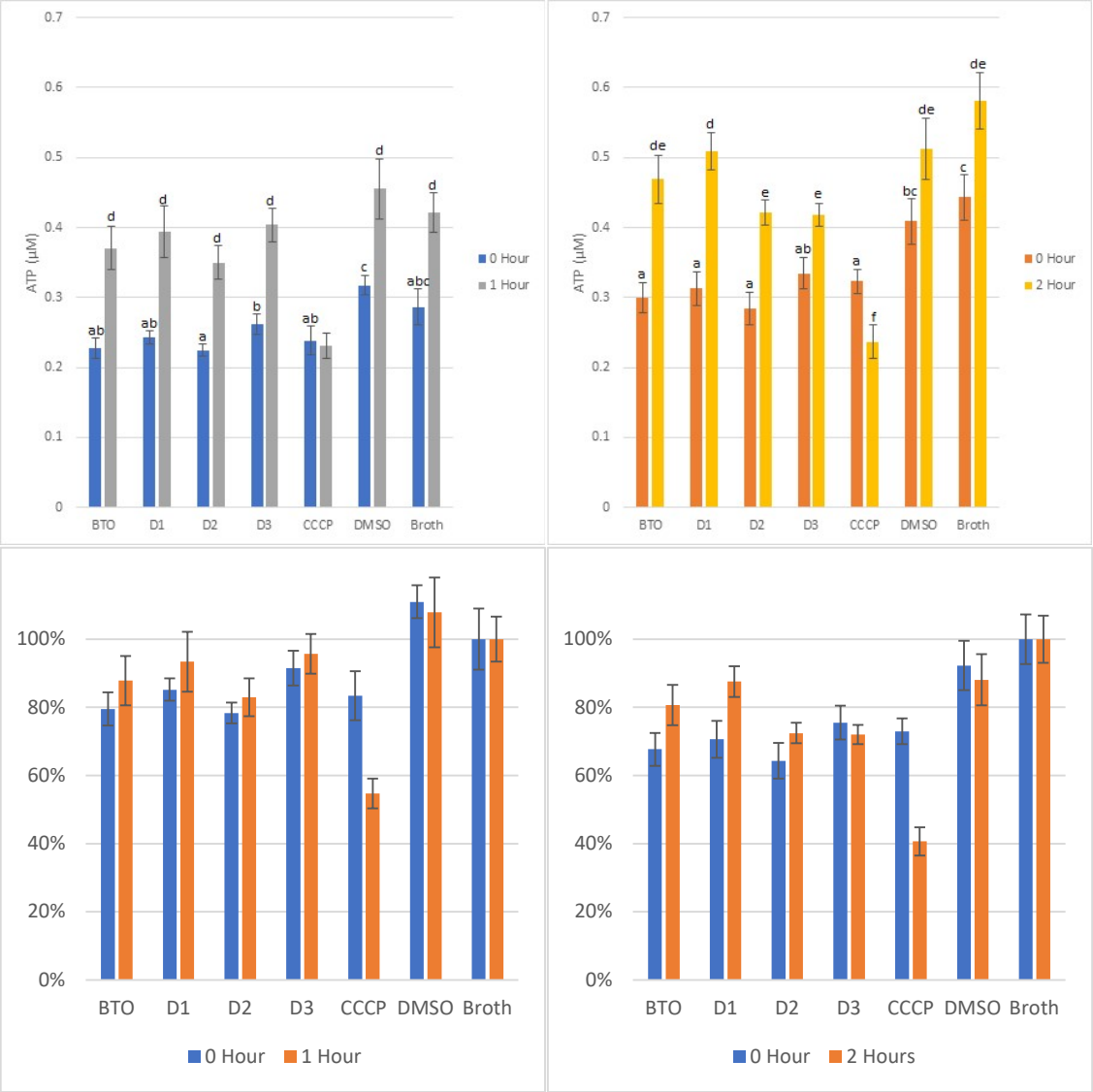


*Figure 13 – Results of initial ATP concentration assay. Values are compared for cultures treated with BTO, Dimer 1 (D1), Dimer 2 (D2), Dimer 3 (D3), CCCP, DMSO and an untreated culture (Broth). ATP concentrations in cultures grown for 1 (upper left) and 2 (upper right) hours. ANOVA was applied to determine if groups were statistically significant. Data sets are grouped based on post-hoc t-tests with Bonferroni correction. Groups marked with the same letter are not statistically different. The change in relative ATP concentration, normalised to the Broth value is compared for 1 hour (lower left) and 2 hours (lower right).*

