TÉCNICO LISBOA

Antibacterial Mode of Action of BMX-11, an antifoulant from bacterial origin

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Thesis to obtain the Master of Science Degree in

Microbiology

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December, 2017

Resumo

Ao longo das últimas cinco décadas, várias espécies bacterianas de relevância clínica desenvolveram mecanismos de resistência em resposta à implementação do uso de a antibióticos, limitando a eficácia de tratamentos atualmente disponíveis. Assim, é de elevada importância o estudo, a descoberta e desenvolvimento/optimização de novos antibióticos.

Esta tese tem como objectivo o estudo do modo de ação antibacteriana do BMX-11, um novo composto activo, através da realização de testes antimicrobianos contra um painel de estirpes bacterianas de relevância, na presença de BMX-11. Foram usadas diversas técnicas como a microdiluição de agentes activos, o estudo da taxa de morte e a monitorização da viabilidade celular em agar e por citometria de fluxo, de forma a definir CMI's (Concentrações Mínimas Inibitórias), CMB's (Concentrações Mínimas Bactericidas), as taxas de morte, a estudar a integridade e a polarização da membrana celular, e o tipo de acção como bactericida ou bacteriostático e como dependente da concentração e/ou do tempo.

O BMX-11 apresentou efeito bactericida contra estirpes de *Cobetia marina, Escherichia coli, Enterococcus faecalis* e *Vibrio vulnificus* e bacteriostático contra *Listeria monocytogenes* e *Staphylococcus aureus*. Mostrou também ter ação dependente da concentração contra todas as estirpes em estudo e mostrou afectar a permeabilidade e a polaridade da membrana celular.

Palavras-chave: modo de acção, BMX-11, antimicrobiano, antibacteriano, antibiótico, CMI, CMB, taxa de morte, citometria de fluxo, bactericida, bacteriostático, bactéria.

Abstract

Over the last five decades, the development of bacterial resistance mechanisms to antibiotics have spread among several clinically important bacterial species, limiting the effectiveness of currently available treatments. There is a pressure over the quest for the development of new antibiotics.

This thesis aimed at the study of the antibacterial mode of action of BMX-11, a new active compound, which was achieved through microbial testing against key reference bacterial strains and techniques such as broth microdilution, time-kill curves and flow cytometry, in order to study the BMX-11 effect as concentration and/or time dependent and its bactericidal vs bacteriostatic behaviour to define its MIC (Minimal Inhibitory Concentration), MBC (Minimal Bactericidal Concentration), time-kill kinetics and effect over membrane integrity and polarization.

It was found that BMX-11 has a bactericidal effect against *Cobetia marina, Escherichia coli, Enterococcus faecalis* and *Vibrio vulnificus*, and a bacteriostatic effect against *Listeria monocytogenes* and *Staphylococcus aureus*. BMX-11 displayed concentration-dependent action against all used strains and showed to be able to damage cell membrane by affecting membrane permeability and membrane polarization.

Key-words: mode of action, BMX-11, antimicrobial, antibiotic, MIC, MBC, time-kill, flow-cytometry, microdilution, bactericidal, bacteriostatic, bacteria.

Acknowledgements

I would like to use this opportunity to express my gratitude to my supervisor, Dr. Patrick Freire, for all his support and guidance throughout this journey. I am really thankful for the opportunity I was given, for his invaluably constructive criticism, patience, and friendly advice.

I would also like to express appreciation to my supervisor from IST, Professor Isabel Sá-Correia, for her support along the development of this thesis. Without her counselling, this thesis would not have been possible.

My thanks also to Professor Ana Tenreiro for all the dispensed time on the flow cytometric assays and for the patience to repeat the trials over and over until they finally worked out.

I am thankful to Dr. Inês Guinote for all her illuminating views, explanations on a number of issues related to the project and for always being available. Also, a special thanks to the remaining BioMimetx team Dr. Gonçalo Costa, Susana Saraiva and Tânia Chança for all the support, for making it possible, and for all the monitoring, care and camaraderie.

I would also like to thank all the Lab Bugworkers | M&B-BioISI team, specially to Filipa Antunes, for providing all the facilities and conductive conditions required for this project.

Finally, I'd like to thank my family and friends for all the incentive and support.

Thank you,

Raquel

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List of abbreviations

ATCC	american type culture collection
AFM	atomic force microscopy
ASW	artificial sea water
ATP	adenosine triphosphate
BCP	bacterial cytological profiling
CECT	colección española de cultivos tipo
Cf	final concentration
CFU	colony forming unit
Ci	initial concentration
CLSI	clinical and laboratory standards institute
DNA	deoxyribonucleic acid
DSMZ	german collection of microorganisms and cell cultures
EtBr	ethidium bromide
FI	fluorescence intensity
FSC	forward-scattered light
LB	lysogeny broth or luria bertani broth or luria broth
MB	marine broth
MBC	minimal bactericidal concentration
MIC	minimal inhibitory concentration
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NCCLS	national committee for clinical laboratory standards
NCTC	national collection of type cultures
OD	optical density
PBS	phosphate buffered saline
PI	propidium iodide
RNA	ribonucleic acid
SD	standard deviation
SEM	scanning electron microscopy
SSC	side-scattered light
TEM	transmission electron microscopy
TLC	thin-layer chromatography
TLC-MS	thin-layer chromatography-mass spectrometry
tRNA	transfer ribonucleic acid
Vf	final volume
Vi	initial volume

1 Introduction

Over the years, the development of bacterial resistance mechanisms to antibiotics has spread among several clinically important bacterial species, limiting the effectiveness of currently available treatments. Antimicrobial resistance is responsible for an estimated 25,000 deaths per year in the EU and 700,000 deaths per year worldwide, according to the EU Commission (https://ec.europa.eu/health/amr/antimicrobial-resistance_en). As it constitutes a major public health concern, pressure is being done for the development of new antibiotics (Kaufman, 2011).

This thesis focuses on the key points for drug discovery and microbiological testing of new antimicrobials, term which includes not only antibiotics, but also synthetically produced compounds (Kaufman, 2011). The global objective of this work is the determination of the mode of action of the newly discovered antimicrobial BMX-11, which was achieved performing a panoply of techniques and using key reference bacterial strains.

1.1 BMX-11

1.1.1 Discovery, description and production

BMX-11 is a novel proprietary antimicrobial owned by BioMimetx, S.A. This company is a global biotech start-up that stands on R&D work related to environmental bacteria with high levels of resistance to last generation antibiotics. BMX-11 is a new antibiotic candidate, that like many others, was discovered using a screening process, in which bacterial isolates were cultured and tested for production of diffusible products that inhibit the growth of test organisms. After this screening, an interesting bacterial isolate belonging to the *Pseudomonas* genus was selected based on its capacity to produce large amounts of compounds with biocidal activity, a mixture which would later be named BMX-11. Thus, like other drugs, BMX-11 is produced from antibiotic material secreted during bacterial fermentation (Kaufman, 2011), together with a purification and isolation downstream process.

Studies of BMX-11 carried out by BioMimetx team, indicate that it combines antimicrobial, antifungal and algaecide effects (“http://biomimetx.com,” 2017) so, the company decided to direct its efforts to the development of a “green” and biological anti-fouling coating solution, using BMX-11 as an active antifoulant additive, to be applied on vessels and submerse marine structures paints to prevent biofouling accumulation. Despite current direction of the BioMimetx product towards an anti-fouling application (biofouling is the natural mechanism of adhesion of marine organisms to submerged structures), BMX-11 has a clear potential to be used in the development of a wide range of products with applications in diverse sectors such as industrial biotech, agriculture or health (“http://biomimetx.com,” 2017), what makes further studies on BMX-11 of high importance to the future development of the company plans. This thesis main focus is over BMX-11 antibacterial effect.

1.1.2 The origin of BMX-11

BMX-11 is produced by an environmental isolate of *Pseudomonas spp* genus, related to both *Pseudomonas aeruginosa* and to *Pseudomonas putida*.

Species of *Pseudomonas* genus (Migula 1894) (http://www.bacterio.net/pseudomonas.html), are aerobic Gram-negative rods, with facultatively anaerobic metabolism. They are catalase positive (Chun, Rhee, Han, & Kyung Sook Bae, 2001), and don´t produce Xanthomonadins, a class of yellow pigments produced by phytopathogenic *Xanthomonas*, which are associated with the outer membrane of the bacterial cell wall (Poplawsky, Urban, & Chun, 2000; Brenner, Krieg, Staley, & Garrity, 2005). Some *Pseudomonas* spp. show the characteristic property to synthesize yellow-green, fluorescent, water soluble pigments (pyoverdine or pseudobactin), that are components of bacterial siderophores, when under iron stress. Siderophores are molecules related to pathogenicity and host infection, and are required for bacterial iron transport (Laguerre, Rigottier - Gois, & Lemanceau, 1994).

Pseudomonas aeruginosa was first described by Schroeter in 1872 and is the type species of the genus (http://www.bacterio.net/pseudomonas.html). It is a common soil and water inhabitant, and an opportunistic human pathogen (Brenner, Krieg, Staley, & Garrity, 2005, p.42; Pierson & Pierson, 2010), with its optimum growth at 37ºC although it can grow at temperatures up to 42ºC. *P. aeruginosa* is motile by means of a single polar flagellum and it is capable of denitrification and gelatin hidrolyzation. The egg-yolk reaction is negative (Brenner *et al*., 2005). *P. aeruginosa* can produce, between other pigments, *Pseudomonas* blue protein phenazine (pyocyanin, pyorubin, chlororaphin, oxiphenazin), and pyoverdine (Brenner *et al*., 2005; Pierson & Pierson, 2010; Li *et al*., 2011; Morohoshi *et al*., 2013). Iron and osmolarity can induce the expression of virulence determinants (Palleroni, 2015 p.6).. Some strains can also produce rhamnolipids (biosurfactant glycolipids) (Tuleva, Ivanov, & Christova, 2002), which although without antimicrobial activity, have emulsifying, demulsifying and detergent characteristics (between other), which makes them able to the attachment and removal of microorganisms from surfaces (Vijayakumar & Saravanan, 2015).

Pseudomonas putida, first described by Trevisan, in 1889 (http://www.bacterio.net/pseudomonas.html) are rods or cocci (pleomorphic), motile by means of more than one polar lophotrichous flagella (Chun *et al*., 2001), with optimum growth between 25 and 30ºC and unable to grow at 41ºC. What classically defines this species is its incapacity to hydrolyze gelatin and inability to produce any phenazine pigments, denitrify, or to give an egg-yolk reaction (Brenner, Krieg, Staley, & Garrity, 2005, p. 66), although it produces pyoverdine (Brenner, Krieg, Staley, & Garrity, 2005 p. 66).

Pseudomonas spp may produce many interesting compounds with antimicrobial properties, such as pyoverdine and pseudobactin, yellow-green, fluorescent, siderophore pigments (Laguerre *et al*., 1994), pseudomonine and pyochelin, salicylic acids-containing siderophores (Li *et al*., 2011), phenazine, a secondary metabolite with antifungal activity (Morohoshi *et al*., 2013; Li *et al*., 2011) and promysalin, a novel type of amphipathic salicylic acid-containing and intragenus-specific antibiotic (Li *et al*., 2011). Phloroglucinols, which are used in the synthesis of pharmaceuticals, pyoluteorin, an antifungal

compound, and cyclic lipopeptides, a promising class of natural products, are also antibiotics produced by *Pseudomonas spp*, as well as pyrrolnitrin, which has been used in the development of the phenylpyrrole class of agricultural fungicides (Li *et al*., 2011) and polyketide mupirocin, a commercial antibiotic (Li *et al*., 2011).

1.1.3 The study of BMX-11 mode of action, the thesis objective

This master's thesis objective is to study the BMX-11 mode of action against a relevant panel of Gram-negative and Gram-positive bacteria by understanding and quantifying BMX-11 capacity to inhibit and to kill bacteria. Additionally, a second goal aimed at knowing BMX-11 mode of action as bactericidal vs bacteriostatic, with concentration- or time-dependent biocidal activity, and structural/functional cell targets.

1.2 Mode of action

1.2.1 What is the mode of action of a biocide/antibacterial?

Briefly, the mode of action of an antibacterial agent is the mechanism that it utilizes to interfere with bacterial cell biological mechanisms. Different antibiotics have different modes of action, owing to the nature of their structure and degree of affinity to certain specific cell targets (Chifiriuc *et al*., 2016). Antibiotic targeting may explore the differences of cell structure between different types of cells (prokaryotic vs eukaryotic; Gram-negative vs Gram-positive; etc.) (Kaufman, 2011) or target enzymes that are either unique to the prokaryotic cell or sufficiently different such that toxicity to the mammalian host is low (Mcdermott, Walker, & White, 2003).

1.2.2 What cell structures/functions may be affected by an antibacterial?

Antibacterial agents can interfere with basic and essential cell structures/mechanisms, as showed in fig. 1, that include inhibition/disruption of cell membranes integrity, cell wall synthesis, protein synthesis, nucleic acid synthesis and inhibition of other metabolic processes such as folate synthesis (Mcdermott *et al*., 2003; Hancock, 2005; Kaufman, 2011).

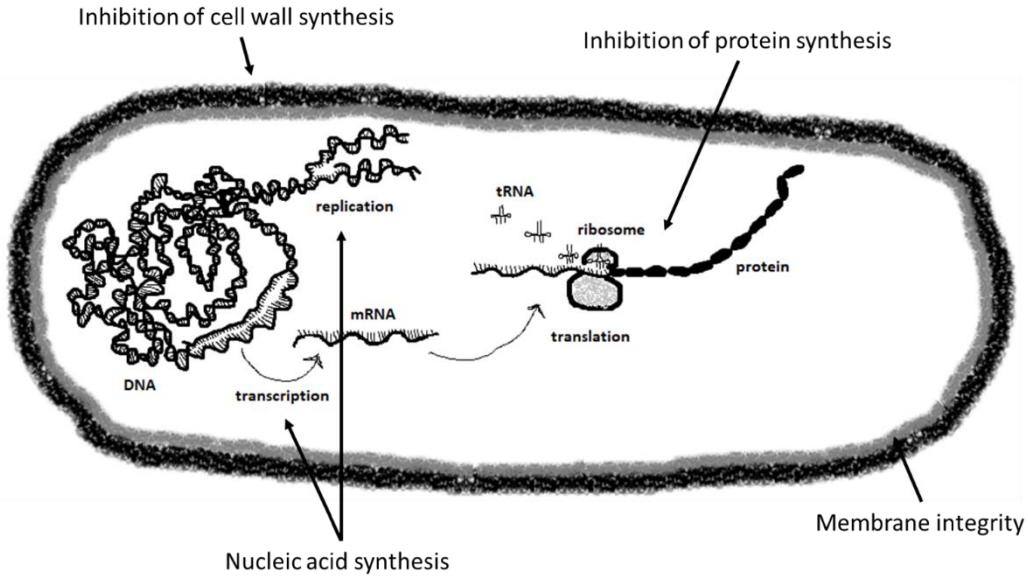


Figure 1. Possible cellular structural/functional targets of antibiotics within the bacterial cell. Adapted from (Chifiriuc *et al*., n.d.; Silva, Gramaxo, Baldaia & Félix, 2008, p. 28).

1.2.2.1 Inhibition of bacterial cell wall synthesis

The bacterial cell wall has a main unique constituent, the peptidoglycan (murein), despite differing in the quantity and in its location within the cell envelope between the two bacterial cell types Gram-positive and Gram-negative (Mcdermott *et al.*, 2003; J.Silhavy, Kahne, & Walker, 2010). Antibiotics can differently act by interrupting peptidoglycan synthesis, assembly or arrangement (Kaufman, 2011). Depending on the drug agent, the effects can be different: some inhibit cell division, leading to long filamentous forms, whereas others lead to the formation of deficient cell walls that easily lyse (Mcdermott *et al*., 2003; Kaufman, 2011). Drugs that destroy the cell in this way generally have a bactericidal effect (Kaufman, 2011).

Peptidoglycan biosynthesis comprises four major steps: (1) synthesis of precursors in the cytoplasm; (2) transport of precursors across the cytoplasmic membrane; (3) insertion of glycan units into the cell wall; and (4) transpeptidation and maturation (Mcdermott *et al*., 2003). β-lactams for example inhibiting steps 3 and 4 of peptidoglycan biosynthesis by inactivating penicillin-binding proteins (PBP's) thus preventing peptidoglycan maturation (Mcdermott *et al*., 2003; Hancock, 2005). Glycopeptides (e.g.: vancomycin, teicoplanin), also act on steps 3 and 4, by binding to D-Ala-D-Ala terminus of peptidoglycan chains, blocking transglycosylation and transpeptidation and inhibiting the growth of the peptidoglycan chain (Mcdermott *et al*., 2003; Hancock, 2005).

1.2.2.2 Inhibition of bacterial DNA synthesis/nucleic acid metabolism

DNA is present in the nucleoid, in the form of one or more chromosomes containing the genetic information required for cell structures and functions, and eventually in other DNA structures, known as plasmids (Kaufman, 2011).

Antibiotics that inhibit bacterial DNA synthesis/nucleic acid metabolism also tend to be bactericidal in action (Kaufman, 2011). For example, quinolones consist in nucleic acids metabolism inhibitors that bind to DNA topoisomerase II (DNA gyrase) and DNA topoisomerase IV (that catalyses supercoiling of DNA, a vital process in cellular metabolism) inhibiting their activity (Mcdermott *et al*., 2003; Kaufman, 2011; Hancock, 2005). Rifamycins (e.g.: Rifampicin) in the other hand, inhibit the initiation of bacterial transcription by binding to the bacterial RNA polymerase (Mcdermott *et al*., 2003; Kaufman, 2011). Metronidazole disrupts bacterial DNA through a chemical reaction that occurs in the absence of oxygen (which means the antibiotic is effective only against anaerobic bacteria) (Kaufman, 2011), and sulfonamides binds to dihydropteroate synthetase enzyme active site, blocking the formation of nucleotide precursors (Mcdermott *et al*., 2003; Hancock, 2005; Kaufman, 2011).

1.2.2.3 Inhibition of bacterial protein synthesis

Protein biosynthesis occurs in the ribosomes by decoding mRNA, a process called translation (Laursen, Sørensen, Mortensen, & Sperling-Petersen, 2005). The existing difference between prokaryotic and eukaryotic ribosomes allows diverse antibacterials to act selectively (Mcdermott *et al*., 2003; Kaufman, 2011).

Translation is conceptually divided into four phases: initiation, elongation, termination, and ribosome recycling. During the initiation the two ribosomal subunits are assembled on the translation initiation region (TIR) of the mRNA. In the elongation phase, the mRNA sequence is decoded by RNA polymerase and a polypeptide chain is synthesized until a stop codon on the mRNA is encountered. Termination occurs when synthesized protein are released from the ribosome (Laursen *et al*., 2005). Details on the process of protein synthesis can be found in Patrick F. McDermott (2003) article.

Examples of antibiotics that inhibit the protein synthesis are aminoglycosides such as streptomycin or gentamicin, that i) can interfere with proofreading, resulting in the misreading of mRNAs and consequently in deficient proteins, ii) can block the formation of the initiation complex, or iii) can inhibit the translocation step (Mcdermott *et al*., 2003; Hancock, 2005; Kaufman, 2011). In the other hand, chloramphenicol for example binds to the 70S ribosome, inhibiting peptide bonds formation (Mcdermott *et al*., 2003; Hancock, 2005). Glycylcyclines inhibits the binding of aminoacyl transfer RNAs to the ribosomal acceptor site, interrupting the elongation step of protein synthesis (Mcdermott *et al*., 2003; Hancock, 2005), and macrolides (e.g. erythromycin and clarithromycin) cause tRNA molecules to dissociate from the ribosomes, inhibiting the ribosomes from functioning (Mcdermott *et al*., 2003; Hancock, 2005; Kaufman, 2011).

1.2.2.4 Disruption of cell membranes

Cell membranes constitute barriers that play a very important role in the maintenance of cellular structure and homeostasis, as they control the flow (input and output) of intracellular and extracellular components. Damaging of cell membranes can compromise cell's survival (Lopez-romero, González-ríos, Borges, & Simões, 2015).

Daptomycin is an example of antibiotic that interacts with cell membrane (Hancock, 2005), as well as cationic peptides, that damage cell membrane or act on internal targets (Hancock, 2005).

1.2.2.5 Inhibition of other metabolic processes

Some antibiotics act on other essential cellular processes such as folate synthesis. Antibiotics like trimethoprim and sulfonamides inhibit folate synthesis and consequently the manufacture of DNA (Kaufman, 2011). Acting separately, sulphonamides and trimethoprim are bacteriostatic (Kaufman, 2011) but combined, as co-trimoxazole, work synergistically and are usually bactericidal (Mcdermott *et al*., 2003; Hancock, 2005; Kaufman, 2011).

Daptomycin is an example of a lipopeptide antibiotic able to act by several ways. It interacts to and enters membranes subsequently causing ion movements and depolarisation (cells can no longer synthesise ATP or take up some nutrients needed for growth) thus affecting membrane integrity, inhibition of protein, RNA, DNA, peptidoglycan, lipoteichoic acid, and lipid biosynthesis. Daptomycin causes rapid bactericidal activity without cell lysis and is effective at all growth phases (Hancock, 2005).

1.2.3 What response mechanisms can bacteria display?

Bacteria have the ability to develop resistance mechanisms and adapt to antibiotics. Some organisms are inherently resistant, while others develop resistance through mutation or by receiving resistant-encoding genetic material from different strains (Kaufman, 2011). In this thesis bacterial mechanisms of resistance to antimicrobial agents will not be described, since it falls out of its scope, but there are several excellent reviews about the subject such as Mcdermott *et al*., 2003, Hancock, 2005, Kaufman, 2011, Poole, 2012 and Cornforth & Foster, 2013.

1.3 Approaches on how to study a new antimicrobial: Available approaches/methods used to study new antimicrobial's mode of action

To adequately use an antibiotic against a bacterial population, it is important to know its range of action, known as spectrum of activity (Hancock, 2005; Faleiro M.L., 2011). Antibiotics can have mechanisms of action of directed-spectrum/narrow-spectrum or broad-spectrum (Hancock, 2005). There is no clear definition of the significance of these terms, however, the broader the spectrum the

more species of bacteria the antibiotic can kill. Generally, a broad-spectrum antibiotic would be able to impair a range of Gram-positive and Gram-negative organisms (Kaufman, 2011).

Several methods can be used for the identification of antimicrobial targets (and therefore its spectrum of activity) such as the method that follow.

1.3.1 Diffusion methods

Diffusion methods, more detailed in Balouiri *et al.* (2016), are used to study antimicrobial susceptibility after bacterial incubation in the presence of antimicrobials (Choma & Grzelak, 2011; Balouiri *et al*., 2016) and provide qualitative results by categorizing bacteria as susceptible, intermediate or resistant (Balouiri *et al*., 2016).

Disc diffusion method is one example of this type of method, and consists in placing filter paper discs soaked with different drugs or concentrations of drug, on solid media where previously were spread test bacteria. After petri dishes are incubated at the correct temperature for enough time, the diameter of the inhibition zone is measured and compared with that of the standard antibiotics to determine a relative activity (Karsha & Lakshmi, 2010; Choma & Grzelak, 2011; Balouiri *et al*., 2016).