## Intracellular reactive oxygen species assay

Intracellular ROS generation was assessed using cells pre-treated with 2',7'-Dichlorodihydrofluorescein diacetate. In general, only positive controls showed higher ROS generation, 1.5-fold at the final measurement. Similarly to the resazurin assay, the background fluorescence did influence the results, with dimers 2 & 3 showing 'negative' fluorescence when adjusted to controls (See Figure 14).

Comparison of uninoculated controls and controls inoculated with undyed bacteria revealed that the presence of the bacteria themselves altered the fluorescence of the sample. The difference was not noticeable for BTO, while dimer 1 showed higher fluorescence for inoculated samples, with dimer 3 showing even higher difference. Dimer 2 however showed higher fluorescence for uninoculated samples (See *Figure 15*).

An initial measurement of excitation and emission spectra was made, for excitation wavelengths of 488nm and 530nm, and emission wavelengths 530nm and 585nm, corresponding to the excitation and emission wavelengths used in the reactive oxygen species assay and resazurin assay, respectively. This measurement was done at the initial point of mixing, at which no difference was observed between NYG medium with or without compounds at relevant wavelengths. (data not shown).

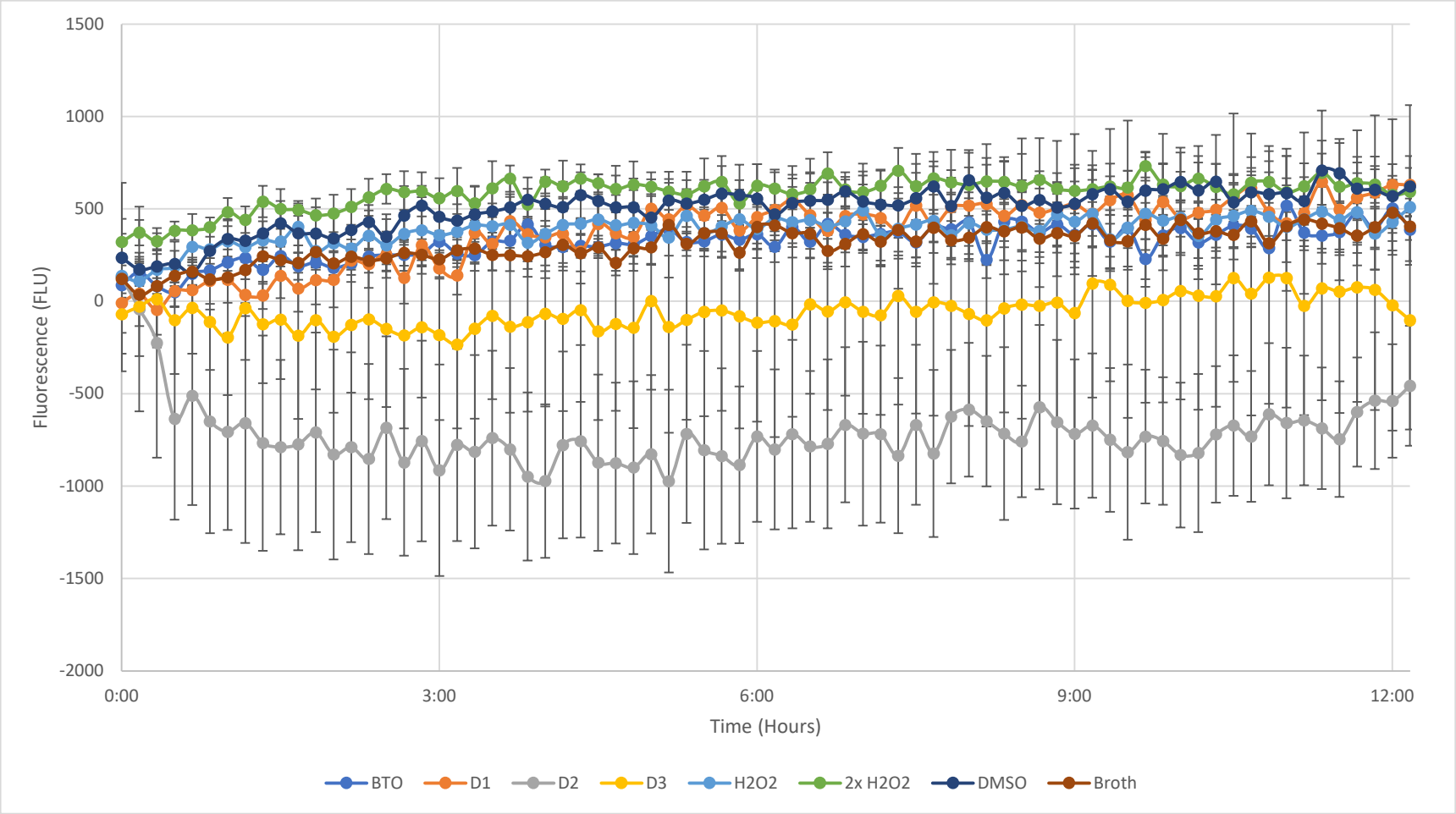


*Figure 14 - Fluorescence of treated samples, normalised to controls inoculated with undyed cells. Values are compared for cultures treated with BTO, Dimer 1 (D1), Dimer 2 (D2), Dimer 3 (D3), DMSO and an untreated culture (Broth). Two concentrations of positive control sample were used, 10mM (H2O2) and 20 mM (2x H2O2).*