1.3.2 Thin-layer chromatography (TLC) - bioautography

Thin-layer chromatography (TLC) such as agar diffusion, direct bioautography and agar overlay assays (Balouiri *et al*., 2016) allow to visualize, separate or purify and in some instances characterize antibacterial compounds (Betina, 1973; Cheng, Huang, & Shiea, 2011). The separation of the chemical compounds on a TLC plate is quantified in terms of the value of R_F (distance of analyte migration/distance of mobile phase migration) (Cheng *et al*., 2011).

Bioautography is a microbiological screening method that allows screening selected bacteria for antibacterial susceptibility by exposing them to obtained chromatograms (Choma & Grzelak, 2011; Kagan & Flythe, 2014).

TLC-bioautography consists in first analysing the antibacterial by separating its components using a TLC plate and an incubation system and then in exposing bacteria to the chromatogram, by placing the TLC plate chromatogram onto agar inoculated petri dishes with test bacteria. After incubating the petri dishes with TLC plates to allow diffusion, petri dishes alone are incubated to allow bacterial growth. The zones of the petri dishes with no bacterial growth may appear in the places of contact with antibacterial specific component/chromatograph band (Choma & Grzelak, 2011; Kagan & Flythe, 2014; Balouiri *et al*., 2016). Quantitative TLC measurements can allow to identify analytes if, when comparing their R_F values and spectra to that of standard compounds, similarities were found (Kawathekar, 2013; Tlc, 2017). If identification is not possible, more specific techniques should be performed, such as MS – Mass Spectrometry- detection (Kawathekar, 2013).

1.3.3 Thin-layer chromatography-mass spectrometry (TLC-MS)

As TLC method does not allow to identify and characterize analyte's structural properties, a combination of TLC with mass spectrometry (MS) can be performed to study detected bands in TLC chromatograms (Kawathekar, 2013), by measuring mass-to-charge (m/z) ratios of ions (Cheng *et al*., 2011). The antibacterial compound characterization is of importance since it allows further, more directed and in-depth studies of the antibacterial mode of action. TLC-MS techniques can be indirect, comprising scraping, extracting, purifying, and concentrating the analyte from the TLC plate and then analysing it using mass spectrometer′s ion source, or can be direct, over the TLC plate, and according to the ion source, TLC–MS can be vacuum-based TLC–MS or ambient TLC–MS (Cheng *et al*., 2011).

1.3.4 Study of bacterial physiological indexes

To better discriminate an antibacterial mode of action, several bacterial physiological indexes can be studied such as: the determination of the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC), membrane permeabilization changes, intracellular potassium (K+) release rates, physicochemical surface properties (like hydrophobicity and/or PI uptake), and surface charge (Borges, Ferreira, Saavedra, & Simões, 2013; Lopez-romero *et al*., 2015).

1.3.5 Potassium (K+) leakage determination by atomic absorption spectroscopy

As the internal environment of cells is generally rich in *K*+, and consequently its presence in the extracellular medium is an indication of cytoplasmic membrane damage/permeability that possibly leads to cell death, the potassium leakage is important to study. Potassium leakage, in filtrated samples (after liquid suspension being incubated in the presence of the antibacterial) can be analysed using atomic absorption spectroscopy and suitable software (Lopez-romero *et al*., 2015).

Other option to study the potassium leakage is by measuring total ion concentration of free potassium in cell suspensions after incubation with the test antimicrobial and sonication, using a potassium ion selective electrode (Cox *et al*., 2000).

1.3.6 ATP bioluminescence assay

ATP bioluminescence assay is based in the measurement of adenosine triphosphate (ATP) produced by bacteria. ATP bioluminescence assay allows establishment of linear relationship between ATP production and cell viability and has a large range of applications, such as cytotoxicity test, evaluation of the impact of biofilms, and drug screening and antibacterial testing. As ATP is present in somewhat constant amount in cells, and when a cell dies, the intracellular ATP amount declines rapidly, the number of cells in a bacterial population can be estimated. The ATP measurement can be done by using D- luciferin, that is converted into oxyluciferin by luciferase in the presence of ATP, consequently

emitting light. The quantity of the emitted light is measured by a luminometer and can be converted into RLU/mole (relative light units) of ATP (Chen & Cushion, 1994; Balouiri *et al*., 2016).

1.3.7 Automated Ethidium Bromide method for analysis of EB efflux or accumulation

Ethidium bromide (EB) efflux or accumulation may be studied to assess the effect of an antibacterial compound on bacterial efflux pump systems. The automated EB method, described in detail in (Miguel Viveiros, Liliana Rodrigues, Marta Martins, Isabel Couto, Gabriella Spengler, Ana Martins, 2011), allows the antibacterial activity study, in real-time, by analysing bacterial cultures in the presence of EB and of several antibacterial concentrations, using a thermocycler. The analysis is based on the comparison between the maximum concentration of EB that a bacterium can extrude from cell and the EB concentration that is actually extruded from cells in the presence of several specific antibacterial concentrations. The greater the level of EB accumulated, the greater the efflux pump system inhibition by the antibacterial (Varga *et al*., 2012).

1.3.8 Dilution methods

Either agar (agar dilution) or broth dilution methods, as described on M26-A guideline of NCCLS and on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Balouiri *et al*., 2016), may be used to quantify antimicrobial activity against bacteria (Choma & Grzelak, 2011; Balouiri *et al*., 2016), namely to determining eventually MIC (and MBC) values (Balouiri *et al*., 2016). Broth dilution antimicrobial susceptibility testing methods involve the preparation of two-fold dilutions of the antimicrobial agent in liquid growth medium dispensed in tubes (macrodilution) or in wells of 96-well microtitration plates (microdilution), which are then inoculated with a logarithmic phase adjusted microbial inoculum so that the final inoculum concentration is of about 5 x 10^5 CFU/mL (Barry *et al*., 1999). After incubation, MIC is defined by the unaided eye or by using viewing devices as the first dilution at which no growth occurs. Moreover, colorimetric methods based on the use of dye reagents such as Alamar blue dye (resazurin), an effective growth indicator, can also be used for this purpose (Balouiri *et al*., 2016). The minimum inhibitory concentration (MIC), usually expressed in µg/mL or mg/L, is defined as the lowest concentration of antimicrobial agent able to prevent a clear bacterial suspension of 10^5 CFU/mL to become turbid after overnight incubation (Balouiri, Sadiki, & Ibnsouda, 2016).

After the value of the MIC is determined, MBC can be measured by collecting, serially diluting and plating samples onto agar plates and by counting CFU (Cell Forming Units) (Barry *et al*., 1999; Balouiri *et al*., 2016). The minimum bactericidal concentration (MBC) also known as the minimum lethal concentration (MLC) (Balouiri *et al*., 2016), is defined as the lowest concentration of antibacterial agent needed to reduce the number of viable cells of the final inoculum by ≥ 3 $\log_{10}$ in CFU/mL, or otherwise the lowest concentration needed to kill 99.9 % of bacteria (Sherris, 1986; Barry *et al*., 1999; Saravolatz *et al*., 2012; Balouiri *et al*., 2016). This percentage is calculated based on colony counts from the control well, and MBC definition takes into account the rejection values determined by Pearson *et al*. (1980).

Methods to study the antimicrobial spectrum, determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) indexes, allow quantification of antimicrobial activity and to classify its action as bacteriostatic or bactericidal (Hancock, 2005). If antibiotic action against bacteria reaches values that only inhibit the bacterial growth, it means that the antibiotic is bacteriostatic (Faleiro M.L., 2011; Kaufman, 2011) for that bacteria, and therefore has a reversible impact since, after neutralization of the agent, the microbial cells generally can recover their reproductive capacity (Faleiro M.L., 2011). If it achieves values that may be used at either high concentrations or are inherently more aggressive, and their action results in a decline in the number of bacterial cells, the agent is bactericide for that bacteria (Faleiro M.L., 2011; Kaufman, 2011). Therefore, it means it has a permanent effect, as even after the neutralization of the agent, the microbial cells are unable of grow and reproduce (Faleiro M.L., 2011). Furthermore, the bactericidal nature of an antibiotic is not an intrinsic property of a given antibiotic but may be influenced by the target species and/or the drug concentration (Hancock, 2005).

The antibiotic tolerance can also be important to study while studying an antibacterial action. The term tolerant has been applied to define bacterial strains or mutants which show unusual numbers of survivors in the presence of (supposedly) lethal antibacterial concentrations. In some cases, the definition as tolerant organisms is extended to all the members of a species or group of organisms (Sherris, 1986). An organism is defined as tolerant when it has MBC/MIC ratios of ≥32 (Sherris, 1986; Denys, Grover, O'Hanley, & Stephens, 2011). In another hand, there also are two poorly explained related phenomena sometimes confused with tolerance, that are the persistence phenomenon and the paradoxical effect. The persistence phenomenon consists in the escape of a small proportion of the inoculum to the antibacterial killing action, consequently leading to an unusual number of survivors (Sherris, 1986), and paradoxical effect (Sherris, 1986), consists in the progressive increasing of the proportion of survivor cells of some strains on plate counts, for at least three consecutive higher concentrations than MIC (Barry *et al*., 1999).

1.3.9 Time-kill studies

To best understand the antimicrobial action, time-kill methods may be performed as described in the M26-A guideline of NCCLS. Time-kill methods allow to define antibacterials as bactericidal or bacteriostatic, (Balouiri *et al*., 2016; Sticker, n.d., p. 308) and gives information about the dynamic interaction between the antimicrobial agent and the microbial strain over time (Balouiri *et al*., 2016). Time-kill method also allows to assess synergistic (e.g. penicillin or ceftriaxone plus gentamicin) or antagonistic (e.g. penicillin plus a tetracycline) effects of two antimicrobials (Coudron & Stratton, 1995; MacGowan *et al*., 1996; Levison, 2004) however this type of studies have not been performed in this thesis. In addition, time-kill test reveals a time-dependent or a concentration-dependent antimicrobial effect (Balouiri *et al*., 2016). In concentration-dependent killing, the killing increases with increased drug concentrations. In time-dependent killing, the killing continues only as long as the concentrations are in excess of the MIC (Pfaller, Sheehan, & Rex, 2004).

Time-kill tests are performed using several antibacterial concentrations, for example 1/2MIC, MIC, MBC and 2MBC (Balouiri *et al*., 2016; Sticker, n.d., p. 308) and a diluted bacterial suspension in the mid logarithmic growth phase (Sticker, n.d., p. 308) in order to achieve a final concentration of $5x10^5$ CFU/mL (Balouiri *et al*., 2016). In defined time intervals samples are collected, serially diluted (to allow to obtain countable numbers of colonies) and each one of the diluted fractions is plated (Balouiri *et al*., 2016). After incubation, colony count determinations are done to know the number of remaining bacteria in each sample and the rate of antimicrobial agent killing action are determined by plotting each sample over time (Balouiri *et al*., 2016; Sticker, n.d., p. 308). Generally, ≥3 $\log_{10}$ reduction in test bacterial counts as compared with the final (starting) growth control indicates an bactericidal action (Sticker, n.d., p. 308). The bactericidal effect is equivalent to a lethality percentage of 90% for 6 h, which is equivalent to 99.9% of lethality for 24 h (Pfaller *et al*., 2004; Balouiri *et al*., 2016).

1.3.10 Flow cytometry

Flow cytometry is an effective tool for antibacterial testing (Balouiri *et al*., 2016). Flow cyto-fluorometric methods allow the estimation of the impact of an antibacterial agent by characterizing individual bacterial cells in terms of viability, DNA content, protein content and enzyme activity, among others (Rights, n.d.; Balouiri *et al*., 2016). The detection of damaged cells by this approach depends on the use of appropriate staining dyes and optimization of the technical procedures (Balouiri *et al*., 2016).

As the cytoplasmic membrane plays a very important role in the maintenance of cellular homeostasis (as it controls the input and output of intracellular and extracellular components) (Lopez-romero *et al*., 2015), the study of membrane characteristics is of concern. One characteristic of concern is the charge of the cell surfaces, as at normal physiological conditions bacterial cells have a negative surface charge, due to the presence of anionic groups (e.g., carboxyl and phosphate) in the membrane. Nonetheless, the magnitude of the charge varies with the species and can be affected by several conditions, including the age of the culture, ionic strength, and pH (Lopez-romero *et al*., 2015). Other aspect of concern is cytoplasmic membrane integrity/permeabilization. Membrane integrity/permeabilization can be detected for example based on the use of DNA stain SYTOX Green once cannot enter intact cells , (Nonejuie, Burkart, Pogliano, & Pogliano, 2013), or based on the uptake of propidium iodide (PI), a fluorescent and intercalating agent, widely used as DNA stain, to which intact cell membrane is usually impermeable, (Cox *et al*., 2000; Lopez-romero *et al*., 2015; Balouiri *et al.*, 2016). The mixture of two nucleic acid-binding stains can also be used to determine membrane integrity/permeabilization and both viable and unviable counts of bacteria (Lopez-romero *et al*., 2015; Balouiri *et al.*, 2016). One example of common utilized mixtures is SYTO 9 stain plus propidium iodide (PI) stain, that compete for binding to the bacterial nucleic acid. SYTO 9 is generally membrane permeable, so labels both damaged and intact cells, whereas propidium iodide only penetrates cells with damaged membranes, reducing SYTO 9 fluorescence thus, bacteria with intact membranes fluoresce green, and bacteria with damaged membranes fluoresce red (Kort, Keijser, Caspers, Schuren, & Montijn, 2008; Nobmann, Bourke, Dunne, & Henehan, 2010). In addition, three subpopulations (dead, viable and injured cells) can be clearly discriminated by this method. The injured cells, described as stressed cells exhibiting cellular

components damages and subsequent impairment of reproductive growth, may be of importance to quantify if cell recovery becomes possible (Balouiri *et al*., 2016). Another aspect of concern about cytoplasmic membrane is the membrane polarization and its proton motive force (PMF). Since maintenance of an appropriately energised cytoplasmic membrane is fundamental to the survival and growth of bacterial cells (Hancock, 2005). However depolarisation is not lethal itself: in non-growing cells, cytoplasmic membrane depolarisation occurred after cell death, indicating that under these conditions it may not be the direct cause of cell death (Hancock, 2005). For instance, the proton motive force (PMF) can be detected by using sensitive dye $DiBAC_4(3)$.

1.3.11 Microscopy

Microscopic methods may also be performed to study antibacterial mode of action on test bacteria.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) are microscopic methods that can be used for antibacterial mode of action studies in fixed test bacteria previously incubated with antibacterial compound (Lv *et al*., 2014). SEM and TEM provide analysis/observation on morphological and ultrastructural changes in bacteria cells, such as damages on cell membranes and cell wall structure, induced by antibacterial agents (Hartmann *et al*., 2010; Lv *et al.*, 2014; Mishra & Chauhan, 2016; Mary, Nongkhlaw, & Joshi, 2017). Electron microscopy analyse the interaction between electron beams and the specimen, in order to create an image, based on the collected radiation (Mishra & Chauhan, 2016). Images are obtained, in SEM, by scanning the raster scan pattern of an electron beam, and in TEM, images are obtained by the translocation of an electron beam through an ultra-thin specimen (Mishra & Chauhan, 2016). So, SEM are focused on the sample's surface and TEM are focused in inside or beyond the surface. SEM is advantageous as it is three-dimensional and the preparation of samples is relatively easier, compared to the TEM (Panda, 2014).

Atomic Force Microscopy (AFM) is another technique that can be used for antibacterial study. AFM allows quantitatively study sub molecular morphological properties and function of bacterial surfaces as a measurement of the interaction forces between AFM tip and cell surface (Haase, Pelling, & Haase, 2015; Mishra & Chauhan, 2016). AFM has the advantages of allowing to perform imaging of live bacteria, in aqueous solution, at real-time and in situ, in terms of elasticity and rigidity properties of bacterial surfaces. AFM allow to analyse samples without being previous stained, moreover, contrast can be enhanced if using stains (Mishra & Chauhan, 2016).

1.3.12 Bacterial cytological profiling (BCP)

Cytological profiling (CP) is a screening technique based on microscopic imaging to search and, if possible, identify compounds in a mixture of compounds (Michael, Glassey, Stuart, & Lokey, 2013). "Bacterial cytological profiling" (BCP) is a rapid and powerful approach for identifying cellular pathways affected by antibacterial molecules. BCP can distinguish between inhibitors that affect different cellular pathways as well as different targets within the same pathway, allowing accurately prediction of the

antibacterial mode of action. Briefly, the BCP method consists in performing BCP on a collection of antibiotics in order to obtain a library of antibiotics mode of action, to can then compare those results with BCP experiment on the test antibiotic (Nonejuie, Burkart, Pogliano, & Pogliano, 2013).

The method analysis comprises several comparisons of the test antibacterial, with several concentrations of antibiotics of each class (e.g. tetracycline, rifampicin, ciprofloxacin, triclosan, and ampicillin), known to inhibit several major pathways such as translation, transcription, DNA replication, lipid synthesis, and peptidoglycan synthesis. To quantitatively analyse results, the cell morphologies resulting from treatment with each antibiotic are measured, and principal component analysis (PCA) are performed to group cells presenting similar morphologies. This method may allow the determination of the test antibacterial class and subclass. Thus, BCP method can discriminate between molecules that have a similar structure but different effects, or different structures and same effect, and different effects exerting by same individual molecule (Nonejuie, Burkart, Pogliano, & Pogliano, 2013; Quach, Sakoulas, Nizet, Pogliano, & Pogliano, 2016).

Summing up, BCP can be used for primary screening of compounds to identify molecules that target specific cellular pathways, though BCP does not identify the target, so, complementary approaches such as isolating resistant mutants or screening a large collection of sensitized strains should be performed (Nonejuie *et al*., 2013).

Molecular studies may also be performed for in-depth know the antibacterial mode of action such as described on Davis, Dibner, & Battey (1986), however this thesis doesn't include any molecular studies.

1.4 Used methods

Some of the methods cited above were used in this thesis, with some modifications, to study the BMX-11 mode of action, like the broth microdilution method, used to study MIC and MBC (Barry *et al*., 1999), the time-kill method (Barry *et al*., 1999), and flow cytometry using SYTOX Green, SYTO 9 +PI and $DiBAC_4(3)$ stains.

1.5 Model species

As antibiotics may have different modes of action and may act in different cell structures/functions depending on cell characteristics (Hooper, 2001; Mcdermott *et al*., 2003; Hancock, 2005; Manuscript, 2009; Kaufman, 2011; Chifiriuc *et al*., 2016). In this master's thesis BMX-11 was tested against several relevant Gram-positive and Gram-negative bacteria mentioned below in table 1. In addition to the information given by table 1, *C. marina* was used in this master's thesis as a marine, Gram-negative, bacteria model; *Enterococcus faecalis* is the type species of the genus (http://www.bacterio.net/enterococcus.html), and was used as model of Gram-positive bacteria, as well as *S. aureus*; *E. coli* was used as a classic Gram-negative model*; Listeria monocytogenes is* the type

species of the genus (http://www.bacterio.net/listeria.html) and was used as a representative of pathogenic Gram-positive bacteria; and *Vibrio vulnificus* was used as a model of pathogenic Gram-negative marine bacteria.

Table 1. Strains used, its collection and characteristic properties.

	Cobetia marina DSMZ 4741	*Enterococcus faecalis* ATCC 29212	*E. coli* ATCC 25922	*Listeria monocytogenes* CECT 4031	*Staphylococcus aureus* NCTC 8325	*Vibrio vulnificus* CECT 5165
Habitat	marine environments	human/animal gastrointestinal flora; food	lower-gut human/animal flora	environment; alimentary tract of human and animals	human/animal skin and mucosal flora	marine/aquatic environments
Gram stain	Gram-negative	Gram-positive	Gram-negative	Gram-positive	Gram-positive	Gram-negative
Morphology	straight, rod-shaped that occurs singly and in pairs	coccus that occurs singly, in pairs, or in short chains	straight cylindrical rodshaped	bacilli	coccus	straight or curved rodshaped
Motility	most are motile by 2 to 5 peritrichous flagella	usually non-motile	motile by peritrichous flagella	motile	non-motile	motile by single polar flagellum
Metabolic appearance	aerobic	aerobic or anaerobic facultative	aerobic or facultative anaerobic	aerobic or facultative anaerobic	aerobic or facultative anaerobic	aerobic or anaerobic facultative
Growth conditions	37 ºC; 7.5 pH; 5 % NaCl	5 to 65 ºC; 4.5 to 10.0 pH; high NaCl concentration	21 to 37 ºC; 5.0 to 9.0 pH	tolerate high concentrations of salt and refrigeration temperatures	30 to 37 °C; 6.0 to 7.0 pH; NaCl up to 25 %	>20 °C; 5 to 20 % NaCl
Other		emerged as multi-resistant nosocomial pathogen in immunocompromised and critically ill patients; belongs to lactic acid bacteria (LAB) group and to Lancefield group D	usually commensal but some biotypes are pathogenic	facultative intracellular bacterial pathogen; capable of cause listeriosis; produce virulence factors (hemolysin, phospholipase, lecithinase, and internalins); is saprophytic	opportunistic pathogen; produce virulence factors e.g. staphylococcal enterotoxins; toxic shock syndrome toxin-1 (TSST-1). Is coagulase positive (CoPS)	nitrogen fixation; able to cause clinical infections; produce cytolytic and cytotoxic extracellular factors

Sources: *C. marina*: bacterio.net, Arahal et al., 2002; Romanenko, Tanaka, Svetashev, & Falsen, 2013);
E. faecalis: bacterio.net, (Schleifer & Kilpper-Balz, 1984; Fisher & Phillips, 2009; Garrido, Gálvez, & Pulido, 2014);
E. coli: (Diamant et al., 2004; Scheutz & Strockbine, 2005; Chaudhuri & Henderson, 2012);
L. monocytogenes: (Ramana & Mohanty, 2013; Kathariou & Pine, 1991; Mcfeeters, 1996);
S. aureus: (Nobmann et al., 2010; Sarkar *et al.*, 2016; Mader et al., 2016; Sticker, n.d.; Klaschik *et al.*, 2015; Mehta, McClure, Mangold, & Peterson, 2015), (Rice, Peralta, Bast, Azavedo, & Mcgavin, 2001; Naicker, Karayem, Hoek, Harvey, & Wasserman, 2016; Mader *et al.*, 2016; Veh et al., 2015; Lina, Pie, Godail-gamot, & Peter, 1985; Becker, Heilmann, & Peters, 2014; Tam, Schilling, & Nikolaou, 2005; Valero *et al.*, 2009);
V. vulnificus: (Kaper, Lockman, Remmers, Kristensen, & Colwell, 1983; Tison & Kelly, 1986; Kaper *et al.*, 1983; Sgorbati et al., 2001).

1.6 Outline

In this master's thesis, the mode of action of the antibacterial mixture BMX-11 will be studied, using a set of different approaches as described above. Briefly, this study starts by establishing the growth curves profiles for target bacteria, and then determining the MIC and MBC thresholds by using the microdilution assay as described above. After both values for MIC and MBC were defined, we performed time-kill curves using the time-kill method. Time-kill curves were used to validate the MIC and MBC values, to understand the antibacterial effect over time and to better determine if BMX-11 antibacterial impact is time and/or concentration dependent.

After these tests, a range of concentrations of BMX-11 was tested to study its effect on the viability of different target bacteria using flow cytometry. In addition to bacteria viability on its own, other possible mechanisms of BMX-11 were studied, by using staining dyes. We used the SYTOX Green staining dye to assess the effect of BMX-11 on membrane permeability; SYTO 9 nucleic acid stain + propidium iodide (PI) also to assess membrane permeability; and $DiBAC_4(3)$ staining dye to assess membrane polarization. The phenotypic changes in bacteria were also followed by microscopic observation.

2 Materials and Methods

For the totality of the methods below, the procedures were repeated at least three times to address the inter-experiment variability, and samples were collected in triplicates in order to validate the intra-experiment variability.

2.1 Bacterial strains and growth media

In this study, a panel of bacterial species (table 2) belonging to different genus of proteobacteria phylum and gammaproteobacteria class (*E. coli, C. marina and V. vulnificus*), and firmicutes phylum and bacilli class (*E. faecalis, L. monocytogenes and S. aureus*) were used. These species were used, in order to represent different groups of microorganisms such as model organisms, Gram-negative, Gram-positive, pathogenic bacteria and marine bacteria. Therefore *E. coli* is used as model organism for gram-negative species and *S. aureus* for gram-positive species, *L. monocytogenes* for pathogenic species, *V. vulnificus* for sensitive marine species and *C. marina* for biofilm forming marine species. All bacteria used were obtained from collections, namely American Type Culture Collection (ATCC), Spanish Type Culture Collection(CECT), German Collection of Microorganisms and Cell Cultures (DSMZ) and National Collection of Type Cultures (NCTC).

All the strains in question were grown in the respective liquid/solid media (table 2), at 37 ºC and 150 rpm agitation for all bacteria strains, with the exception for *Cobetia marina*, which was grown at 28ºC and 160 rpm. For the incubation performed at 37ºC with agitation, Forma Orbital Shaker (Thermo electron corporation, U.S.) was used; for the 28 ºC incubation with agitation, MAXQ 4000 orbital shaker (Thermo scientific, U.S.) was used.

Table 2. The six used strains for the majority of the methods, its respective collection, collection number, incubation temperature and the liquid and solid media (with its respective brand) used for each strain.

Species	Strain name	Liquid/solid media	Incubation temperature
Cobetia marina	DSMZ 4741	Marine broth (Difco™, France); marine broth+agar	28 ºC
Enterococcus faecalis	ATCC 29212	Trypto-casein soy broth (Biokar); LA	37 ºC
Escherichia coli	ATCC 25922	LB broth (nzytech, Portugal); LA	37 ºC
Listeria monocytogenes	CECT 4031	Brain heart broth (Biokar, France); brain heart broth+agar	37 ºC
Staphylococcus aureus	NCTC 8325	LB broth (nzytech, Portugal); LA	37 ºC
Vibrio vulnificus	CECT 5165	Alkaline peptone water (APW); Trypto-casein soy broth+agar (Biokar, France)	37 ºC

2.2 Antimicrobial agent (BMX-11)

BMX-11 was stored in a powdered state at -20 ºC. For the study of *E. coli* growth curves, BMX-11 was used directly (powdered). For the remaining methods, it was used in solution in sterile ultrapure H_2O which has been filtered with a 0.20 µm filter.

2.3 E. coli growth curves

2.3.1 OD_{600nm} study

The effect of several BMX-11 concentrations was assessed on *E. coli* mid-logarithmic growth phase cultures and the effect of 15000 µg/mL was assessed on stationary growth phase cultures by optical density measurements using a 600 nm filter. The procedure follows:

Day 1: From -80 ºC log phase stored *E. coli* culture, 2 streaks were done onto LA petri dishes and 1 onto Blue Eosin Methylene Agar. The plates were incubated overnight at 37 ºC, and in the next day one isolated CFU from 1 LA petri dish was used to inoculate one 50 mL Erlenmeyer containing 10mL of LB medium. This pre-inoculum was incubated overnight at 37 ºC and 150 rpm.

Day 3: First, OD_{600nm} was measured using UV1101 Biotech Photometer (WPA, UK) with a 600nm filter and afterward one new diluted culture with an OD_{600nm} of 0.05 was prepared in a 500 mL Erlenmeyer containing 240 mL of LB medium. The volume of pre-inoculum to be added to the 240 mL of medium was calculated as follows:

$$Ci\ x\ Vi\ =\ Cf\ x\ Vf$$

$$\Leftrightarrow Pre-inoculum\ OD600nm\ x\ Vi\ (mL) =\ 0.05\ x\ 240mL \quad (1)$$

$$\Leftrightarrow Vi\ =\ 12\ /\ pre-inoculum\ OD600nm$$

The 240 mL culture was divided into 4 Erlenmeyers of 250 mL (60 mL transferred to each one) and incubated at 37 ºC and 150 rpm – 2 cultures would be the test cultures, and other 2, the control cultures.

To study BMX-11 effect at 5000 µg/mL and 10000 µg/mL on exponential phase, *E. coli* cultures were previously grown and after 3.5 hours BMX-11 was added. To study BMX-11 effect at 15000 µg/mL and 30000 µg/mL, cultures were previously grown and after 4.5 hours BMX-11 was added. The effect of BMX-11 was monitored for 7 and 6 hours respectively, after it was added, and for 4 to 6 hours after 22 hours of growth.

To study the BMX-11 effect on stationary phase, prior to exposure to BMX-11, *E. coli* cultures were incubated for 15.8 h. Then, BMX-11 was added to the test cultures to that to be at 15000 µg/mL - this was the concentration of choice since it exhibited good results on mid-logarithmic phase study. The growth was monitored for 6 hours after the addition of BMX-11 and for 3 hours after 38 hours of growth.

Data was analysed in real time using Microsoft Excel. Averages and standard deviations were calculated and graphics of OD_{600nm}/mL of each culture against time were drawn.

2.3.2 Viability study

Viability study on logarithmic phase *E. coli* cultures at 15000 µg/mL of BMX-11 (and growth control cultures) were performed in parallel with the OD measurements by plate culture. Around the growth times 1 h, 2 h, 3 h and 5 h after BMX-11 was added, samples of 1 mL were collected, serially diluted (1:10) from 10^0 to 10^{-9} and 100 µL of each dilution were plated in triplicate in onto LA petri dishes. Inocula were spread with glass spheres till it dries and plates were incubated overnight at 37 ºC.

In the next day, the number of CFU was counted, transformed in CFU/mL and growth curves of $\log_{10}$ CFU/mL against time, with respective averages and standard deviations were drawn.

2.4 Minimal Inhibitory Concentration (MIC)

The broth microdilution method used for determining bactericidal activity were adapted from (Wikler *et al*., 2009). Eleven two-fold dilutions of BMX-11 ranging from 120000 µg/mL to 117 µg/mL or from 15000 µg/mL to 15 µg/mL against the 6 previous mentioned strains were tested.

2.4.1 Inoculum preparation

Inocula for MIC's test were prepared following these steps:

In the "day 1", pre-inoculum composed of 10 mL medium plus 200 µL aliquot of -80 ºC log phase stored culture were launched in one 50 mL Erlenmeyer and incubated overnight.

In the "day 2" one culture was launched in one 50 mL Erlenmeyer with 4.5 mL medium plus 500 µL of overnight culture – "10^{-1} culture" - and after incubating for 1:30 h was diluted and a new culture was launched - "10^{-2} culture" – and incubated for 1:30 h.

After previous mentioned incubations, the optical density of 1mL sample was read by means of UV1101 Biotech Photometer (WPA, UK) with a 600 nm filter. Afterward, the necessary calculations* were done so that after diluting the culture on 8 mL medium, the microbial suspension was at twice ($1x10^6$ cells/mL) the desired final concentration ($5x10^5$ cells/mL).

*Calculations/analytical methods:

- According to M07-A8 document of CLSI, cultures are diluted to the equivalent of 0.5 McFarland standard, then a 1:20 dilution is made, and 10 µL of this suspension are pipetted into the microplate wells containing 0.1 mL of medium.

- Knowing that 0.5 McFarland ⇔ 0.08 to 0.13 OD_{600nm} (CLSI M07-A8 approved standard) and that the desired was to use 0.5 mL of inoculum in the wells (instead of 10 µL):

$$OD600nm\ x\ pre\ inoculum\ volume = 0.08/20\ x\ 8000\mu L$$

$$\Leftrightarrow pre\ inoculum\ vol. = 32\mu L/OD600nm \text{ (if only use 10µL)} \quad (2)$$

$pre\ inoculum\ vol. = 32/OD600nm/5 = 6.4/OD600nm$ (5x diluted to could use 500µL)

2.4.2 BMX-11 solution preparation

For each 48-well plate, 2 mL of BMX-11 solution at twice the major desired test concentration were prepared.

2.4.3 48-well plate preparation and optical density measurement

48-well plate preparation was done in accordance with CLSI document M07-A8 with some modifications. Briefly, all the wells except A1-A4 were filled with 200 µL sterile ultrapure H_2O and A1-A4 were filled with 400 µL BMX-11 solution at 240000 µg/mL (or 15000 µg/mL, depending on the tested strain). Next, two-fold serially dilutions were made by transferring 200 µL from each well. Finally, 200 µL of previous prepared inoculum were pipetted into all the wells except that of the negative controls. The 48-well plate is schematized in figure 2.

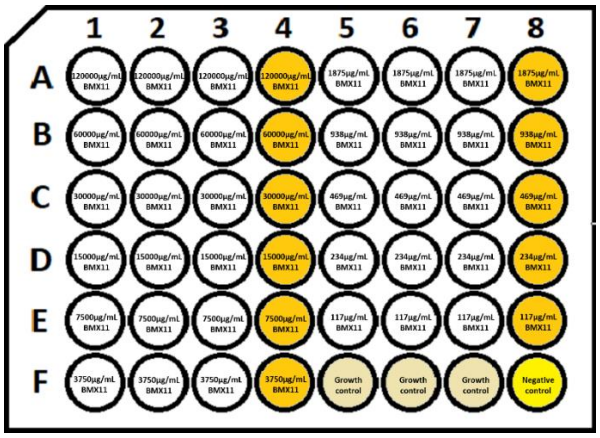


Figure 2. Scheme of a prepared 48-well microplate, with the eleven BMX-11 test concentrations, negative controls of each concentration in 4th and 8th columns, negative control of the test in the F8 well and growth controls of the test in the F5, F6 and F7 wells.

Samples of final inoculum suspensions (in growth control wells) were plated to confirm if it closely approximates to 5 x 10^5 CFU/mL.

The microplate incubated statically at 37 ºC, during 16 h (with the exception for *C.marina* which incubated at 28 ºC) and then OD was read in multimode detector Zenyth 3100 (anthos, Austria) with a 595 nm filter and using Multimode Detection Sofware v. 2.1.0.17 (Beckman Coulter, Inc., USA). The data was recorded in .xls format and analysed using Microsoft Excel. Graphics were drawn by plotting OD_{595nm} against BMX-11 serial concentrations. Averages, standard deviations and theoretical and real MIC's value were determined.

2.5 Bioscreen analysis

Another procedure using the broth microdilution method was performed (on *E. coli,* to test the method and) on *C. marina,* to that measurements could be under agitation and consequently eliminate lack of precision in OD readings due to the formation of agglomerates by *C. marina*. The assay was performed using Bioscreen C™ (Thermo Fisher Scientific, USA) automated microbiology growth curve analysis system and respective Honeycomb microplates (10x10 wells). The Honeycomb microplate preparation was like the 48-well microplate preparation but using half of the volumes. Honeycomb microplates incubated with intensive agitation during 24 h at 37 ºC for *E. coli* and during 16 h at 28 ºC for *C.marina* and OD_{600nm} measurements were automatically done at each 30 min.

In the next day, turbidimetric data was extracted from Growth Curves software (v. 2.11, Transgalactic Ltd., Finland) and analysed using Microsoft Excel. Graphics plotting OD_{600nm} against time were drawn and averages, standard deviations and MIC were determined.

2.6 Minimal Bactericidal Concentration (MBC)

MBCs can only be determined by first performing a MIC assay. MBC test were adapted from (Barry *et al*., 1999) and was performed by the three below mentioned ways. The procedures were as follows:

Method 1:

After the MIC assay was done, the first 4 highest BMX-11 dilutions above those where no visible growth was seen and the next highest dilution in which there was visible growth were selected to test/plate. From each well of each concentration, duplicate samplings of 100 µL of several (1:10) dilutions were plated onto suitable petri dishes with sterile glass spheres. Larger dilutions were plated from the control wells (“growth control” in the figure 3). All the resulting petri dishes were incubated overnight. Note that for *C. marina* it was used a spreader instead of spheres to spread the inoculum.

Method 2:

From each well of each selected concentration and of growth control’s wells, 200 µL were collected and serially (1:10) diluted. With a suitable multichannel micropipette, 5 µL droplets of each dilution from each well were spotted, onto suitable square solid media petri dishes. After letting the droplets to dry, the resulting petri dishes were incubated overnight at 37 ºC. This procedure was not suitable for *C. marina*.

Method 3:

For *C. marina*’s, platings from Honeycomb microplate were done. The total content of each well of selected BMX-11 concentration, were mixed into 2 mL microtubes and serial dilutions of 1:10 were done in marine broth. 100 µL of each dilution were plated in duplicate using glass spheres. The resulting petri dishes had statically incubated overnight at 28 ºC.

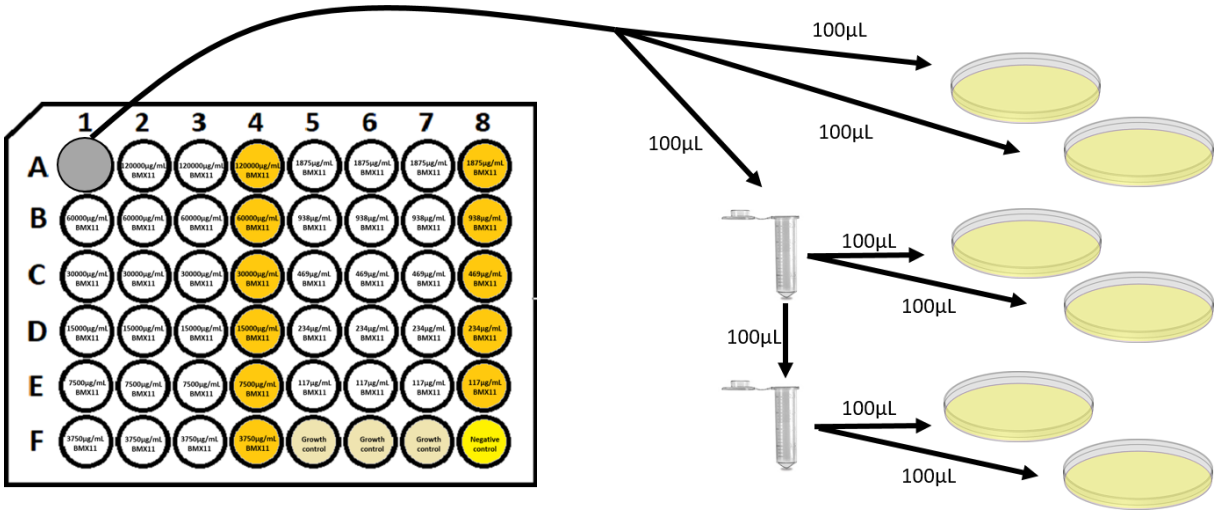


Figure 3. Scheme of dilutions and platings to do from 1 example well of 48-well microplate. From each well of each selected concentration, duplicate samplings were done by plating 100 µL onto suitable solid media petri dishes; 100 µL were diluted into 2 mL microtube containing 900 µL of medium (=10^{-1} dilution) and from this, 100 µL were diluted into another microtube containing 900 µL of medium (=10^{-2} dilution). From the "10^{-1}" and "10^{-2}" microtubes, duplicate samplings were done by plating 100 µL onto another suitable solid media petri dishes.

For all method, colony forming units (CFU) were counted in the next day, the numbers were transformed in CFU/mL and a graphic of CFU/mL against BMX-11 concentrations (µg/mL) were drawn. For MBC determination, percentages and $\log_{10}$ decreasing in growth in relation to the final inoculum were calculated.

2.7 Time-kill method

MICs were prior determined so time-kill assays can be performed based on and adapted from (Barry *et al*. (1999) and from Foerster, Unemo, Hathaway, Low, & Althaus (2015). For each of the 6 previous mentioned strains, the respective BMX-11 concentrations MBC, 2MIC, MIC and ½MIC were tested.

2.7.1 Inoculum preparation

Inoculum was prepared as before.

2.7.2 BMX-11 solutions preparation

For each 96-well plate, 4 mL solutions of BMX-11 at twice the maximum concentrations to be tested for each strain (2xMBC, 4xMIC) were prepared.

2.7.3 96-well plate preparation

First, the wells of the 5th, 6th, 11th and 12th columns were filled with 100 µL sterile ultrapure H_2O. Next, the wells of the 1st and 7th columns were filled with 100 µL of BMX-11 filtered solution at 2xMBC concentration and those of the 2nd and the 8th were filled with 200 µL of the 4xMIC solution. 100 µL of each well with 4xMIC solution were transferred to the next column and 100 µL of each well of this column were transferred to the next column in order to be (1:2) serially diluted. (All the wells with the exception of those of the last 2 lines were then filled with 100 µL pre-inoculum and the wells of the last 2 lines (G and H) with 100 µL of medium. After the first sampling (0 h sampling time), the 96-well plate were statically incubated during 24 h.

2.7.4 Sampling

As presented in the figure 4, samples were collected at incubation's times 0, 1, 2, 4, 16 and 24 hours. At each sampling time the total content from each well of an entirely line was transferred (1st line was collected at 0 h sampling time, 2nd line was collected at 1 h sampling time and so on) to the first line of one new 96-well plate. That way, each 96-well plate's line has given rise to one new 96-well plate. At each sampling time, OD_{595nm} of new 96-well plate was read in the multimode detector Zenyth 3100 (anthos, Austria). The results were recorded in .xls format and analysed.

After OD_{595nm} were read, the remaining wells of the new 96-well plates were filled with 180 µL of medium and then serially dilutions of 1:10 were made, with a microchannel micropipette. 5 µL of each well of each dilution were then spotted onto suitable square solid media petri dishes, allowed to dry under sterility and then the petri dishes were statically incubated overnight. In the next day, colony forming units (CFU) were counted, the numbers were transformed in CFU/mL and averages and standard deviations were calculated.

Graphics plotting OD against time, and graphics plotting CFU/mL against time were drawn. $\log_{10}$ reduction in CFU/mL at designated timepoints were considered and compared with the CFU/mL at the start of testing.

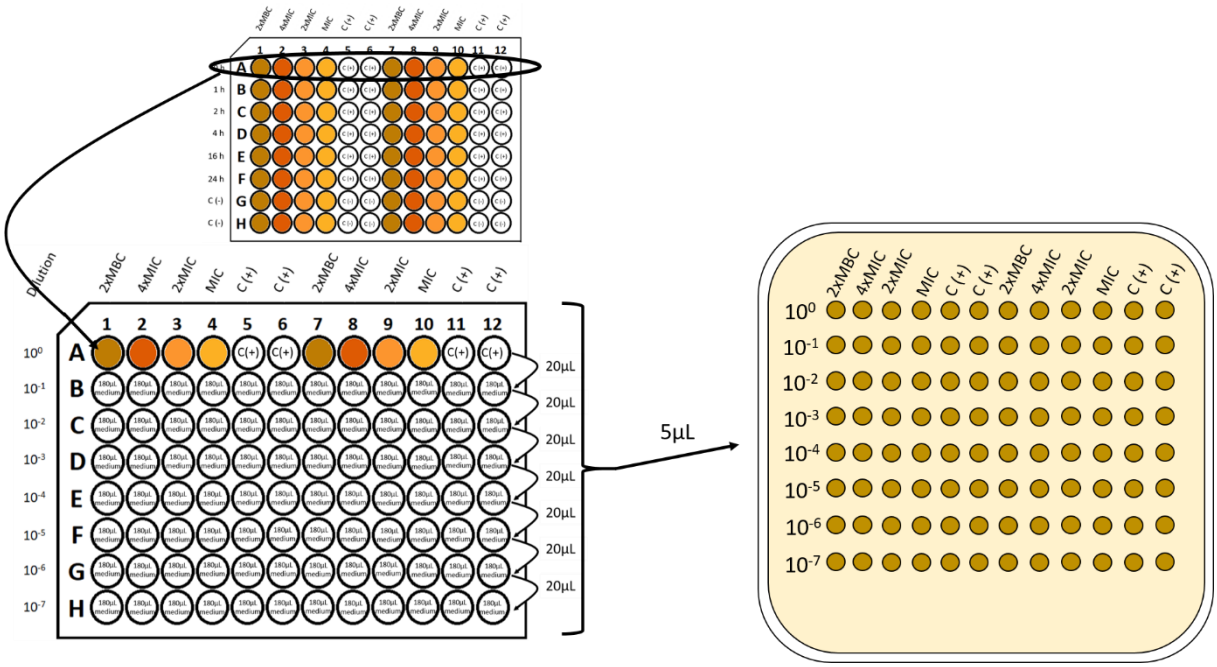


Figure 4. Example of one time-kill method sampling time. Time-kill 96-well microplate is presented at the top, where one line per each sampling time is showed. The 96-well microplate at the bottom represents the serial dilutions performed at each sampling time by the transference of 20 µL between lines, starting in the 1st line. At the bottom right corner, is presented the inoculation of one square petri dish with 5 µL of each well of each dilution as had been done for each sampling time.

2.8 Flow cytometry

Flow cytometry assays were performed on *E. coli* and on *C. marina* using the CyFlow® Space flow cytometer (Sysmex Partec GmbH, Germany) equipped with a 20 mW blue solid-state laser at 488 nm. All parameters (FSC - forward-scattered light, SSC - side-scattered light, and emitted fluorescence) were represented in logarithmic scale. Green fluorescence was detected approximately at 530 nm and was displayed by the FL1 channel, and red fluorescence was detected approximately at 640 nm and was displayed by the FL3 channel (Philippe Lebaron, Parthuisot, Curie, & Cedex, 1998).

2.8.1 Method validation

Prior to the assays themselves, instrument adjustment and validation of the method were done by analysing non-treated cultures of each strain, prepared as described below. Two samples of 15 mL were collected, centrifuged during 5 min at 3000 rpm in Centrifuge 5702 R (eppendorf, Germany), the supernatants were discarded, and pellets were resuspended in 5 mL of PSB (in the case of *E. coli*) or in artificial sea water (Tropic Marin®, USA) at half the concentration (in the case of *C. marina*). For *C. marina*, the centrifugation step was repeated. One of the samples was stored in ice (LC – live cells) and from the other sample, 1.5 mL were collected and placed on Thermo-Shaker (Grant Instruments, UK) previous heated at 80 ºC, during 20 min, to kill the cells by heat.

Suitable dilutions of each sample were done (in PBS if *E. coli* or in artificial sea water at half the concentration if *C. marina)* and mixtures of known concentrations of live and dead cells – 100 % LC; 50 % LC – 50 % KC; 75 % LC – 25 % KC; 25 % LC – 75 % KC; 100 % KC - were analysed by flow cytometry for viability assessment using 4 fluorophores: SYTOX® Green, SYTO® 9+PI and $DiBAC_4(3)$ (life technologies™, Netherlands). Each measurement was performed on 1.5 mL samples to which was added 1µL of fluorophore.