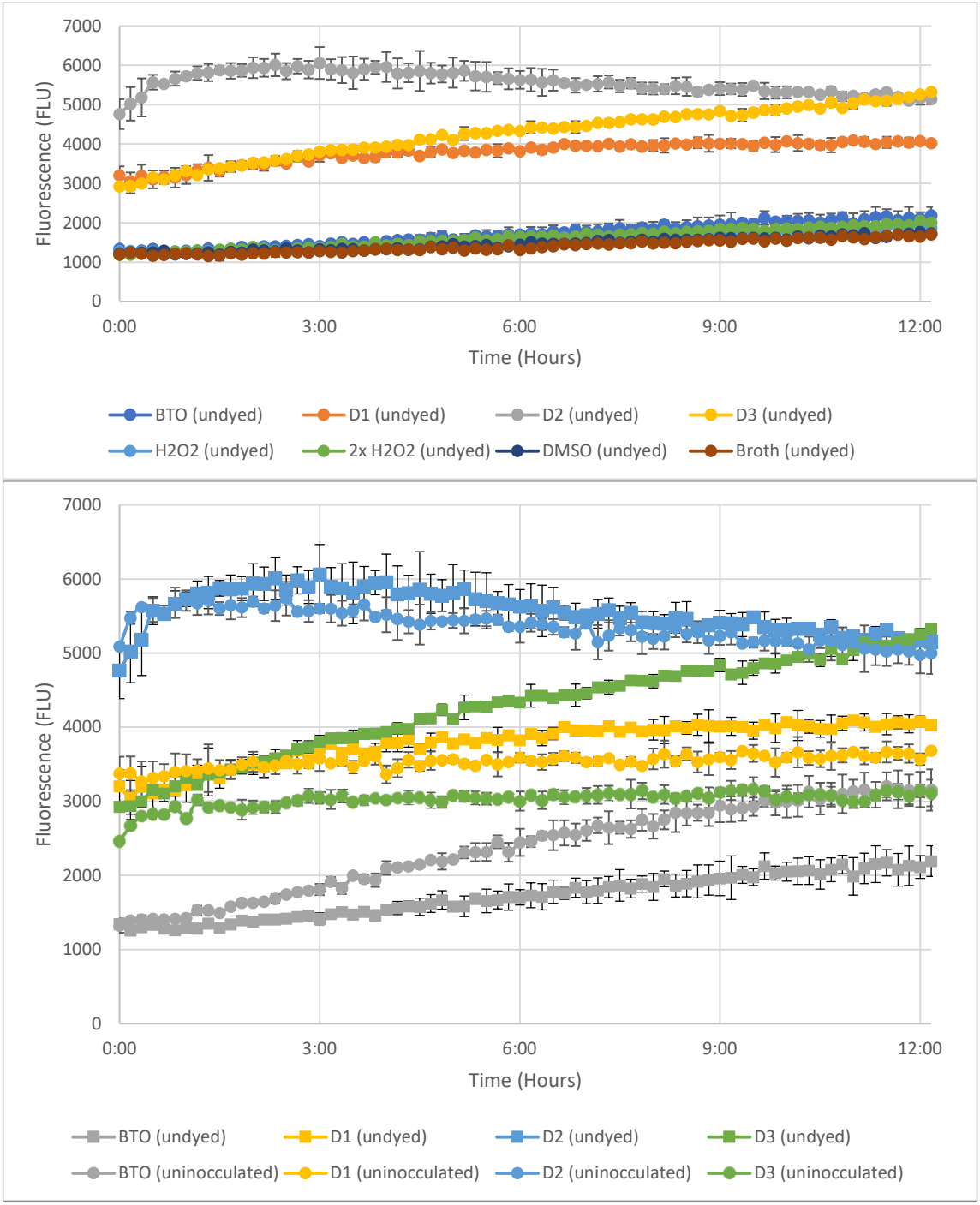


*Figure 15 - (Upper) Background fluorescence of controls for ROS assay. Controls were samples inoculated with undyed bacteria. (Lower) Comparison of fluorescence of media treated with compound, inoculated with undyed cells, and uninoculated.*

# Discussion

## Activity of compounds compared to other anti-microbial compounds

*Table 3 – Summary of MIC and MBC values of compounds, compared to previously tested compounds heptyl-2,4-dihydroxybenzoate (Nazaré et al., 2018) and n-heptyl gallate (Silva et al., 2013). MIC values of the control antibiotic kanamycin and commonly used copper compound copper oxychloride also provided (Nazaré et al., 2018).*

| Compound | MIC (µg/mL) | MIC (µM) | MBC (µg/mL) | MBC (µM) |
|---|---|---|---|---|
| BTO | 6.25 | 49.6 | 12.5 | 99.1 |
| Dimer 1 | 25 | 99.9 | 25 | 99.9 |
| Dimer 2 | 50 | 201.5 | 100 | 402.9 |
| Dimer 3 | 50 | 214.7 | 50 | 214.7 |
| heptyl-2,4-dihydroxybenzoate | 13.2 | 52.3 | 25 | 99.1 |
| n-heptyl gallate | 31.2 | 116.3 | 31.2 | 116.3 |
| Copper oxychloride | 43.1 | 99.5 | | |
| Kanamycin | 19.9 | 40 | | |

BTO had a low MIC value, which compares positively to previously tested compounds as well as to the control antibiotic kanamycin. For comparison, two of the compounds with the lowest MIC from previous studies were heptyl-2,4-dihydroxybenzoate with MIC of 13.2 µg/mL (Nazaré et al., 2018) and n-heptyl gallate with a MIC value of 31.2 µg/mL (Silva et al., 2013). Its MBC value, while higher than the MIC value, was still similar or lower than previously reported compounds. The comparatively low molar mass of BTO does also factor in the low MIC mass value, but it still compares favourably when comparing MIC and MBC values in molarities.

The dimers were not as active, with higher MIC and MBC values. Dimer 1 was the most active, with MIC and MBC value of 25 µg/mL, while dimer 2 the least with MIC value of 50 µg/mL and MBC of 100 µg/mL. Dimer 1 still compares favourably to previously tested compounds as well as copper oxychloride, a common copper compound used in agriculture (Nazaré et al., 2018).

A question arising from previous studies was whether it was BTO itself which contributed this anti-bacterial activity, or its dimerization and other reaction products. Initial pilot showed BTO had higher antibacterial activity than a mix of dimers 1 & 2. Furthermore, the antibacterial activity of BTO decreased with pre-incubation in media, indicating that BTO itself is the active compound in BTO-treated samples. Unlike BTO, the antibacterial activity of the dimers did not decrease with pre-incubation time.