2.8.2 Test cultures preparation

Cultures were launched in one sterile Erlenmeyer of 50 mL containing 15 mL of medium plus 200 µL aliquot of -80 ºC log phase stored culture and incubated for 14 hours. Then, two new cultures were launched into two 100 mL Erlenmeyers, each containing 27 mL of LB broth plus 3 mL of previous culture and were incubated. *E. coli* cultures were incubated for one hour and half and *C. marina* incubated for two hours. Afterwards 3 mL of sterile ultrapure water were added to one culture (the growth control culture) and 3 mL of previous prepared BMX-11 solution was added to the other culture (the test culture) - this is the time zero. At this time (0 h), the first sampling was performed (and cultures continued incubating for 24 hours).

2.8.3 BMX-11 solution preparation

BMX-11 solution was prepared in 3 mL of sterile ultrapure H_2O so that the total 32.5 mL of culture was at 30000 µg/mL.

2.8.4 Sampling

2.8.4.1 *E. coli* tests

Samples of 1.5 mL of each culture were collected at 0, 2, 3, 4, 5, 7 and 24 hours of growth. Samples were centrifuged in Centrifuge 5424 R (eppendorf, Germany) for 5 minutes, at 4 ºC and 8000 rpm. From sampling time 5 h, samples were only centrifuged for 3 minutes. After centrifugation, the supernatant was discarded, and the pellet resuspended in 0.5 mL of phosphate-buffered saline (PBS) by carefully pipetting. The resulting suspension was then suitable diluted in PBS (to reach concentrations between 1 to $3x10^6$ cells/mL).

2.8.4.2 *C. marina* tests

Samples of 1.5 mL of each culture were collected at 0, 1, 2, 4, 6, 8 and 24 hours of growth. Samples were centrifuged for 3 minutes, at 4 ºC and 8000 rpm, supernatant was discarded and the pellet resuspended in 0.5 mL of artificial sea water (Tropic Marin®, USA) at half the concentration by carefully

pipetting. This step was repeated and therefore the resulting suspension was diluted in artificial sea water at half the concentration according to the necessity of the flow cytometer. *C. marina* cells were washed in artificial sea water (ASW) once previous flow cytometric studies by BioMimetx team and studies by others (Christophis & Rosenhahn, 2012) figured out that bacteria can grow in ASW, that assays in ASW revealed more reproducible comparing to that in MB and that the washing of cells in ASW allows removal of excess extracellular polymeric substances (EPS).

2.8.5 Incubation with fluorophores

850 µL of each sample were collected into polystyrene flow cytometry tubes to be analysed for cell counts and 3 subsamples of 1.5 mL of each sample were collected into cytometer tubes for viabilities measurements.

For viability assessment, 1 µL of one fluorophore (or 1+1 µL if SYTO® 9+PI) was added to each subsample, incubated with, and analysed in the flow cytometer after light vortexing.

There were used 4 different fluorophores:

SYTOX® Green nucleic acid stain (life technologies™, Netherlands), which is impermeable to live cells but that can penetrate cells with compromised membranes, works as nuclear and chromosome stain (T. Fisher https://www.thermofisher.com/pt/en/home/life-science/cell-analysis/fluorophores/sytox-green-stain.html). It is an indicator of dead cells, with excitation maximum at 504 nm and emission maximum at 523 nm, fluorescing bright green (https://www.thermofisher.com/pt/en/home/life-science/cell-analysis/fluorophores/sytox-green-stain.html). It was used at 0.02 µM, a little lower concentration than the manufacturer recommendation (0.5 - 5 µM) in the protocol “SYTOX ® Green Nucleic Acid Stain” (2006), and was incubated for 10 min under no light conditions for membrane permeabilization evaluation;

SYTO® 9 green-fluorescent nucleic acid stain (life technologies™, Netherlands), is cell membranes permeable nucleic acid stain with high affinity for DNA, working as nuclear and chromosome stain of both live and dead cells. It is green-fluorescent, with excitation maximum at 483 nm and emission maximum at 503 (“SYTO ® Green-Fluorescent Nucleic Acid Stains,” 2003). It was used at 2.23 µM, in accordance with the manufacturer recommendations (0.05 – 20 µM) in “SYTO ® Green-Fluorescent Nucleic Acid Stains” (2003) as counterstain contrasting with propidium iodide (PI) so both were incubated for 15 min under no light conditions for membrane permeability evaluation;

Propidium iodide (PI) (life technologies™, Netherlands), is a red-fluorescent nuclear and chromosome stain which binds to nucleic acids by intercalating between bases. PI is membrane impermeable so, penetrates and stains only cells with lost membrane integrity, working as an indicator of dead cells. It has an excitation maximum at 535 nm and emission maximum at 617 nm (https://www.thermofisher.com/pt/en/home/life-science/cell-analysis/fluorophores/propidium-iodide.html.). A concentration of 13 µM of PI was used, as a counterstain in a multicolour fluorescent

technique together with SYTO® 9. PI+SYTO® 9 were incubated for 15 min under no light conditions for membrane permeability evaluation.

$DiBAC_4(3)$ (Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol) (life technologies™, Netherlands) is an anionic potential-sensitive probe that enters depolarized cells and binds to intracellular proteins or membranes (as are largely excluded from mitochondria, are primarily sensitive to plasma membrane potential). Increased depolarization results in additional influx of the anionic dye and thus in enhanced fluorescence. $DiBAC_4(3)$ has an excitation maximum of 490 nm and emission maximum of 516 nm (https://www.thermofisher.com/order/catalog/product/B438). A concentration of 12.9 µM of $DiBAC_4(3)$ was used and was incubated for 30 min under no light conditions for membrane polarization evaluation.

In figure 5 is shown the excitation and emission wavelengths and respective intensity, in percentage, of each fluorophore.

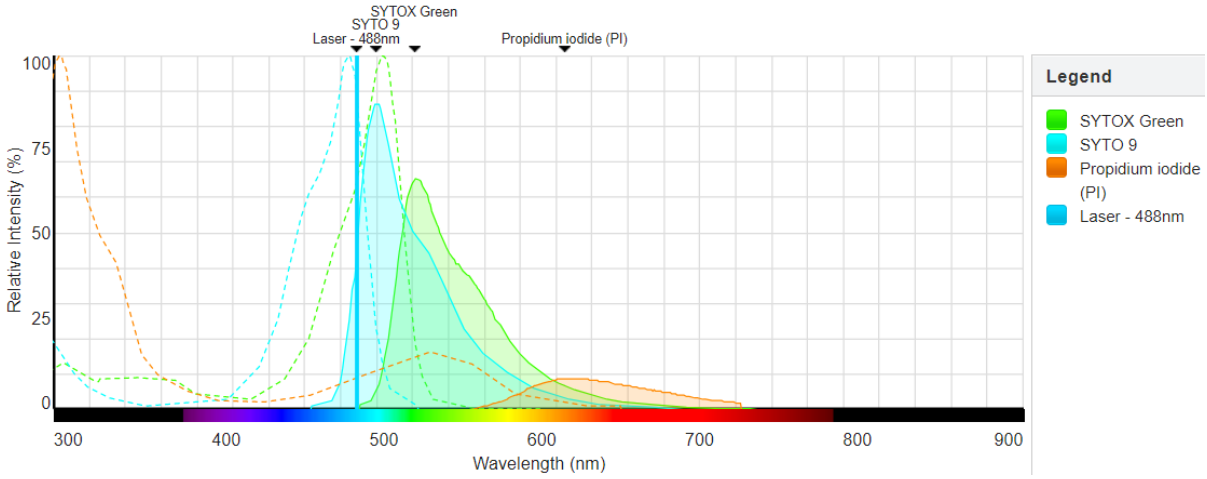


Figure 5. Flow cytometry laser and fluorophores wavelengths. Vertical solid blue line represents the blue solid-state laser of 488 nm; dotted curves represent excitation wavelengths and solid curves represent emission wavelengths of SYTOX® Green nucleic acid stain (green), SYTO® 9 green-fluorescent nucleic acid stain (blue) and propidium iodide (PI) fluorophores (orange). Source: https://www.thermofisher.com/pt/en/ (“Fluorescence SpectraViewer,”).

2.8.6 Flow cytometric analysis

The analysis in the flow cytometer were performed as soon as possible in parallel with the sampling. Three independent intra and inter experiment replicas of each measurement analysis were done.

Data analysis was done by using FloMax®, the Windows™-based flow cytometry software and Microsoft Excel. Quantitative assessment of each bacterial subpopulation was performed by counting the number of events included inside each corresponding gate. Total counts were displayed as dot plot graphics, and emitted fluorescent data were displayed as histograms and as dual parameter dot plot graphics. Geometric mean, mode, counts, and parent frequency were calculated for the various parameters assessed. Comparison between control and test cultures and among these, between viable and non-viable were done. Also, percentages of viability in relation to the control culture were calculated

and compared with results from the following assays (OD and plate culture measurements).

2.9 OD_{600nm} at flow cytometry sampling times

To complement flow cytometry results of total cell counts, samples of 0.5 mL of each culture were collected at each sampling time and analysed for OD_{600nm} using the UV1101 Biotech Photometer (WPA, UK) device. After all samplings, means and standard deviations were calculated. Graphics of OD_{600nm}/mL were plotted against time and were transformed in cells/mL by the equivalence 1 OD=$8x10^8$ cells/mL (Myers, Curtis, & Curtis, 2013) to then can be compared with flow cytometric data.

As *C. marina* produce agglomerates, its results relying in OD measurements were not as reliable as desired.

2.10 Viabilities at flow cytometry sampling times

To complement flow cytometry results of viable cell counts, samples of each culture at each flow cytometry sampling time were analysed by plate culture method.

First, test cultures were launch and prepared as described above (in “Flow cytometry - test cultures preparation”), then samples were collected as follows.

While for *E. coli* measurements, samples of 1 mL from each culture (test and control) at each sampling time were collected, for *C. marina* measurements, samples of 1.5 mL from each culture (test and control) at each sampling time were collected into two 2 mL microtubes, centrifuged during 3 min at 4 ºC, 8000 rpm, supernatant discarded, pellet resuspended in 0.5 mL artificial sea water (Tropic Marin®, USA) and the procedure was again repeated. Afterwards, samples were divided into 3 wells of a 48-well plate and (1:10) serially diluted. 5 µL droplets of each well of each dilution were spotted onto suitable solid media petri dishes using a suitable multichannel micropipette. Drops dried under sterility conditions, and then petri dishes were incubated overnight. In the next day, colony forming units (CFU) were counted, calculations for CFU/mL were made and a graphic of CFU/mL against time was drawn using Microsoft Excel. The analysis of the results integrated its comparison with flow cytometric data and with OD data.

3 Results and discussion

3.1 *E. coli* growth curves in OD_{600nm}

The effect of several BMX-11 concentrations was assessed on *E. coli* mid-logarithmic and stationary growth phases by optical density measurements using a 600 nm filter. The aim of the study was to understand the range of concentrations of BMX-11 necessary to inhibit the *E. coli* growth on growing cultures and to understand whether there are differences between administrating BMX-11 in mid-logarithmic phase or in stationary phase.

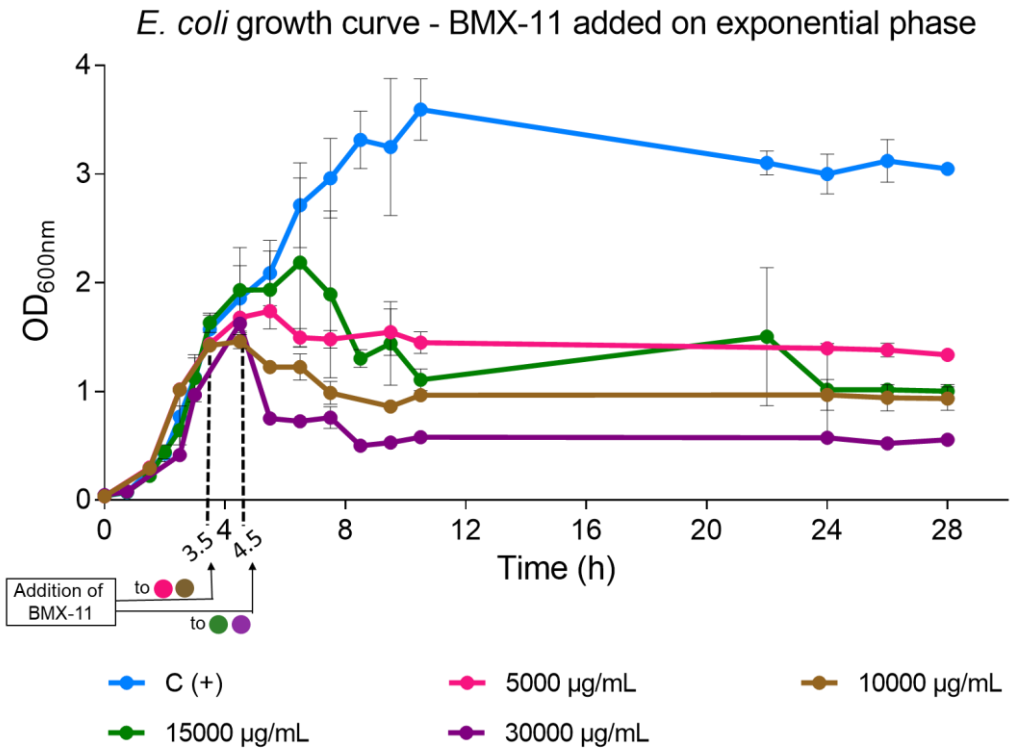


Figure 6. OD_{600nm} measurements of *E. coli* cultures with several BMX-11 concentrations added on exponential phase. BMX-11 was added to 2 cultures at mid-logarithmic phase at time-point 3.5 h so that they were at a concentration of 5000 and 10000 µg/mL, and to other 2 cultures at time-point 4.5 h so that they were at a concentration of 15000 and 30000 µg/mL. Time-points of OD measurements are represented with dots. The blue line represents the E. coli growth control cultures (C (+); without BMX-11), the purple line represents E. coli cultures at the concentration of 30000 µg/mL of BMX-11, the green line represents the ones at 15000 µg/mL BMX-11, the brown line represents the 10000 µg/mL cultures, and the pink line represents the cultures at 5000 µg/mL. OD_{600nm}: optical density measured at 600 nm; error bars: standard deviation.

In the study presented in figure 6 (data in A1 annex), *E. coli* cultures were grown and i) after 3.5 hours BMX-11 was added to cultures in order to be at 5000 µg/mL and at 10000 µg/mL; ii) after 4.5 hours BMX-11 was added to other cultures, in order to be at 15000 µg/mL and at 30000 µg/mL. The growth control culture (C(+), blue in fig. 6) presented distinguishable capacity to grow when compared with all the tested cultures containing BMX-11, reaching a maximum OD of 3.6. Among test cultures, a concentration of 30000 µg/mL of BMX-11 caused the biggest decay in growth, with approximately 84 % less growth than the control cultures, 4 hours after BMX-11 has been administered (time-point 8.5 h in

the fig. 6). The cultures at 15000 µg/mL and 10000 µg/mL of BMX-11 have different responses during the initial hours after BMX-11 was added, with the 15000 µg/mL culture displaying longer delay (2 h comparing to 1 h delay of the 10000 µg/mL culture) but then faster decrease in the growth than the 10000 µg/mL culture. From time-point 10.5 h on, these 2 cultures showed similar responses, stabilizing around an OD of 1, which means that they displayed 68.4 % less growth comparing to the control culture. 5000 µg/mL of BMX-11 still has effect on the *E. coli* exponential growth curve cultures, however this concentration only inhibits the growth (instead of decreasing it), as opposed to the other 3 test cultures. Cultures at 5000 µg/mL of BMX-11 grew approximately 53.4 % less than the control culture.

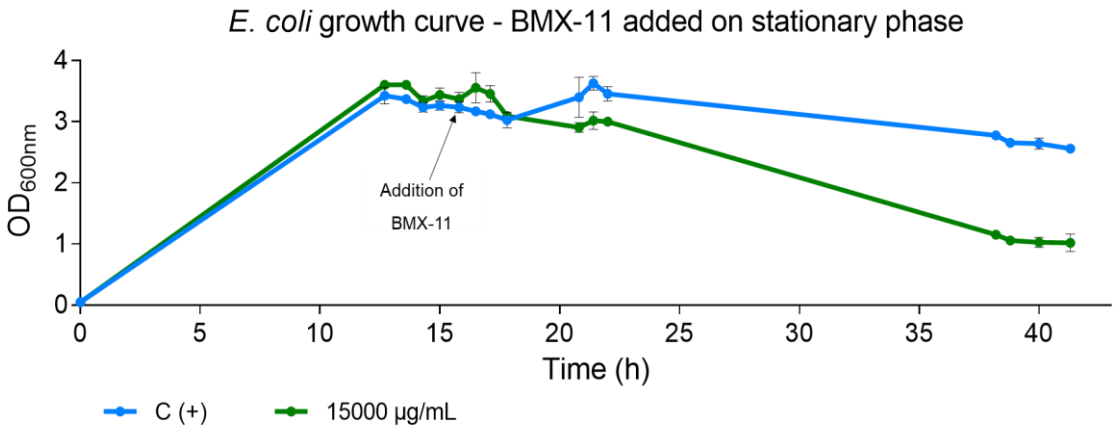


Figure 7. OD_{600nm} measurements of *E. coli* cultures with BMX-11 added on stationary phase. Prior to add BMX-11, *E. coli* cultures were incubated for 15.8 h to be at stationary-phase, then, BMX-11 was added to test cultures so that they were at a concentration of 15000 µg/mL. Time-points of OD measurements are represented with dots. The blue line represents the *E. coli* growth control culture (C (+); without BMX-11), and the green line represents the cultures at 15000 µg/mL. OD_{600nm}: optical density measured at 600 nm; error bars: standard deviation.

As shown by the OD curve in fig. 7 (data in A2 annex), the effect of BMX-11 on *E. coli* stationary growth phase was also assessed. Prior to exposure to BMX-11, *E. coli* cultures were incubated for 16 h to generate high cell density stationary-phase samples. Then, BMX-11 was added to the test cultures to a final concentration of 15000 µg/mL - this was the concentration of choice since it exhibited good results on mid-logarithmic phase study. Comparing to the growth control culture curve, at time-point 20.8 h, the test culture presented a decrease in growth of only about 14.71 % and at time-point 38.8 h a decrease of 60.74 %.

Comparing results obtained from cultures at 15000 µg/mL of BMX-11 in both systems (BMX-11 added in mid-logarithmic and in stationary phases), similar OD values and similar percentages of growth decrease in the last sampling hours were observed - logarithmic phase: 60.74 % lower than control at 28 h and stationary phase: 68.4 % than control at 40 h. However, BMX-11 showed diminished activity on non-growing bacteria in stationary phase, with a slower decreasing effect when added in the stationary phase than in the logarithmic phase - taking 23 and 5 hours, respectively (to reach about 60

% of decreasing effect). Such reduced effect is even more noticeable when analysing results from six hours after BMX-11 was added, showing 69.25 % growth decrease in logarithmic phase comparing to the 13.17 % decrease when added in stationary phase. Reduced antibacterial effect in stationary phase may be due to bacterial physiological state, no longer actively replicating, with the number of viable cells remaining constant. Cells can enter in arrested growth due to the high cell density (Mascio, Alder, & Silverman, 2007; Pletnev, Osterman, Sergiev, Bogdanov, & Dontsova, 2015; Brauner, Fridman, Gefen, & Balaban, 2016), the depletion of nutrients (Faleiro, 2011; Pletnev, Osterman, Sergiev, Bogdanov, & Dontsova, 2015), oxygen (Faleiro M.L., 2011), changes in the pH of the media (Grant & Hung, 2013), or between other, due to the accumulation of toxic products (Faleiro M.L., 2011). This "resting" state is the cause why many antibiotics, especially those that act on the cell wall, lose much of their effectiveness in stationary phase or in conditions where this state is achieved (Dajcs *et al*., 2001).

In addition, bacteria in stationary phase can also exhibit the phenotype of "environmentally induced antibiotic indifference" (characterized by arrested growth and population-wide antibiotic non-inherited tolerance). which can be triggered by external factors that include host factors (if applicable), antibiotic itself, and nutrient deprivation. The growth arrest as a passive survival strategy can be associated with a coordinated transcriptional response to stress, providing protection during antibiotic stress, leading the majority of the population to survive (Grant & Hung, 2013; Brauner *et al*., 2016). Some of the response mechanisms, such as the regulation of efflux pumps, or the production of altered amino acids, may also reduce the effectiveness of the antibiotic (Grant & Hung, 2013; Brauner, Fridman, Gefen, & Balaban, 2016). Moreover, when the environmental stress is removed, the surviving cells can restart growth and recover antibiotic sensitivity, since these modifications are phenotypical (Grant & Hung, 2013).

Vancomycin, daptomycin, teicoplanin, ciprofloxacin, rifampin, rifampicin, netilmicin and fleroxacin are examples of antibiotics that, like BMX-11, showed lower effect when used against bacteria in the stationary phase than in the logarithmic phase, consequently requiring higher doses or increased exposure time to obtain effects similar to exponential phase of growth (Zimmerli, Frei, & Widmer, 1994, Widmer, Frei, Rajacic, & Zimmerli, 2000). For example, when studying bactericidal effect against *S. epidermidis*, rifampin only needed a 2.5 fold increase in concentration to achieve a bactericidal effect on stationary phase similar to the logarithmic phase, while ciprofloxacin needed a 200 fold concentration increase (Widmer *et al*., 2000). Furthermore, ciprofloxacin also constitutes an example of an antibacterial effect dependent on the species of the bacterial strain, as shown by Zeiler and Grohe (1984) that revealed high efficacy of ciprofloxacin on stationary phase *E. coli* cultures while Widmer *et al*. (2000) showed low effect on stationary phase *S. epidermidis* cultures.

In opposition to the examples above, there also are antimicrobials with similar effect on both logarithmic and stationary phases, like lysostaphin, an enzyme capable of lyses *S. aureus* by digesting its cell wall (Dajcs *et al*., 2001), or daptomycin, a lipopeptide (class of bactericidal antibiotics) that targets the bacterial cytoplasmic membrane of Gram-positive bacteria, and which has bactericidal activity against meticillin-susceptible *S. aureus* (MSSA) without cell lysis (Hancock, 2005).

3.2 Viability of *E. coli* exponential-phase cultures in the presence of BMX-11

The effect of BMX-11 was also assessed on *E. coli* logarithmic-phase cultures viability by plate culturing, since from OD measurements it was observed a higher/faster BMX-11 effect on logarithmic-phase cultures than on stationary-phase cultures. These tests were performed to complement the first test (based on OD) and to verify if the results of both tests agree. Colonies were counted from control growth cultures and cultures at 15000 µg/mL of BMX-11 after 1, 2, 3, and 5 hours of the addition of BMX-11 to the test culture. Colony counts from growth control cultures have increased over time, as expected, reaching $2.88x10^9$ CFU/mL 5 hours after the addition of BMX-11. BMX-11 test cultures showed a fast decrease in viability from the first to the second hours after the addition of BMX-11, but stabilizing around $1.75x10^8$ CFU/mL from the second hour of growth on. At time-point 8.5 h (5 h after the addition of BMX-11), test cultures displayed 93 % lower viability than the control cultures.

These OD and viability tests allowed to study BMX-11 antimicrobial effect on grown bacterial cultures. BMX-11 revealed to have a strong effect on *E. coli* growth, exhibiting greater effect when added to cultures at the logarithmic phase than in the stationary phase. In addition, these tests allowed to conclude that OD_{600nm} shows the same trend that CFU/mL counts, showing that BMX-11 is impairing growth of the target bacteria, but also that it has an impact on the viability of the cells.