## Summary of mode of action

*Table 4- Summary of the results of mode-of-action testing.*

| Compound | Membrane permeabilization | Lowered metabolism | Reactive oxygen species formation |
|---|---|---|---|
| BTO | Not observed | Not observed | Inconclusive |
| Dimer 1 | Not observed | Not observed | Inconclusive |
| Dimer 2 | Not observed | Not observed | Inconclusive |
| Dimer 3 | Not observed | Observed* | Inconclusive |

** While ATP concentration was lower for samples treated with dimer 3, it was not considered significantly different from samples treated with other compounds or DMSO control by post-hoc t-test, only from the untreated negative control.*

Overall, the mode of action was not uncovered, neither for BTO nor dimers. None of the compounds did increase the number of permeabilised cells in treated cultures. This is different from previously tested alkylated derivatives of BTO, which do permeabilise the cell membrane of *X. citri*. Alkylated BTO derivatives were able to permeabilise both Gram positive and negative cells within 15 minutes (Cavalca et al., 2021). It is possible that without the hydrophobic tail, the interaction of the compound with the membrane is limited.

Initial respiratory metabolism assays utilizing resazurin, done on BTO and dimer 3, indicated that both compounds led to lower metabolism in the treated cells. The high fluorescence of the uninoculated control wells brings the validity of that conclusion into question. As the background fluorescence from the compound and the fluorescence from the reagent are not necessarily a linear addition, meaning that the background fluorescence may be 'hiding' some of the fluorescence from the dye.

Due to the problems arising from fluorescence measurements, the intracellular ATP concentration assay utilising luminescent reagents was used instead to evaluate effects of the compounds on metabolism. Initial tests did not show significant difference between the cultures treated with compound and controls. Due to the methodology of the initial tests, it was difficult to compare ATP concentrations at different time points, as each test only included an assay at the initial point and after incubation. Differences in concentration at the initial time point between the experiments made the comparison difficult. The optimisation to include snap-freezing of samples mitigated this by having samples taken at different time points from the same culture. For the latter experiment, treatment with dimer 3 lead to lower ATP concentration than other treatments. However, the dimer 3 sample was not significantly different from samples treated with dimers 1 or 2, or the control treated with DMSO. It was however different from samples treated with BTO, positive control treated with CCCP and negative untreated control. The experiment needs to be repeated to confirm the validity of this result.

BTO has been shown to damage mammalian cells by producing radical oxygen species (Lévay and Bodell, 1992; Shen et al., 1996; Zhang et al., 1993). Due to the fluorescence of both undyed inoculated controls and uninoculated controls it was not possible to verify if this was also the case in *X. citri*. Changes in methodology to either accommodate for said fluorescence or side-stepping it would be necessary to elucidate this. If it is shown that intracellular reactive oxygen species are formed in the cells, further experiments could be done to establish if there is a causal link between the radicals and the antibacterial activity of BTO and its derivatives.

## Background fluorescence and side reactions of compounds

Background fluorescence by the tested compounds was a problem in both resazurin assay and reactive oxygen species assay. Therefore, it came as a surprise when excitation and emission range spectra of the compound was measured that not much difference was seen between medium with and without compound. While only initial testing was done in this regard, which without repetition does not offer strong basis to draw conclusions, it can be posited that the compounds themselves are not fluorescent, but rather they react into compounds that are over time. This is in accordance with previous experiments showing that background fluorescence increased over time. Further observation of the colour change of resazurin solutions with different compounds and NYG media provided further evidence that BTO and dimer 1 react with the media, as resazurin in medium with high concentration of those compounds lost colour, while the effect was much smaller where the compounds were dissolved in water instead of medium.

An interesting observation from the controls of the reactive oxygen species assay was that inoculating media with undyed bacteria changed how the fluorescence of the well increased with time, leading to greater or lesser increase depending on the compound. The presence of bacteria in the medium appears to alter the fluorescence of the compound. It is possible that this was due to a change in pH caused by the bacteria, which in turns changes the solubility of the compounds. If the fluorescence is indeed caused by reaction products of the compounds

with the medium, instead of the compound itself, such change in pH may be affecting the rate of such reactions.