3.3 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The capacity of BMX-11 to inhibit growth and to kill bacteria was quantified by integrating MIC and MBC determined values for a panel of 6 distinct bacterial strains. The results obtained from microdilution assays with agitation (only performed for *C. marina* and *E. coli*) are shown in fig. 8 and fig. 9 and from the static assays with the other strains in fig. 10.

3.3.1 Optical density analysis using a continuous, with agitation, system on *C. marina* and *E. coli* cultures

Optical density analysis at 600 nm to test 9 BMX-11 concentrations over time were performed first for *E. coli* as a model bacterium (data in fig. 8 and in A3 annex), and after for *C. marina* (data in fig. 9 and in A4 annex). This test was performed to compare data obtained from static and agitating assays to overcome experimental difficulties found when performing static assays for some bacterial strains, due to aggregation of the cells, and confirm that such approach would allow to have data still comparable with other static tests performed for other bacteria. The average growth at each sampling point comprises the results obtained from 2 inter-experiment replicates with 8 intra-experiment replicates each, without the standard deviations presented on the graph for better clarity.

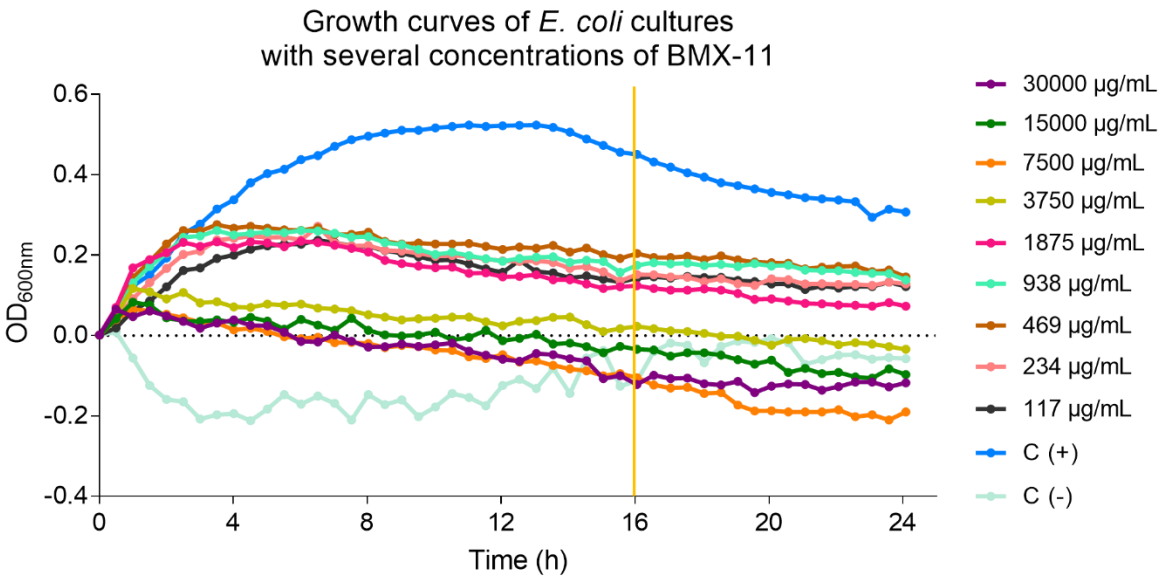


Figure 8. Optical density at 600 nm measurements of *E. coli* growth in the presence of several BMX-11 concentrations over time. Two-fold concentrations of BMX-11 ranging from 117 µg/mL to 30000 µg/mL were added to cultures at the mid-logarithmic phase (time 0) and measurements were taken every half hour for 24 hours. C (+) represents the *E. coli* growth control culture (without BMX-11), and C (-) represents the negative control of the test (without BMX-11 and without bacterial culture). Vertical yellow line defines the time-point at which MIC's were defined; OD_{600nm}: optical density measured at 600 nm.

The monitoring of *E. coli* growth over time in the presence of several two-fold serially diluted BMX-11 concentrations (from 30000 to 117 µg/mL), allowed to generate a more complete knowledge of the BMX-11 capacities to inhibit bacterial growth. By analysing fig. 8 (data in A3 annex), it is possible to observe 3 clearly separated groups of responses depending on the BMX-11 concentration: i) one group was composed only by the control culture, which stands out for high growth, reaching approximately 0.5 of OD ; ii) a second group composed by cultures submitted to the lowest serial dilution series of BMX-11 concentrations, from 117 to 1875 µg/mL, at which growth was slightly inhibited over time, with a reduction of 63.6 % in respect to control cultures at time-point 16 h; iii) and a third group, composed by cultures tested with the highest BMX-11 concentrations, from 3750 to 30000 µg/mL, with growth strongly impaired, and a clear OD decrease over time indicating a significant impact on cell survival, presenting close to of 100 % lower growth than the control at time-point 16 h, with probable destruction of the cells. Since these 3 separated groups were observed, and although the disadvantage of two-fold dilutions of producing large differences at high dilutions (Barry *et al*., 1999), there is an undetermined concentration of BMX-11 between 1875 and 3750 µg/mL, that corresponds to a limit where, from that point on, the BMX-11 effect is no longer dependent on its concentration.

MIC's were determined from static assay after 16 h of growth. In order to cross-check the data obtained from the assays with agitation at 16 h with the data on MIC measurements, values corresponding to the vertical yellow line in fig. 8 (16 h), was analysed and compared with MIC values. From the assays with agitation it was observed that, at time-point of 16 h, cultures at lower and including 1875 µg/mL concentrations, although unquestionably affected by BMX-11, still exhibited growth.

Cultures at the concentration of 3750 µg/mL (light green line in fig. 8) and above are part of the group on which BMX-11 had a high effect on growth, however at this concentration cultures apparently reduced or arrested growth but still exhibited some turbidity (0.023 OD) for 18 hours. The next concentration (7500 µg/mL) exhibited no turbidity at 16 h, so MIC of BMX-11 for *E. coli* is defined as 7500 µg/mL. The obtained MIC is coincident with that obtained from static assays (results presented later).

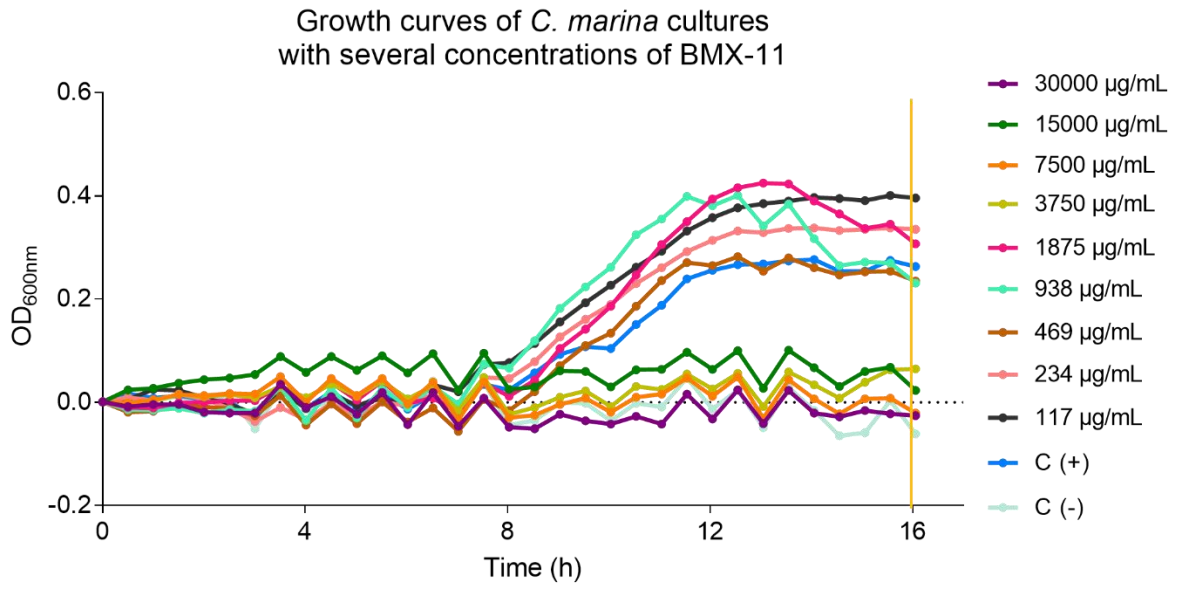


Figure 9. Optical density at 600 nm measurements of *C. marina* growth in the presence of several BMX-11 concentrations over time. Two-fold concentrations of BMX-11 ranging from 117 µg/mL to 30000 µg/mL were added to cultures at the mid-logarithmic phase (time 0) and measurements were taken every half hour for 16 hours. C (+) represents the *C. marina* growth control culture (without BMX-11), and C (-) represents the negative control of the test (without BMX-11 and without bacterial culture). Vertical yellow line defines the time-point at which MIC's were defined; OD_{600nm}: optical density measured at 600 nm.

C. marina growth was also monitored over time in the presence of two-fold BMX-11 concentrations ranging from 117 to 30000 µg/mL. In fig 9 (data in A4 annex), it can be observed that there were 2 distinct groups of cultures for which BMX-11 acts differently depending on its concentration: one composed by the growth control cultures (C (+)) plus the ones from 117 to 1875 µg/mL (the lowest concentrations), which exhibited similar growth capacity, with an average growth at time-point 16 h of 0.316 of OD, and which neither appear to be affected by BMX-11 nor it is possible to distinguish whether cultures were differently affected by the range of BMX-11 concentrations; and a second group composed by cultures at concentrations ranging from 3750 to 30000 µg/mL (the highest), in which growth was greatly inhibited, showing an average growth of 0 of OD at time-point 16 h. Since these 2 separated groups were observed and although the already mentioned disadvantage of the two-fold dilutions, apparently and as before, there is an undetermined concentration of BMX-11 between 1875 and 3750 µg/mL from which and before which the effect is not concentration related. Cultures tested at the concentration of 3750 µg/mL of BMX-11 showed higher growth inhibition, however with still visible growth at 16 h, so, the next concentration – 7500 µg/mL - of BMX-11 for *C. marina* was defined as the MIC, since it was the lowest concentration that presented no visible growth.

Results obtained from this assay (with agitation) agree with those obtained from static assays, being the 7500 µg/mL of BMX-11 the lowest concentration which after 16 hours of incubation had reached no visible growth and therefore is MIC for *C. marina*.

OD measurements of *C. marina* growth obtained from the assay with agitation had imprecisions due to the data acquisition equipment, an issue also detected in larger extent in the static assays. The agitation did not solve the problem found in measuring OD statically, even though it become possible to achieve average values clearly enough to conclude the tests.

3.3.2 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) assays

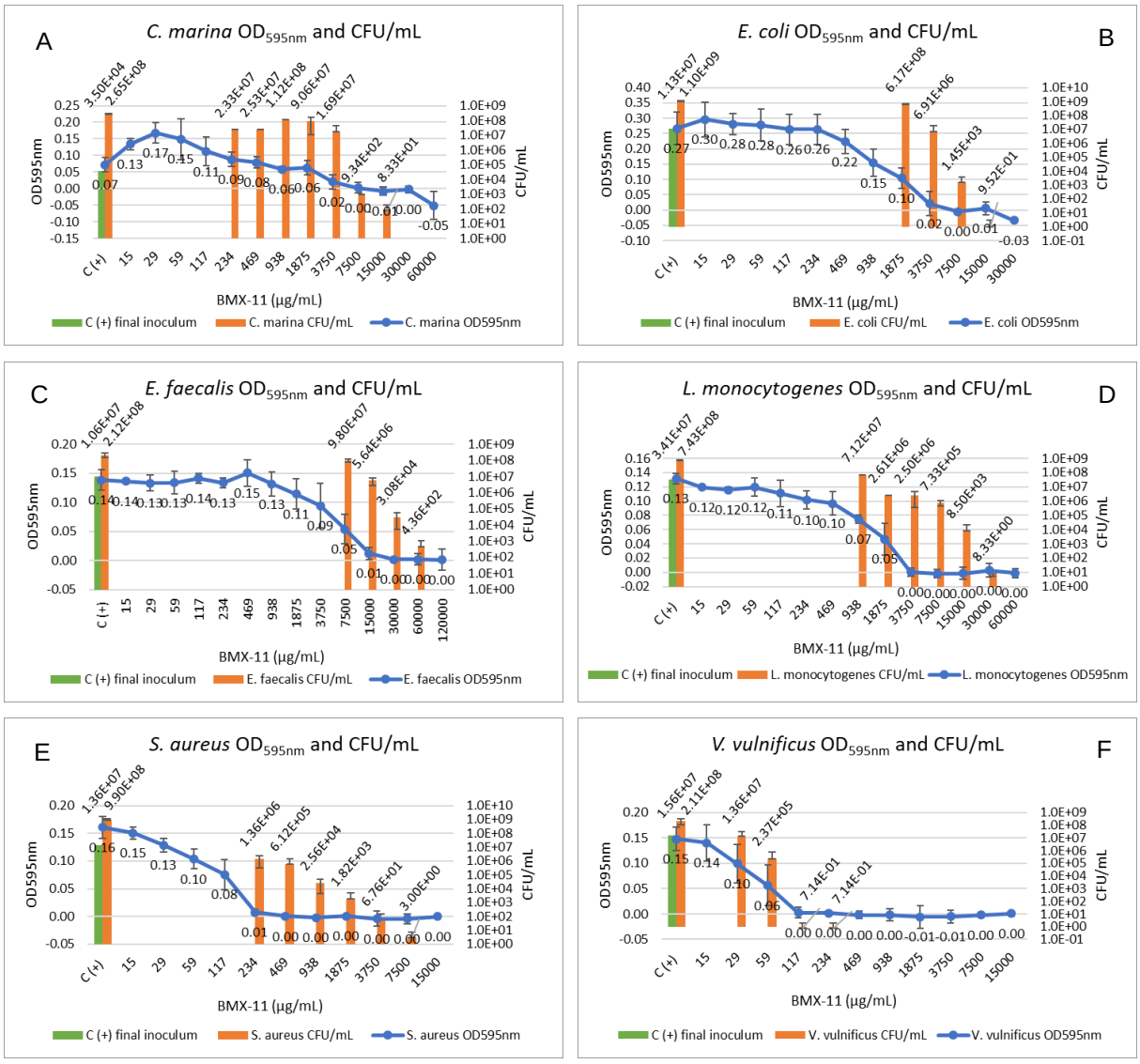


Figure 10. Effect of exposing *C. marina, E. coli, E. faecalis, L. monocytogenes, S. aureus* and *V. vulnificus* cultures to two-fold concentrations of BMX-11 by OD_{595nm} and by CFU/mL counts. OD: optical density measured at 595 nm represented by blue lines with dots and read on left Y axis. CFU/mL counts: orange columns, read on right Y axis. Green columns: CFU/mL counts of final inoculum. Two-fold serial concentrations of BMX-11 were studied, ranging from 15 µg/mL to 15000, 30000, 60000 or 120000 µg/mL depending on the strain in study.

C (+) represents the growth control cultures. A: *C. marina*, B: *E. coli*, C: *E. faecalis*, D: *L. monocytogenes*, E: *S. aureus* and F: *V. vulnificus*. Error bars represent standard deviations.

In fig. 10 is exposed the capacity to grow of 6 strains based on optical density mean measurements at 595 nm (blue line with dots) and on their viabilities, in CFU/mL (orange columns), in the presence of two-fold serial BMX-11 concentration's ranging from 15 µg/mL to 15000, 30000, 60000 or 120000 µg/mL depending on the species. OD measurements were done after 16 h of incubation, as described on M07-A8 approved standard of CLSI, and viable cells were counted manually through bacterial colonies after approximately 18 h of incubation, but checked and remaining unaltered for up to 48 h.

Regarding *C. marina* capacity to grow by OD, it was observed that, in comparison with the control cultures (C(+)), concentrations of BMX-11 ranging from 15 to 469 µg/mL have little or no impact in the growth. With the increasing of BMX-11 concentration, a gradual reduction in OD was observed until a concentration of 7500 µg/mL is used. As MIC is defined as "the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in a broth dilution susceptibility test" (Wikler *et al*., 2009), that concentration for *C. marina* is 7500 µg/mL of BMX-11. Regarding *C. marina* viability as colony counts, little difference was seen among cultures from the concentration of 234 µg/mL to 3750 µg/mL of BMX-11. Starting at 3750 µg/mL, the increase of BMX-11 concentration led to a gradual reduction of colony counts, reaching approximately 83 CFU/mL with 15000 µg/mL of BMX-11 and none CFU/mL with higher concentrations. *C. marina* tests for MIC determination didn't produced OD_{595nm} data sets and viabilities as reliable as for the other strains tested, mainly due to its capacity to form bacterial aggregate, turning imprecise both the serial dilutions of the culture, the OD readings and the CFU/mL counts. Therefore, *C. marina* microbial plating was done using glass spheres for increased spreading capacity, however this procedure proved not to be a good solution since many petri dishes only displayed colonies in agglomerates on the borders of the petri dishes.

Regarding *E. coli* capacity to grow in the presence of BMX-11, it was observed that, from 15 to 234 µg/mL, BMX-11 seems to have little or no effect. Nevertheless, with the increase of concentration, a gradual growth reducing effect was observed until it reaches the maximum inhibition at 7500 µg/mL, concentration from which no turbidity was seen - MIC of BMX-11 for *E. coli* is 7500 µg/mL. Regarding *E. coli* viability, little difference was seen between counts from growth control cultures (C(+)) and up to 1875 µg/mL BMX-11. Starting at 1875 µg/mL, the increase of BMX-11 concentration led to a gradual decrease of colony counts, reaching approximately 1 CFU/mL when at 15000 µg/mL of BMX-11. Cultures at the next two-fold BMX-11 concentration (30000 µg/mL) presented no CFU/mL.

By OD measurements of *E. faecalis* test cultures, BMX-11 at concentrations ranging from 15 to 938 µg/mL seems to have no effect when comparing to the growth control (C(+)). When BMX-11 concentrations increase (above 938 µg/mL), a gradual decrease in growth was observed until it reaches a maximum inhibition at 30000 µg/mL - MIC of BMX-11 for *E. faecalis* is 30000 µg/mL. Respecting to *E. faecalis* CFU/mL counts, little difference was seen between control cultures (C(+)), that produced 2.12×10^8 CFU/mL, and the ones up to 7500 µg/mL of BMX-11, that produced 9.8×10^7 CFU/mL. From the concentration of 7500 µg/mL of BMX-11 on, the increase in concentration led as before to a gradual

decrease of colony counts, until 120000 µg/mL of BMX-11, at which no colonies did grow. Among the 6 strains on test, *E. faecalis* showed to be the most tolerant strain to the BMX-11 action (Levison, 2004). Comparing to the concentrations needed to inhibit growth and to kill the other 5 strains on test, the concentrations of BMX-11 needed to obtain the same results on *E. faecalis* were the highest.

Concerning to *L. monocytogenes* OD measurements, it was observed that, from 15 to 59 µg/mL, BMX-11 has little or no effect. From this concentration on (59 µg/mL), the increasing in concentration leads to a gradual inhibitory effect until it reaches its maximum effect at 3750 µg/mL, concentration from which no turbidity was seen – MIC of BMX-11 for *L. monocytogenes* is 3750 µg/mL. Regarding colony counts, from the lowest BMX-11 concentration to present any impact (938 µg/mL) a gradual decrease was observed until the concentration of 30000 µg/mL was reached, at which point the bacterial culture presented a viability of approximately 8 CFU/mL.

The capacity to grow of *S. aureus* gradually decreased with the increasing of concentration of BMX-11, starting from the lowest concentration on test (15 µg/mL) up until it reaches the maximum growth inhibition at 234 µg/mL - MIC of BMX-11 for *S. aureus* is 234 µg/mL. Unlike for the other strains in study, *S. aureus*, at the concentration of MIC (234 µg/mL), showed a 0.01 OD value (fig. 10 E) however, this MIC value was defined since this OD is a rounded value which does not necessarily reflects growth. Regarding *S. aureus* viability in the presence of BMX-11, a gradual decreasing effect was observed beginning from cultures at the lowest BMX-11 concentration (234 µg/mL) presenting $1.36x10^6$ CFU/mL and reaching only 3 CFU/mL at 7500 µg/mL of BMX-11.

V. vulnificus growth was slowly impaired upon BMX-11 challenge, from 15 to 117 µg/mL. The maximum growth inhibition was observed on cultures with 117 µg/mL of BMX-11, concentration at which no turbidity was seen - MIC of BMX-11 for *V. vulnificus* is 117 µg/mL. From colony counts, a decreasing in the viability of *V. vulnificus* was seen from cultures at 59 µg/mL of BMX-11, with $1.36x10^7$ CFU/mL, to cultures at 117 µg/mL of BMX-11, with approximately 1 CFU/mL. *V. vulnificus* showed to be the most sensitive strain on test: in one hand, because it was the only strain being killed and inhibited from growing in the presence of the same BMX-11 concentration and on the other hand because this was the lowest BMX-11 concentration either determined for MIC or MBC (Levison, 2004).

The performed viability assays (results showed in fig 10), allowed to define MBC's of BMX-11 for the strains in study - the most common estimation of bactericidal activity (Barry *et al*., 1999). MBC is defined as the lowest antimicrobial concentration needed to kill 99.9 % of the final inoculum (indicated as green columns in fig 10), what means a ≥3 $\log_{10}$ drop in CFU/mL of the viable organisms in the final inoculum (Barry *et al*., 1999) after incubation for 16 h (Wikler *et al*., 2009). Taking this into account, CFU/mL reduction in comparison with the final inoculum, in percentage, was calculated and are shown in fig. 11. MBC's were defined not only based on the capacity to kill 99.9 % of the viable cells of the final inoculum but also according to Pearson, Steigbigel, Davis, & Chapman (1980), meaning that the number of colonies in a sample should be compared with a specific value – rejection value – , not exceeding this one, to reliably consider the antimicrobial as lethal. As can be seen in fig. 10, the final inoculums used

were around 1 to $3x10^7$ CFU/mL, so, , only concentrations displaying 10^4 CFU/mL or less (3 log_{10} reduction) may be defined as MBC's, and the rejection value taken into account was 155 CFU/mL (Pearson *et al.*, 1980). In Pearson, *et al*. (1980), 155 CFU/mL is the rejection value to take into account when using final inoculums of $1x10^7$ CFU/mL, single 0.01 mL samples, and pipette with 1 % error.

The lowest antimicrobial concentration needed to kill 99.9 % of the final inoculum of each strain were found, however more CFU/mL than the rejection value was counted for all the strains (except for *V. vulnificus* and *C. marina) so,* MBC's were defined as the next highest concentration - see fig. 11 - this one revealing a number of CFU/mL lower than the rejection value. Unlike for the majority of the strains, the MBC for *C. marina* was defined as being the following lower concentration of the one capable to kill 99.9 % of viable cells. This was done because low numbers of CFU/mL were obtained from the final inoculum due to the aggregates formation, which do not appear to be reliable. *V. vulnificus* also constituted an exception since it was the only strain for which the 99.9 % kill of the final inoculum means a number of CFU/mL lower than the rejection value.

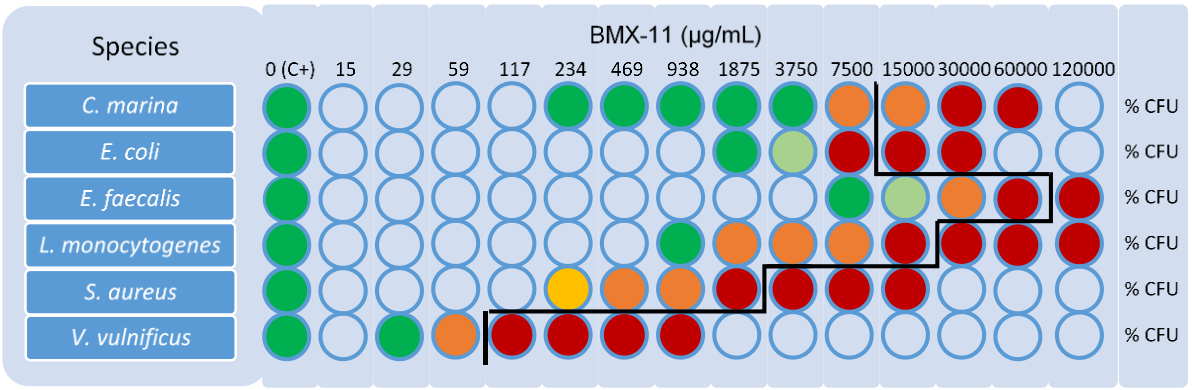


Figure 11. Comparison of viability reduction (CFU/mL), in percentage, within and between strains.. Strains on study are on the left side (*C. marina, E. coli, E. faecalis, L. monocytogenes, S. aureus* and *V. vulnificus*) and tested two-fold serial BMX-11 concentrations, in µg/mL, are on the top. Viability reduction (in percentage) based on CFU/mL counts are shown as circles. Colour scheme of viability reduction: <10%; 10-50%; 51-90%; 90-99; ≥99.9; untested. MBC for each strain is the concentration at the right of the black line. % CFU: percentage of viability reduction relatively to the final inoculums.

Two phenomena that also may interfere with bacterial killing are paradoxical effect and tolerance. Paradoxical effect happens when a concentration higher than the optimal bactericidal concentration is used and the bacteria dies at a reduced rate than the increase in concentration (Pankey & Sabath, 2004). This phenomenon was not tested, however based on the results of viabilities performed (fig. 10), it apparently did not happen, once at concentrations above MBC's, a greater (if possible) decrease in CFU/mL was observed for all the strains. The tolerance phenomenon is defined as an MBC that is ≥32 times the MIC (Pankey & Sabath, 2004). Tolerance has not been tested either, however for MIC's and MBC's determination for *S. aureus* and for *V. vulnificus*, concentrations tested were high enough to reach MBC≥32MIC (fig. 10) but, for these two strains, tolerance was not detected, presenting MBC's

lower than 32 times its MIC's, as indeed observed for the other strains in study (that presented a maximum of MBC≥16MIC).

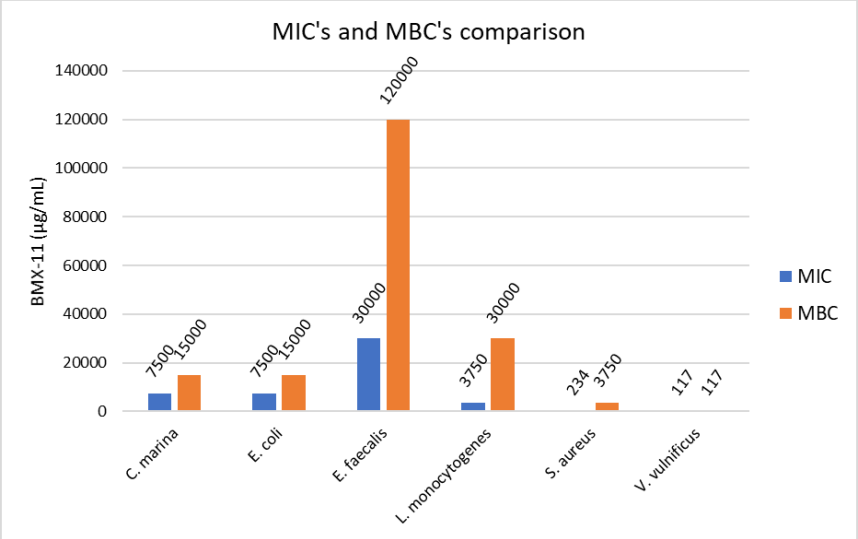


Figure 12. Comparison of MIC's and MBC's of BMX-11 within and between the 6 strains in study. MIC: blue columns; MBC: orange columns.

Summing up, the comparison of MIC's and MBC's within and between the 6 strains in study are shown in fig. 12: for *C. marina* and for *E. coli* MBC=2MIC, for *E. faecalis* MBC=4MIC, for *L. monocytogenes* MBC=8MIC; for *S. aureus* MBC=16MIC and for *V. vulnificus* MBC=MIC. Both to inhibit growth and to kill *E. faecalis*, the highest BMX-11 concentration was needed, as opposed to *V. vulnificus* that showed the lowest MIC and MBC, being the most sensitive of the strains to BMX-11 and being the only one which needed the same concentration both to be inhibited and to be killed. *S. aureus* showed to be the most resistant strain to BMX-11 since the ratio MBC/MIC was the highest.

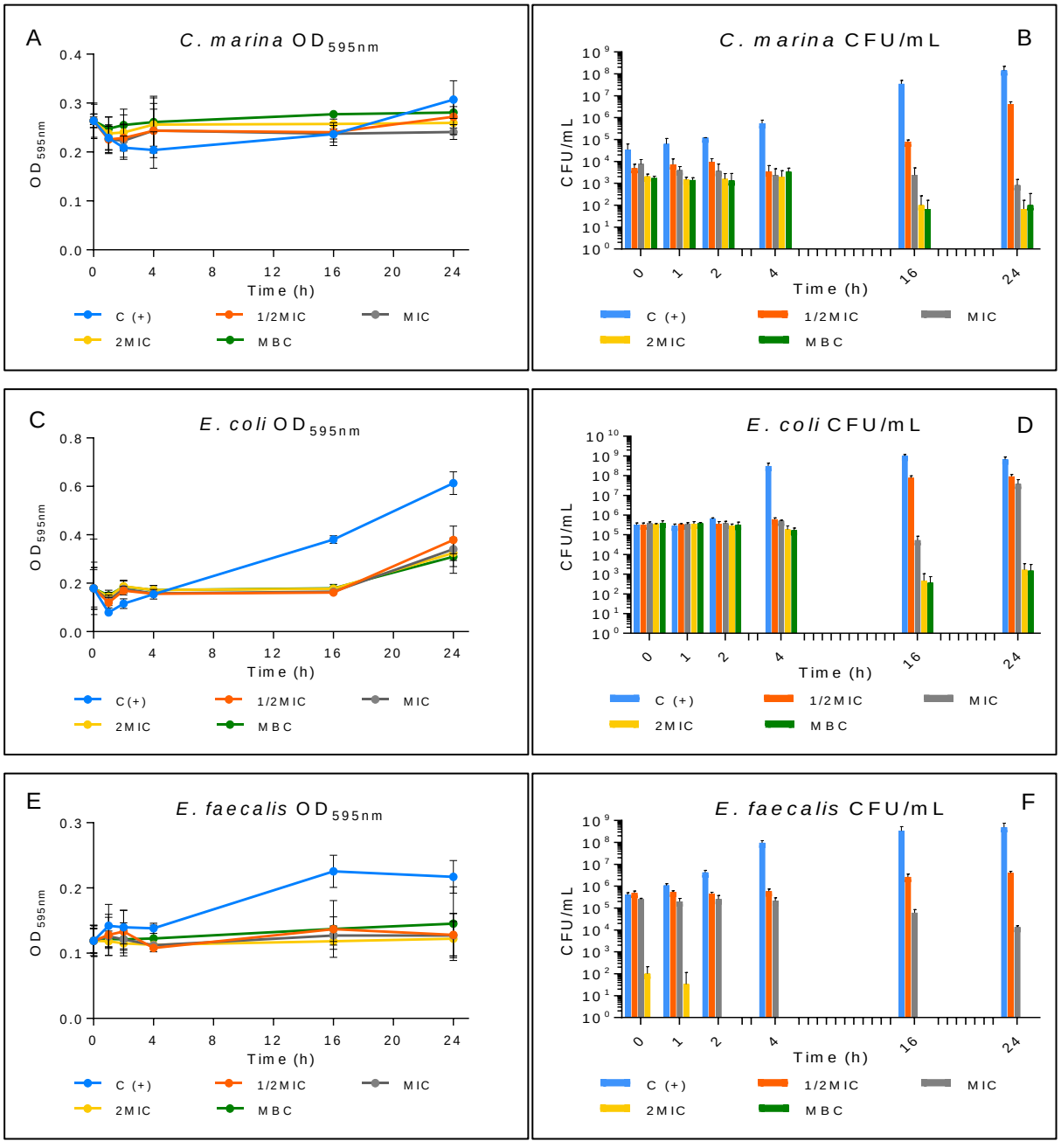
The definition of MIC and MBC are important not only because it allows the quantification of antimicrobial activity but also because it allows the definition of BMX-11 action against a specific bacterium as bacteriostatic or bactericidal. The definition of bacteriostatic is controversial, some define bacteriostatic as maintenance of growth or a reduction of less than 99.9 % of the final inoculum and at which after neutralization of the agent, the microbial cells can resume growth (Pankey & Sabath, 2004; Petersen, Jones, & Bradford, 2007) and others define as a ratio of MBC to MIC of >4 (Levison, 2004; Pankey & Sabath, 2004; Sim *et al.*, 2014). Bactericidal consists in the so-called capacity to reduce in 99.9 % (≥3 $\log_{10}$) the CFU/ml of the original inoculum and which has permanent effect (Barry *et al.*, 1999; Pankey & Sabath, 2004; Faleiro, 2011). Examples of bacteriostatic drugs are macrolides, clindamycin, tetracyclines, sulfonamides, linezolid, and chloramphenicol, and examples of bactericidal drugs are b-lactams, vancomycin, aminoglycosides, fluoroquinolones, daptomycin, and metronidazole (Levison, 2004).

Summing up, BMX-11 showed to be able to inhibit the growth and to kill every single strain in study, being gram-negative, gram-positive, marine or even pathogenic. It showed to be bactericidal to all the strains in study at concentrations equal to or higher than MBC's and to be bacteriostatic for concentrations below MBC's. If we consider the definition of bacteriostatic as a ratio of MBC to MIC of

>4, BMX-11 showed to be bactericidal for all the strains excepting for *L. monocytogenes* and for *S. aureus*.

3.4 Time-kill method

Time-kill assays were performed on the 6 strains in study by means of measuring optical density at 595 nm and of counting CFU/mL (fig. 13 and annex A5). Time-kill assays were performed with the objective of determining the dynamic interaction between BMX-11 concentrations known to be capable of decrease growth – MBC, 2MIC, MIC and 1/2MIC of each strain – and microbial strains, and with the objective of determining its rate of bactericidal activity (Levison, 2004).



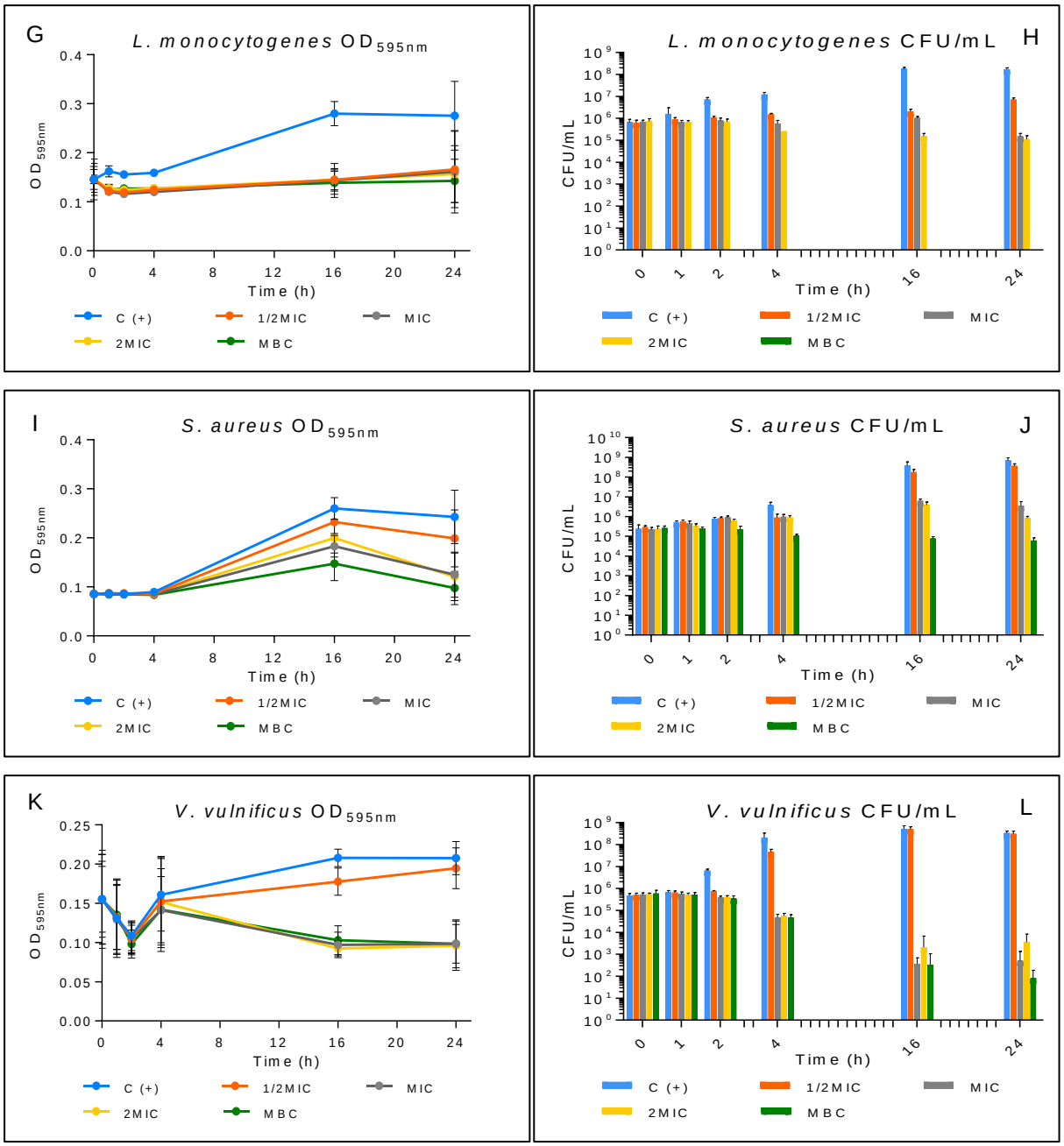


Figure 13. Comparative killing curves by OD_{595nm} and by CFU/mL counts of BMX-11 against the 6 strains on study over time. A, C, E, G, I, K: killing curves by optical density measurements at 595 nm (OD_{595nm}), represented as lines with dots; B, D, F, H, J, L: killing curves by CFU/mL counts (in logarithmic scale), represented by columns. Strains in study: *C. marina, E. coli, E. faecalis, L. monocytogenes, S. aureus and V. vulnificus*. BMX-11 concentrations studied: 0 µg/mL (C (+) – growth control cultures), ½MIC, MIC, 2MIC and MBC. Sampling points: 0, 1, 2, 4, 16 and 24 hours. Error bars represent standard deviations.

OD_{595nm} studies of *C. marina* cultures showed a lack of precision in the measurements, not allowing to take any conclusion about the effect of the BMX-11 concentrations in study. From colony forming units (CFU's) counts, it was observed that the concentration 1/2MIC still allows *C. marina* to grow, however leading to a reduction of almost 3 log_{10} in the number of CFU/mL at 16 h time point comparing to the growth control (C (+)). Cultures upon BMX-11 challenge at MIC showed stable viability over time,

and cultures at 2MIC and MBC reduced viability in more than 3 log_{10} CFU/mL, exhibiting approximately 100 CFU/mL after 16 hours (for *C. marina*, 2MIC=MBC=15000 µg/mL).

By OD_{595nm} measurements of bacterial growth, all the test concentrations of BMX-11 showed similar effect on *E. coli* growth, with the highest concentrations presenting a strong effect only after 24 h. For *E. coli,* 2MIC=MBC so, CFU/mL counts from cultures challenged at these conditions were similar, displaying the highest viability decreasing, with only 400 CFU/mL after 16 h (>6 log_{10} reduction). Cultures at MIC and 1/2MIC presented similar responses at all sampling times except at 16 h, at which cultures at 1/2MIC produced $7.7x10^7$ CFU/mL and cultures at MIC produced $5.3x10^4$ CFU/mL. These results might indicate that one of the tested points (from 1/2MIC or from MIC, at 16h or at 24 h) was an outlier, or, cultures at MIC were capable to resume viability (once showed $5.3x10^4$ CFU/mL at 16 h and $3.8x10^7$ CFU/mL).

For *E. faecalis*, OD was similar among all the BMX-11 concentrations on test, over time, revealing an average of 42.4 % less growth than the control at 16 h. Unlike OD measurements, viability results diverged proportionally with the BMX-11 concentration. Cultures at MBC did not presented colonies at all and cultures at 2MIC presented only about 100 CFU/mL at sampling times 0 and 1 hours (>3 log_{10} decrease). Cultures at MIC and at 1/2MIC showed similar viability at 4 hours (with approximately 2 to 6 $x10^5$ CFU/mL), but dissimilar from there, with cultures at 1/2MIC presenting $1.4x10^4$ CFU/mL (2 log_{10} decrease) and with cultures at MIC presenting $4x10^6$ CFU/mL (≥4 log_{10} decrease), after 24 hours.

OD measurements of bacterial growth of *L. monocytogenes* shows similar responses for all test BMX-11 concentrations only differing a little after 24 hours, presenting on average a maximum of 43 % less growth than the control at 24 h. Based on CFU/mL counts, no CFU was counted from cultures at MBC, while cultures at 2MIC displayed ≥3 log_{10} CFU/mL reduction from 16 h and cultures at MIC displayed ≥3 log_{10} CFU/mL reduction at 24 h. Although the observed proportionality between BMX-11 effect and its lowest test concentrations (1/2MIC, MIC and 2MIC), it seems to exist a concentration between the 2MIC and the MBC which marks the point at which proportionality ceases to exist (2MIC always showed viabilities in the order of 10^5 CFU/mL while MBC did not show CFU at all).

Interestingly, as can be observed in fig. 14, *L. monocytogenes* seems to be affected by antibiotic carryover from 2 h time-point, since no CFU were observed from directly plated samples, while there were countable CFU from the first plated dilution (10^{-1} dilution). Antibiotic carryover may happen when a measurable volume of antibiotic, usually at high concentrations is transferred to the subculture plate. It may be prejudicial for the count of survivors since there may be survivors, however being inhibited by the antibiotic. Antibiotic carryover may be eliminated inactivating the antibiotic on the subculture plate by, for example, flooding the plate with antibiotic inhibitor if it exists (e.g. using β-lactamase against β-lactam antibiotic), by diluting or washing the samples, or what is sometimes enough, let inocula to dry (let the drug to diffuse throughout the agar) and then streaking the sample (Barry *et al*., 1999).

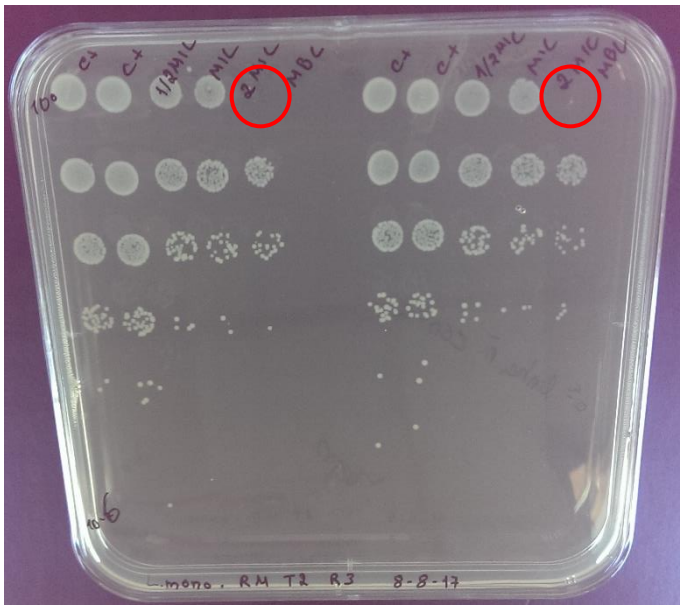


Figure 14. *L. monocytogenes* plate of the time-point 2 of time-kill viabilities test. Two intra-experiment replicas, one at left and one at right, each composed by the plating of 6 cultures: C (+): growth control cultures, and cultures at the concentrations of 1/2MIC, MIC, 2MIC and MBC of BMX-11. In first line, samples were plated directly (10^0 dilution), and in the following lines were 1:10 serially diluted. Red circles show none produced CFU only from directly plated samples at concentrations of 2MIC.

S. aureus OD data displayed from the 4 h time-point showed clear concentration-dependent inhibition of growth, *i.e.* increase in BMX-11 concentration led to proportionally growth decrease – 1/2MIC displayed 18 % less growth than the control, MIC displayed 48.6 %, 2MIC displayed 50.4 % and MBC displayed 59.9 %. For *S. aureus* MIC is much lower than MBC (MIC=234 µg/mL and MBC=16MIC) so, maybe for this reason, CFU/mL counts from the control were similar with the ones from 1/2MIC. As expected (once MIC=234 µg/mL and 2MIC=468 µg/mL are low/close concentrations), cultures at MIC and 2MIC also presented similar viability, with a 2 $\log_{10}$ reduction in CFU/mL after 16 h. Cultures at MBC exhibited a ≥4 $\log_{10}$ decrease in CFU/mL from 16 h. The great observed difference in viability between cultures at MBC and the others is probably due to this concentration being much higher than the others.

OD measurements from *V. vulnificus* growth control cultures and from cultures at 1/2MIC showed related values to each other. Cultures at MIC, 2MIC and MBC showed related values to each other, presenting 53 % less growth than the control. CFU/mL data displayed a similar pattern as that from OD measurements. In one hand 1/2MIC was not enough to decrease viability, with the cultures at this concentration presenting CFU/mL values close to that from control. In the other hand at MIC, 2MIC and MBC conditions, they showed similar reduction in viability between each other, showing 6 to 8 $\log_{10}$ reduction in CFU/mL relatively to control. At the concentration of MBC, *V. vulnificus* displayed the fastest viability reduction in comparison to the all other strains, followed by *E. coli* (also at respective MBC).

Time-kill assays also allow to determine the BMX-11 activity as bactericidal when a ≥3 $\log_{10}$ reduction in CFU/mL at a specific time or when 99.9 % killing is reached, and for that reason, the $\log_{10}$ reduction in CFU/mL were taken into account in the discussion above – this is one of the means that can be used

for comparing time-kill curves (Barry *et al.*, 1999). Other way to study lethal activity is comparing the rate of killing (slope of killing curves) of known concentrations of antibacterial (Barry *et al*., 1999).