## Other possible methods and experiments

Due to the problems caused by background fluorescence in the resazurin and reactive oxygen species assays, alterations or alternative methodologies should be considered. The background fluorescence could be counteracted by measuring fluorescence at various concentration mixes of both the compound and the dye. Analysis of the data could be used to analyse how each factor contributes to total fluorescence. From this, the contribution of only the reaction of interest can be deduced. Preparation and measurement of multiple samples would however increase the workload of the experiment. Furthermore, the interactions leading to fluorescence would be unique for each compound, and as such this setup would have to be repeated for each compound. This setup would also not be compatible with the method used for measuring reactive oxygen species, as it involves washing away the dye before compounds are added, leaving only the dye left in the cells. This would make it difficult to establish any meaningful connection between dye concentration and fluorescence.

Another possible method to counteract fluorescence would be to wash the compounds away before applying dye. This procedure could be described as reversing the method used for reactive oxygen species assay. Samples would be taken from an incubating culture, washed and resuspended in medium with the selected dye. As this method would take a snapshot of the culture, it is not suited for observation of processes such as metabolism. Problems may also arise when attempting to measure reactive oxygen species, as due to their reactivity it is possible they may not be measurable after the washing and dyeing are completed. As such the feasibility of such washing is questionable.

Instead of trying to counteract the background fluorescence, it is also possible to use methods that do not utilise fluorescence at all. For metabolism we already utilised an intracellular ATP assay that relies on luminescence instead of fluorescence. The ROS-Glo™ H2O2 Assay utilises luminescence to measure concentration of hydrogen peroxide ($H_2O_2$) in cells. $H_2O_2$ has a long half-live compared to other reactive oxygen species, and some reactive oxygen species have a tendency to form it, making it a good marker of oxidative stress (Duellman et al., 2013). The primary reactive oxygen species formed by BTO have indeed previously been noted to be hydroxyl radicals and hydrogen peroxide, which does suggest this reagent would indeed be useful in this regard (Nishikawa et al., 2012; Shen et al., 1996).

Instead of attempting to measure reactive oxygen species, their contribution to anti-bacterial activity can be assessed indirectly. This can be done by observing if treatment with antioxidants can counteract the anti-bacterial effects of the compounds. Similar experiments have been used to show that catalase is capable of counteracting chromosomal damage in human lymphocytes (Zhang et al., 1993).

Another way to look indirectly at radical activity is to observe the oxidative stress response of the cell. Various enzymes such as catalase and superoxide dismutase are expressed to defuse the radicals to harmless compounds. Due to their roles as plant pathogens, bacteria of genus *Xanthomonas* have evolved effective means of dealing with $H_2O_2$, which is utilised by plants to fight infections (Loprasert et al., 1996; Tondo et al., 2010). Using RT-PCR to observe if change in transcription is similar for BTO-treated cells and cells treated with ROS-generating compounds might be used to establish a link.

## Possible usage of compounds in agriculture

Before any of the compounds can be used in agriculture, the mode of action would have to be discovered. Further considerations would be overall activity, how long said activity lasts and if the compound demonstrates toxicity to other life as well.

While BTO was initially only considered as a precursor chemical for active compounds, the revelation of its own activity opened up the possibility of it being considered for use against citrus canker. While it is highly active against *X. citri* sp. *citri,* the decrease of said activity with time works against it. Furthermore, BTO is among the metabolites believed to be responsible for the toxicity of benzene in humans. It has been shown to cause multiple types of DNA damage in mammalian cells by generating reactive oxygen species (Lévay and Bodell, 1992; Shen et al., 1996; Zhang et al., 1993). Its alterations of DNA have also been shown to halogenate DNA and affects methylation (Nishikawa et al., 2012; Yu et al., 2019). The former disadvantage might counteract the latter to some degree. If the reaction products of BTO prove to be less toxic, then toxicity would also decrease over time.

While the dimers have lower activity compared to BTO, that activity does not decrease with time. With synthesis of said dimers having only gotten some focus recently, no data exist on whether they are toxic to other life. Toxicity studies are underway among our associates in UNESP in Brazil. The results from those studies will factor into whether the dimers will be looked at as candidates for further work towards making an antibacterial product.

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