All BMX-11 test concentrations presented similar killing kinetic profiles after 2 h on all strains except on *L. monocytogenes*, to which cultures at MBC did not showed CFU at all and on *E. faecalis,* to which 2MIC and MBC had bactericidal effect at the time of BMX-11 addition. In general, from 4 h on, the killing rates of 1/2MIC was lower than at MIC, which was slower than 2MIC which in turn was slower than MBC. In summary and consistent with the ≥3 $\log_{10}$ reduction in CFU/mL, at each respective 2MIC and MBC, BMX-11 was found to be bactericidal towards all tested strains after 24 h exposure. On the other hand, at MIC, BMX-11 was bacteriostatic towards *E. coli* and *S. aureus* and bactericidal towards the remaining strains. In general, killing rates had increased in proportion with the increase of BMX-11 concentration, revealing concentration-dependent bactericidal effect for the total of strains in study (Levison, 2004; Pankey & Sabath, 2004). Examples of antibiotics that display concentration-dependent action are aminoglycosides, daptomycin, fluoroquinolones, metronidazole, possibly the azalide, azithromycin, and ketolides (Barry *et al*., 1999; Levison, 2004; Pankey & Sabath, 2004; Mascio *et al*., 2007). Antibacterial action can also display time-dependent killing. Time-dependent killing only occurs when the use of concentrations above MIC do not result in a proportional increase in killing, originating slow killing rates (Barry *et al*., 1999; Levison, 2004; Pankey & Sabath, 2004), and/or when concentrations above MBC does not enhance bactericidal activity (Levison, 2004). Antibiotics that display time-dependent action are for example β-lactams, vancomycin and oxazolidinones (Barry *et al*., 1999; Levison, 2004; Pankey & Sabath, 2004).

For all the strains on test it would be of interest to sample around the 8th and/or the 10th hours of growth, since a lack of information was observed for the interval between the 4th and the 16th hours. It remains uncertain to what extent the strain continues to develop and at what point growth thrives.

3.5 Flow cytometry

A flow cytometric method was also used to study BMX-11 mode of action, since it has proven to be useful in previous antimicrobial testing (Durodie, Coleman, Simpson, Loughborough, & Winstanley, 1995; Silva & Lourenc, 2011; Dwyer, Camacho, Kohanski, & Callura, 2012; Manoil *et al*., 2014; Brien-simpson, Pantarat, Attard, Walsh, & Reynolds, 2016) and since it allows real-time data display, acquisition and analysis. In other hand, flow cytometry allowed cell viability assessment by the study of morphological changes, by FSC (related to particle size) and SSC (related to cell structure) measurements and, more accurately, by changes in membrane permeability and/or polarization by the exclusion or retention of dyes (Desjardins, Boulos, & Barbeau, 1999; Sgorbati, Brown, & Denis, 2001; Quintas & Tomás, 2014). *C. marina* and *E. coli* cultures were treated with BMX-11 so that they remain at the concentration of 2MBC=30000 µg/mL and were stained with SYTOX® Green, SYTO® 9+PI and $DiBAC_4(3)$ stains. Green emitted fluorescence was detected approximately at 530 nm and was displayed by FL1 channel and red emitted fluorescence were detected approximately at 640 nm and was displayed by FL3 channel. Fluorescence is displayed as a variation of fluorescence intensity. This study had the

aim to compare the percentage of live/dead bacteria (Quintas & Tomás, 2014), to obtain results to be compared with plate counting and OD measurement values, and to know whether BMX-11 have effect on bacterial membrane.

3.5.1 Validation of the method

The validation of the method/calibration of the instrument was critical to ensure that the results were suitable for their intended purpose. The validation was made by analysing non-treated *C. marina* and *E. coli* cultures before and after been killed by heat in order to be used as references for the determination of the detection ranges. For measurements, known percentages of live (LC - live cells) vs dead (KC - killed cells) bacteria were mixed and analysed: 100 % LC; 100 % KC; 75% LC, 25 % KC; 50 % LC, 50 % KC and 25 % LC, 75 % KC. Bacteria were stained with SYTOX® Green, SYTO® 9+PI and $DiBAC_4(3)$. This procedure was performed for both *E. coli* and *C. marina* cells, however due to similar results were obtained, only the ones from *C. marina* are presented/discussed.

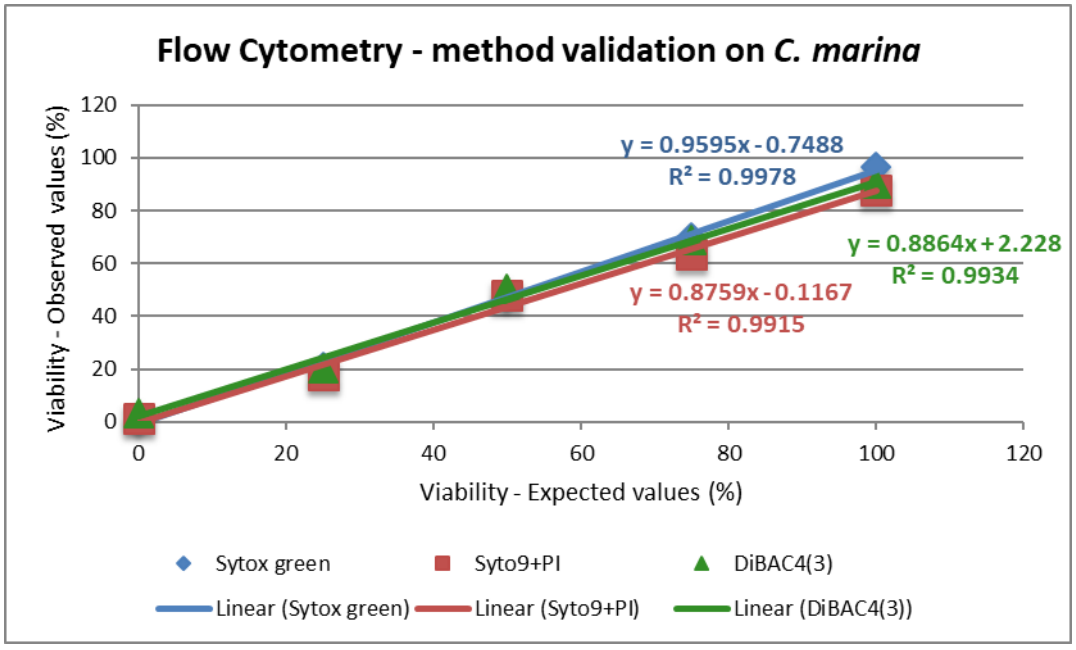


Figure 15. Linear regression for comparison between observed and expected viability from flow cytometry, for validation of the method. Mixtures of 0, 25, 50, 75 and 100 % of live cells with dead cells were analysed. ◆ Percentage of viable cells detected using SYTOX Green; ■ percentage of viable cells detected using SYTO9+PI; ▲ percentage of viable cells detected using $DiBAC_4(3)$. Regression lines obtained for each fluorophore have the same colour. R^2 is the coefficient of determination. Error bars represent standard deviations.

In fig.15 are showed the results of the method validation on *C. marina*, comprising the comparison of expected viability (%) and observed viability (%). SYTOX® Green showed to be the best fluorophore to explore viability, since it showed the highest coefficient of determination value (R^2=0.998), indicating an almost perfect linear relation between expected and observed viability values. SYTO® 9+PI and $DiBAC_4(3)$ staining showed similar R^2 values, of 0.9809 and 0.9807 respectively, also evidencing

reliability in the utilization of the fluorophores. Standard deviation between observed and expected values (showed as error bars in the fig.15), indicates SYTOX® Green as showing the lowest variation, and $DiBAC_4(3)$ showing lightly less variation than SYTO® 9+PI. Despite noticed (little) variations, high similar viability values were obtained from cells stained with all three fluorophores to validate the method. The agreement between data obtained from staining with SYTOX® Green and SYTO® 9+PI, with data obtained from the voltage sensitive dye $DiBAC_4(3)$, makes sense, since changes in membrane ion permeability are enough to have an effect in bacteria viability by membrane depolarization (Perry, Norman, Barbieri, Brown, & Harris, 2011; Winkel, Gray, Seistrup, Hamoen, & Denham, 2016).

The efficiency of used stains for viability assessment in stress conditions was checked, as has been prior reported for several microorganisms (Wickens, Pinney, & Mason, 2000; Decker, 2001; Kort *et al*., 2008).

3.5.2 Impact of BMX-11 on *E. coli* (30000 µg/mL)

In addition to the analysis presented below, in annexes A6.1, A6.2 and A6.3 are presented the calculated means and standard deviations of obtained FSC geometric mean and mode, and quadrants frequency of parent relating to each of the fluorophores used.

The number of cells/mL was assessed and control cells have increased from T0 ($1.33x10^9$ cells/mL) to T4 ($2.08x10^9$ cells/mL), have stagnated until T7 ($1.91x10^9$ cells/mL) and increased until T24 ($2.67x10^9$ cells/mL). Test cells have slowly decrease soon from T0 ($1.50x10^9$ cells/mL) to T7 ($5.03x10^8$ cells/mL) and to T24 ($9.89x10^7$ cells/mL). This data is presented in A8 annex and will be discussed and compared with data from other methods further ahead.

In fig. 16 are displayed FSC (forward-scattered light; at left) and SSC (side-scattered light; at right) measurements which represent the variation of *E. coli* cells in size (by FSC) and in complexity (by SSC) over time (h) (Desjardins, Boulos, & Barbeau, 1999; Sgorbati, Brown, & Denis, 2001; Quintas & Tomás, 2014). In graphics of FSC and in graphics of SSC at the right ("SSC test" in fig. 16), are shown the measurements performed on *E. coli* cells treated with 30000 µg/mL of BMX-11. SSC graphics at the left ("SSC control" in fig. 16) display *E. coli* control cells variation over time. FSC analysis were also done on *E. coli* control cells (without BMX-11), however FSC measurements have not undergone many changes, so only data from LC and from KC are shown.

FCS of untreated cells (LC in fig. 16) and of killed cells (KC in fig. 16) showed similar modal values (close to 0.5). With exception of T0 measurements, that showed a modal value of 1.93, treated cells (Tnumber in fig. 16) displayed similar FSC pattern (mode of 1.16) over time, with modal values slightly higher than those of live and killed cells. BMX-11 seems to have effect on *E. coli* cells size, making them larger.

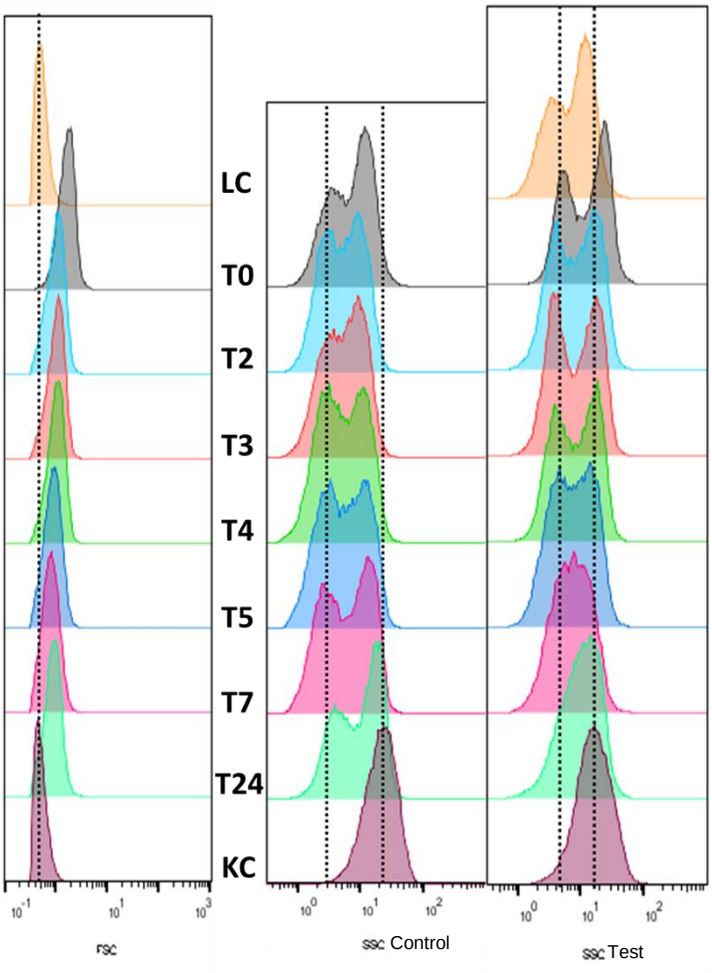


Figure 16. Changes in size (at left; FSC) and changes in complexity (at right; SSC) of *E. coli* cells over time. FSC: forward-scattered light, displays changes in size of cells treated with 30000 µg/mL of BMX-11. SSC: side-scattered light, displays changes in complexity of: untreated *E. coli cells* (control), at left and of treated *E. coli* cells with 30000 µg/mL of BMX-11 (test), at right. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum counts from the reference controls of live cells (LC) and killed cells (KC).

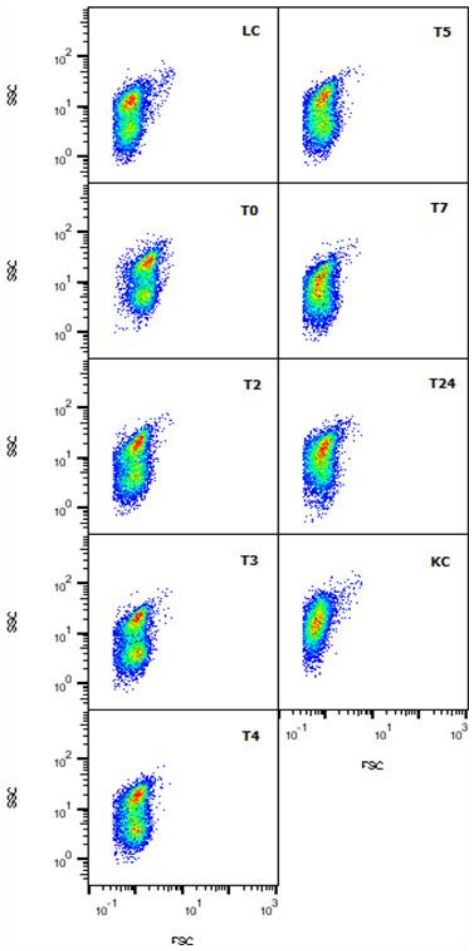


Figure 17. Changes in complexity against changes in size of *E. coli* cells in the presence of 30000 µg/mL of BMX-11 over time. FSC: forward-scattered light, displays changes in size of cells; SSC: side-scattered light, displays changes in complexity of cells. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells.

SSC (at right in fig. 16) is proportional to cell granularity or internal complexity (Becton Dickinson and Company, 2002; Tzur, Moore, Jorgensen, Shapiro, & Kirschner, 2011).Two SSC peaks (one of 2.8 and other of 13, approximately), more or less proportional to each other were observed in respect to control sample at all sampling times (SSC control graphics in fig. 16), in respect to live cells (LC), and in respect to test cells from T0 to T5. In opposition, test cells from T7 to T24 and killed cells (KC) showed only one peak of 8, 13 and 16, respectively. Test cells at T0 showed two peaks (modal values of 5.5 and of 25), however both slightly more complex and distinct from each other than that of LC (modal values of 3.5 and of 12). Over time, the peak of less complexity increased, and up for T5, the two peaks had merged into one of 8.5 modal value at T7 and of 14 at T24, this last being more similar to that of the KC (of 16). The observed changes in complexity demonstrated the existence of both test and control heterogeneous cell population. This heterogeneity may be due to cells were dividing so, exhibiting different

complexities/size regarding to mother and daughter cells. From T4, test cells showed a deviation of the pattern observed in previous time samplings and in control cells. From T4, it seems that test cells were losing complexity, perhaps losing its capacity to reproduce, what suggest that BMX-11 also affects the complexity of cells.

In the fig. 17 is showed the dispersion of size relatively to complexity over time. It is possible to observe two distinct subpopulations at T0 with the same size but different complexities, being the largest subpopulation (representing 58.6 % against 41.4 % of the smallest) the more complex one (what is seen by the red centre in fig. 17, T0). The same two subpopulations were observed from T2 to T5 and, from T5, they have merged into only one of greater variety in terms of complexity.

Changes in morphology, and consequently cell viability, can be detected by analysing forward and side-scattered characteristics as were done, but this can be a crude measurement of viability (Quintas & Tomás, 2014) so, membrane permeability and polarity analysis were performed using fluorescent dyes, since it offers the possibility to compare the percentage of live/dead distribution of the bacteria, before and after treatment with antimicrobials (Quintas & Tomás, 2014).

In figures 18, 19 and 20 are shown half offset histograms displaying the emitted fluorescence by treated (Tnumber) and untreated (LC and KC) cells after being stained with SYTOX® Green (fig. 18), SYTO® 9+PI (fig. 19) and $DiBAC_4(3)$ (fig. 20). Although results are not showed, similar analysis were performed over time on untreated cultures which were used as a control of the assay. Emitted fluorescence by treated (with BMX-11) *E. coli* cells were detected and are displayed in the figures (18, 19 and 20) by the FL1 channel (at the left of each figure), which displays the emitted green fluorescence, and by the FL3 channel (at the right of each figure), which displays the emitted red fluorescence. Emitted fluorescence from cells both stained by SYTOX® Green and by $DiBAC_4(3)$ is mainly detected by FL1 (green fluorescence), however its comparison with the FL3 (red fluorescence) provided interesting information, so, measurements by this channel were also showed. Emitted fluorescence from cells stained by SYTO 9 is mainly detected by FL1 (green fluorescence), and from cells stained with PI is mainly detected by FL3 (red fluorescence). Both FL1 and FL3 channels detected fluorescence at all sampling times (a graphic is always shown) however only the most intense fluorescence is of concern. The observed lowest intense fluorescence is due to surface binding of the dyes which show emitted low but detectable fluorescence (P Lebaron, Catala, Parthuisot, & Oce, 1998).

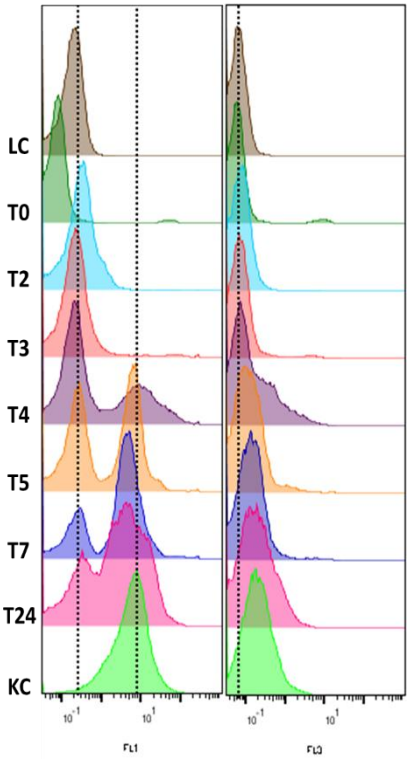


Figure 18. Viability assessment of *E. coli* cells in the presence of 30000 µg/mL of BMX-11, over time (h), using SYTOX® Green stain. FL1 (at left) displays viability changes assessed by emitted green fluorescence; FL3 (at right) displays viability changes assessed by emitted red fluorescence. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum peaks of live cells (LC) and killed cells (KC).

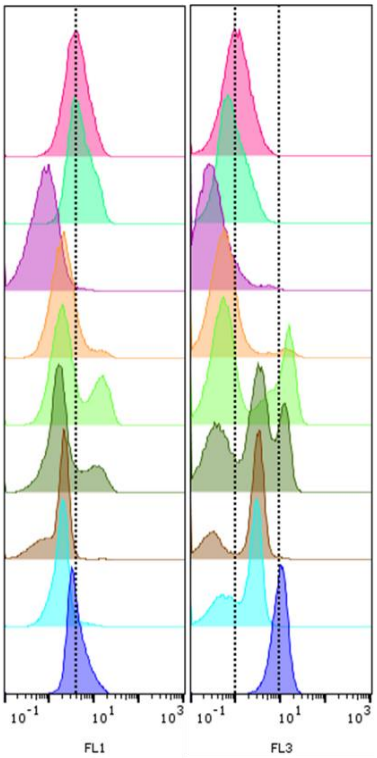


Figure 19. Viability assessment of *E. coli* cells in the presence of 30000 µg/mL of BMX-11, over time (h), using SYTO9® + PI stains. FL1 (at left) displays viability changes assessed by emitted green fluorescence; FL3 (at right) displays viability changes assessed by emitted red fluorescence. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum peaks of live cells (LC) and killed cells (KC).

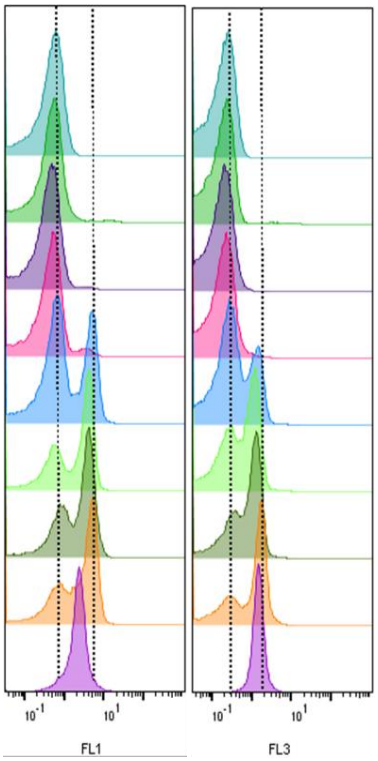


Figure 20. Viability assessment of *E. coli* cells in the presence of 30000 µg/mL of BMX-11, over time (h), using DiBAC$_4$(3) stain. FL1 (at left) displays viability changes assessed by emitted green fluorescence; FL3 (at right) displays viability changes assessed by emitted red fluorescence. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum peaks of live cells (LC) and killed cells (KC).

The inclusion of SYTOX® Green stain by cells allows viability assessment by membrane permeabilization (Decker, 2001). Taking this into account, and as was expected, FL1 analysis (fig. 18; left) at T0 showed only one population exhibiting low intensity of (green) fluorescence (mode of 0.09 FI), and representing 98 % of events, which means that, by comparing to LC and to KC pattern, represents cells with impermeable membranes so were viable ("SYTOX ® Green Nucleic Acid Stain," 2006). This viability pattern was observed until T3 and, up to T4, this viable subpopulation (modal value of 0.23 FI; 55.3 % of events) had decreased while other subpopulation with high fluorescence intensity (modal value of 9 FI; 34.6 % of events), representing cells with permeable membranes thus unviable/dead, had emerged. At T7, viable subpopulation showed 24.8 % of the events and unviable/dead subpopulation of cells showed 67.8 % of the events. At T24 only 23.5 % of events/cells were membrane impermeable thus viable, and most of cells (61.0 %) were membrane permeable (unviable/dead), however this last

had a modal peak of higher range of fluorescence intensity (from 1.6 to 16 FI). This variation in emitted fluorescence was maybe due to most of cells had already died or disintegrated perhaps due to increased turgor pressure, then remaining few cells to be stained and subsisting cellular remaining in the culture media being measured and hindering precise measurements (Ramani & Chaturvedi, 2000), which causes the results from T24 not reliable as the previous ones. This hypothesis is supported by performed microscopic observations (results not presented), in which was seen cells with strange morphologies and sizes (e.g. very thin and slender) and cellular remains at T24, which weren´t observed at previous sampling times. In other hand, the red emitted light by cells stained with SYTOX® Green, which is displayed by FL3, does not provide additional information.

The analysis of SYTO® 9+PI staining of cells also allows viability assessment by membrane permeabilization (Kort, Keijser, Caspers, Schuren, & Montijn, 2008). In figure 19, is displayed the response of *E. coli* cells treated with 30000 µg/mL of BMX-11, over time, which were stained with SYTO® 9+PI and analysed for emitted green (displayed at left, by FL1) and red (displayed at right, by FL3) fluorescence. The emission of intense green fluorescence by cells (seen in FL1) means that they were only stained by SYTO® 9 so, were membrane impermeable, thus viable, while the emission of intense red fluorescence (seen in FL3), means that propidium iodide (PI) had the capacity to enter and stain the nucleic acids so, were membrane permeable, thus unviable (Quintas & Tomás, 2014).

From FL1 detection of SYTO 9+PI staining of treated *E. coli* cells it was observed a similar fluorescence intensity pattern among LC, T0 and KC, which showed a modal value of 4 FI. At T2 it was displayed the lowest fluorescence intensity peak (0.9 FI). From T3, the fluorescence intensity (FI) slightly increased again, showing values of 2.2 FI which lasted until T24. In addition, from T3 to T5, a second subpopulation with higher fluorescence intensity of 15 FI had emerged. These detected changes in FI by FL1 simply means that cells differ a little in emitting fluorescence maybe due to differences in DNA content (Mascio, Alder, & Silverman, 2007), and are due to there existed a little reflex of what is detected/displayed by FL3. These changes don't have translation in viability, once SYTO 9 can stain nucleic acids both of viable and unviable cells ("SYTO ® Green-Fluorescent Nucleic Acid Stains," 2003).

In relation to the detection of red emitted light by FL3 channel, at T0 it was observed a low FI peak (of 0.7 FI) corresponding to 99.4 % of events and which was similar to that of LC. From T2 to T4, FL3 showed a subpopulation with slightly lower FI (0.27 to 0.6 FI) than in T0, and showed the appearance of a second subpopulation presenting the highest observed FI (of 16 FI) among all sampling times, representing 2.23 % of the events at T2, 7.68 at T3 and 37.6 % at T4. The major variation among the two subpopulations were observed between T3 and T4, at which the subpopulation of lower FI, representing the membrane impermeable so, viable cells, changed from being represented by 89.3 % of the events at T3, to be represented by 60.2 % at T4. At T5 a third subpopulation, with a medium range of 3.5 FI had emerged, representing 44 % of the events against 20.2 % represented in high FI subpopulation and 32.7 % in low FI subpopulation. At T7 and T24 the FI medium range subpopulation was the best represented (69 to 66 % of events), comparatively to the subpopulation of lowest FI, which comprised only 26 to 32 % of events, and comparatively to the subpopulation of highest FI, which had disappeared between T5 and T7 sampling times. Summing up, three subpopulations were observed:

one with lowest FI, similar to LC so, representing the cells with impermeable membranes, thus viable cells; a second subpopulation with the highest FI values, higher than that of the KC, T7 and T24, so by comparison, appear to be slightly membrane permeable/damaged and probably representing the injured cells; and a third subpopulation with medium FI values which, by comparison among subpopulations of T4, T5, T7, T24 and KC, representing the cells with membrane permeable so, unviable/dead cells.

$DiBAC_4(3)$ staining allows viability assessment by membrane polarization, with high fluorescence intensity indicating membrane depolarization/low membrane potential (Davey & Kell, 1996; Caron, Stephens, & Badley, 1998; Winkel *et al*., 2016). From FL1 channel (fig. 20; left) one population similar among LC, T0, T2 and T3 was observed, presenting a modal peak of 0.5-0.6 FI, which by comparison between each other and with KC, is knowing to represent the population of well-energized/polarized membranes, thus viable cells. From T3, a subpopulation with highest FI (4 to 5 FI) so, of non-polarized membranes, had emerged and had increased over time until T24. The greatest variation in polarization, with non-polarized membranes subpopulation reaching almost the same counts that polarized membranes subpopulation was occurred between T3 and T4. Between T3 and T4 polarized membranes subpopulation varied from representing 73.2 to 50.9 % of the events and non-polarized membranes subpopulation varied from representing 5.01 to 34.4 % of the events. At T24, the subpopulation with lowest FI, representing the well-energized/polarized membranes thus viable cells, represents 28.7 % of events and the subpopulation with highest FI, representing the non-energized/depolarized membranes thus unviable cells, represents 61.2 % of events. Through FL3 channel (fig. 20, right), variations of FI similar to those of FL1 channel were observed.

In pseudocolour density dot plots of fig. 21, fig. 22 and fig. 23 are showed over time changes in FL1 against FL3 of *E. coli* cells treated with 30000 µg/mL of BMX-11 and stained with SYTOX® Green, SYTO® 9+PI and $DiBAC_4(3)$, by this order.

From SYTOX® Green point of view (fig. 21) there is not much else to add to what was already described for fig. 18: at T0 it was observed one subpopulation in the fourth quadrant (Q4), representing viable cells; over time, the emitted fluorescence intensity had increased gradually, being displayed another subpopulation in the third quadrant (Q3) concerning the permeable membrane thus unviable/dead cells. At T0, there was 98.2 % of events (cells) in the 4^{th} quadrant (Q4) and 1.78 % in the 3^{rd} quadrant (Q3). At T5, the two populations are almost constituted by the same percentage of events (cells), presenting 51.0 % of events in Q4 and 49.0 % of events in Q3. At T7, the population in Q3 (membrane permeable cells) was more represented (67.4 %) than that of Q4 (32.7 %) and at T24, there was 40.9 % of events (cells) in Q4 and 59.2 % of events (cells) in the Q3, meaning that 59.2 % of cells were membrane permeable thus are unviable/dead.

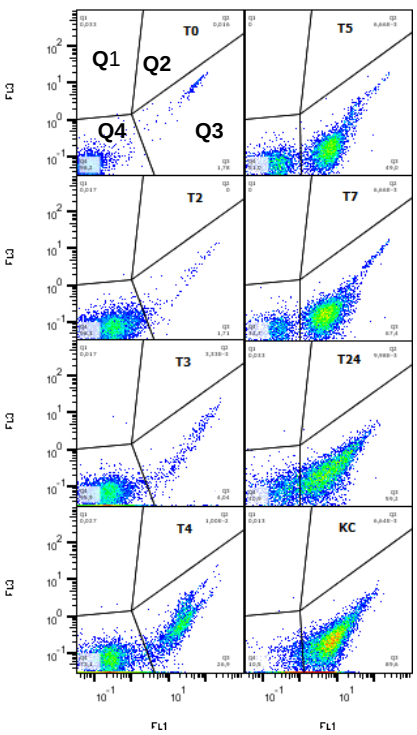


Figure 21. Pseudocolour density dot plots. Comparison of changes in *E. coli* cells in the presence of 30000 µg/mL of BMX-11 over time (h) stained with SYTOX® Green and recorded by FL1 and FL3. FL1 displays viability changes assessed from emitted green fluorescence; FL3 displays viability changes assessed from emitted red fluorescence. Tnumber: sampling time in hours; Q1, Q2, Q3 and Q4: quadrants; KC: reference control of killed cells.

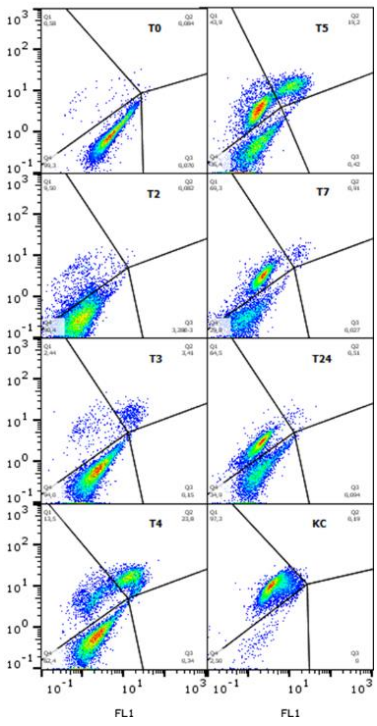


Figure 22. Pseudocolour density dot plots. Comparison of changes in *E. coli* cells in the presence of 30000 µg/mL of BMX-11 over time (h) stained with SYTO® 9 + PI and recorded by FL1 and FL3. FL1 displays viability changes assessed from emitted green fluorescence; FL3 displays viability changes assessed from emitted red fluorescence. Tnumber: sampling time in hours; Q1, Q2, Q3 and Q4: quadrants; KC: reference control of killed cells.

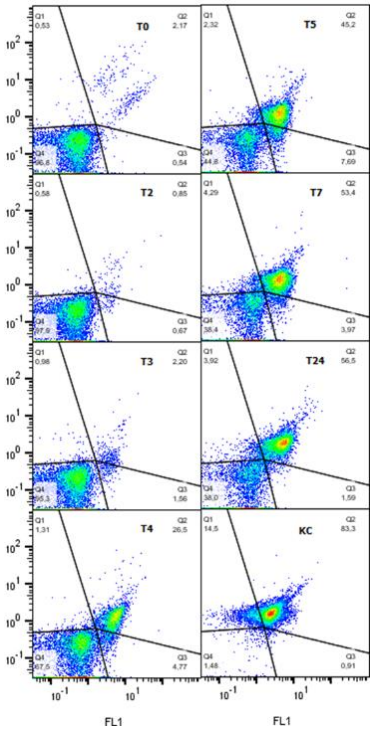


Figure 23. Pseudocolour density dot plots. Comparison of changes in *E. coli* cells in the presence of 30000 µg/mL of BMX-11 over time (h)stained with $DiBAC_4(3)$ and recorded by FL1 and FL3. FL1 displays viability changes assessed from emitted green fluorescence; FL3 displays viability changes assessed from emitted red fluorescence. Tnumber: sampling time in hours; Q1, Q2, Q3 and Q4: quadrants; KC: reference control of killed cells.

In the density dot plot graphics in fig. 22 is shown the emitted fluorescence intensity by cells loaded with SYTO® 9+PI and which was detected by FL1 (green fluorescence) in relation to FL3 (red fluorescence) channel. At T0 only one distinct subpopulation was observed, in the 4th quadrant (Q4), which represented 99.3 % of the events against 0.44 % of dispersed events in the 1st quadrant (Q1), so, events of Q4 are relating to the impermeable membranes subpopulation, thus viable cells. At T2, the number of events in Q1 increased a little (represented by 9.5 %) while at T3 had emerged a third subpopulation in Q2 exhibiting 3.41 % of the events against 2.44 % in Q1 and 94 % in Q4. Populations in the 1st and in the 2nd quadrant are in respect to cells with damaged membranes, since a red fluorescence intensity (seen from FL3) is higher than in the other quadrants. Between T4 and T5 it was possible to see a reduction of the population regarding to cells with integral/impermeable membranes thus viable (in Q4; from 62.4 to 36.4 % of events), in opposition to the increase of the subpopulation in Q1 (from 13.5 to 43.9 %) and to the stabilization (around 21 %) of the subpopulation in Q2, these last representing the cells with damaged/permeable membranes. At T7, a greatest increase of the subpopulation in Q1 was observed, exhibiting 69.3 % of the events, while at Q2 almost none (0.91 %) were observed and only 29.8 % were observed at Q4. At T24 it was observed 64.5 % of the events in

the 1st quadrant, 0.51 % in the 2nd and 34.9 % in the 4th. By comparing the evolution of the three subpopulations over time and with that of KC, it seems that: events that occurred in Q1 represent the cells with permeable membranes thus unviable/dead and which were not able to resume growth; events that occurred in Q2 represent the injured cells, meaning that they were unable to reproduce due to damage of cellular components and that may recover or die; and events that occurred in the Q4 represent viable cells which has integral and impermeable membranes (Sgorbati *et al*., 2001; Serio, Marsilio, & Suzzi, 2008).

The pseudocolour density dot plot graphics displaying emitted fluorescence from cells stained with $DiBAC_4(3)$ (fig. 23) shows, as those from cells stained with SYTOX Green, only one or two populations. At T0 and T2, the minority of events (1.51 % in average) are displayed in the 2nd quadrant (Q2) and the majority (97.35 % in average) of events in the 4th quadrant (Q4), therefore this last representing cells with polarized cell membranes and viable. Up to T3, the population in Q2 had increased over time, occurring the greatest variation in polarization between T3 and T4, with cells with polarized membranes displacing to being non-polarized (at T3 Q2=2.2 % and Q4=95.3 %; at T4 Q2=26.5 % and Q4=67.5 %). At T7, there were 53.4 % of events in Q2 and 38.4 % in Q4, and at T24 there were 56.5 % of events in Q2 and 38.0 % in Q4, what means that almost no cell polarized/energized membranes exist.

Although it seems that a considerable percentage of viable cells remain at T24, independently of the fluorophore in use, it should be taken in consideration that the fluorescence displayed in the graphics were emitted by cells that exist at the time of the sampling, which must be compared to the total counts of the control cultures at the same sampling time (analysis that are presented further ahead). As the total cell counts per mL had decreased between T7 and T24, (i.e. some cells may have disintegrated) and those from control didn't, having for example 30 % of viable cells, it actually means that the accurate values would be in fact a smaller percentage in comparison to the control.

3.5.3 Impact of BMX-11 on *C. marina* (30000 µg/mL)

In addition to the analysis presented below, in annexes A7.1, A7.2 and A7.3 are presented the calculated means and standard deviations of obtained FSC geometric mean and mode, and quadrants frequency of parent relating to each of the fluorophores used.

The number of cells/mL were assessed and test cells have little increased in number between T0 ($1.14x10^8$ cells/mL) and T1 ($2.93x10^8$ cells/mL) but have stagnated until T8 ($2.02x10^8$ cells/mL) and decreased between T8 and T24 ($1.07x10^8$ cells/mL). Control cells have increased its number from T0 ($1.41x10^8$ cells/mL) to T6 ($1.87x10^9$ cells/mL) and decreased from here to T24 ($4.34x10^8$ cells/mL). This data is presented in A9 annex and will be discussed and compared with data from other methods further ahead.

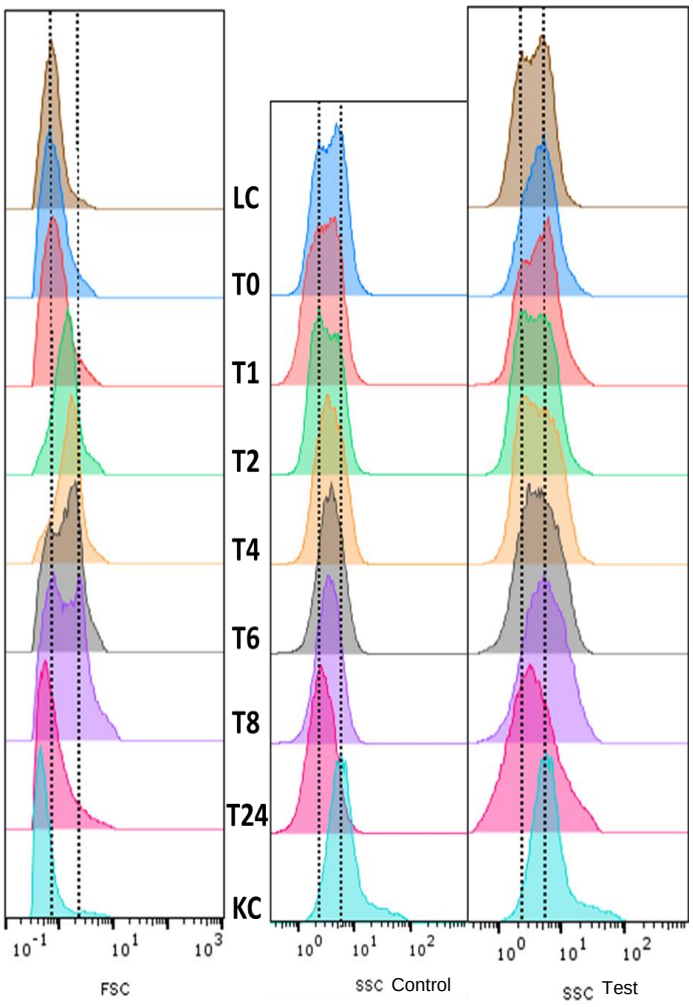


Figure 24. Changes in size (at left; FSC) and changes in complexity (at right; SSC) of *C. marina* cells over time. FSC: forward-scattered light, displays changes in size of cells treated with 30000 µg/mL of BMX-11. SSC: side-scattered light, displays changes in complexity of: untreated *C. marina cells* (control), at left and of treated *C. marina* cells with 30000 µg/mL of BMX-11 (test), at right. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum counts from the reference controls of live cells (LC) and killed cells (KC).

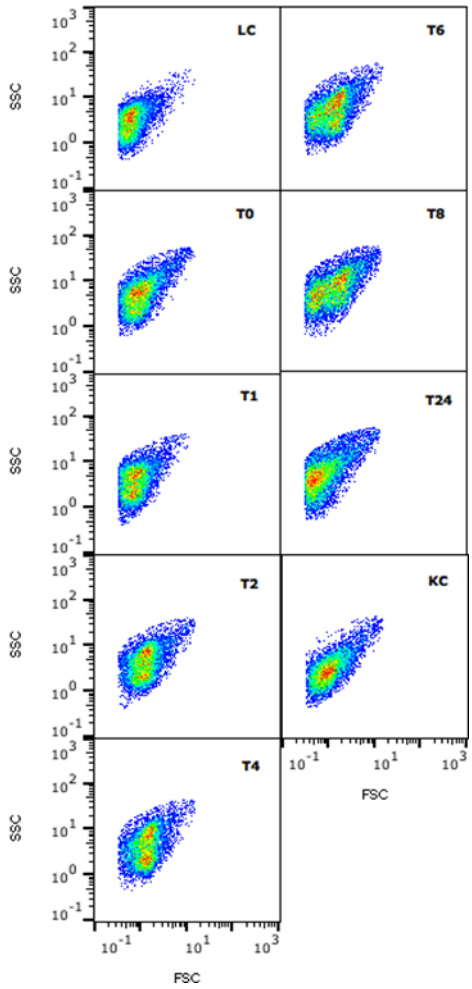


Figure 25. Changes in complexity against changes in size of *C. marina* cells in the presence of 30000 µg/mL of BMX-11 over time. FSC: forward-scattered light, displays changes in size of cells; SSC: side-scattered light, displays changes in complexity of cells. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells.

In fig. 24, are displayed FSC (forward-scattered light), and SSC (side-scattered light) measurements of *C. marina* cells, representing its variation of size and of complexity, respectively, over time (h) (Desjardins, Boulos, & Barbeau, 1999; Sgorbati, Brown, & Denis, 2001; Quintas & Tomás, 2014). In graphics of FSC and in graphics of SSC at the right ("SSC test" in fig. 24), are shown the measurements performed on *C. marina* cells treated with 30000 µg/mL of BMX-11. SSC graphics at the left ("SSC control" in fig. 24) display *C. marina* control cells variation over time. FSC analysis were also done on *C. marina* control cells (without BMX-11), however FSC measurements have not undergone many changes, so only data from LC and from KC are shown.

FCS of untreated cells (LC in fig. 24), of killed cells (KC in fig. 24) and of treated cells at T0, T1 and T24 showed similar peaks (varying from 0.5 of KC to 0.8). From T2 it was observed a displacement of the previous peak to the right (from 0.7 modal value to 1.5), i.e. to represent slightly larger cells. At T6 and at T8 were observed two proportional peaks: one of the same size of that from T0 (of 0.7 of mode) and other of the same size of the one which has appeared from T2 (of 2.0 of mode). At T24 the peak in respect to larger cells was not observed. BMX-11 seems to have effect on *C. marina* cells size, making them larger. At T24 only the peak related to smaller cells was seen, maybe because most of cells have already died and/or disintegrated (Ramani & Chaturvedi, 2000) and those that subsisted were not affected by BMX-11 by the same way or as fast.

In relation to complexity (seen in SSC), control sample showed two closely peaks with modal values of 2.4 and 4.8 at T0, T1 and T2. Between T4 and T8, control sample showed only one peak of 3.8 modal value but which covers the range of both the peak displayed at T24 (of little less complexity; mode of 2.6) and the peak displayed by KC (5.7 of mode; which correspond to one of the peaks of T0). The test samples showed two peaks or only one but more comprehensive from T0 to T6, with modal values ranging approximately from 1.5 to 11. At T8 showed only one peak with a modal value (of 5.5) similar to that of KC and at T24 only one peak very embracing (of 3.1 mode). Summing up, complexity from both control and test samples was very similar between T0 and T2, however it varied a little among samples between T4 and T24, at which control sample displayed a narrower peak than test sample. This data, although a bit dubious, suggests that BMX-11 can affect the complexity of *C. marina* cells.

In the fig. 25 is showed the dispersion in size in respect to the variation in complexity of *C. marina* cells treated with 30000 µg/mL of BMX-11 over time. It is possible to observe most cells belonging to a single population at T0, followed by the emergence of a subpopulation with the same size and less complexity, and later, at T6 and T8 the emergence of other subpopulation(s) a bit bigger. These changes in size and complexity over time may be due to already death and/or injured cells and cells that are still alive but affected, coexisting (Renggli, Keck, & Jenal, 2013; Saint-ruf *et al*., 2016).

As viability assessment by changes in morphology can be a crude measurement (Quintas & Tomás, 2014), membrane permeability and polarization were also studied. In fig. 26, fig. 27 and fig. 28 is displayed the fluorescence detected by FL1 (green fluorescence, at the left of each figure) and FL3 (red fluorescence, at the right of each figure), which were emitted by *C. marina* cells treated with BMX-11

and stained with SYTOX® Green, SYTO® 9+PI and $DiBAC_4(3)$. Emitted fluorescence from cells both stained by SYTOX® Green and by $DiBAC_4(3)$ is mainly detected by FL1 channel (that displays the emitted green fluorescence), however its comparison with the FL3 channel (that displays the emitted red fluorescence) provided interesting information, so, measurements by this channel are also shown.

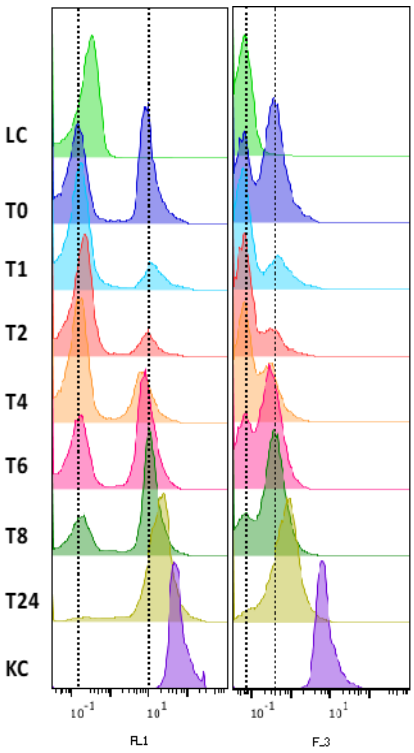


Figure 26. Viability assessment of *C. marina* cells in the presence of 30000 µg/mL of BMX-11, over time (h), using SYTOX® Green stain. FL1 (at left) displays viability changes assessed by emitted green fluorescence; FL3 (at right) displays viability changes assessed by emitted red fluorescence. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum peaks of live cells (LC) and killed cells (KC).

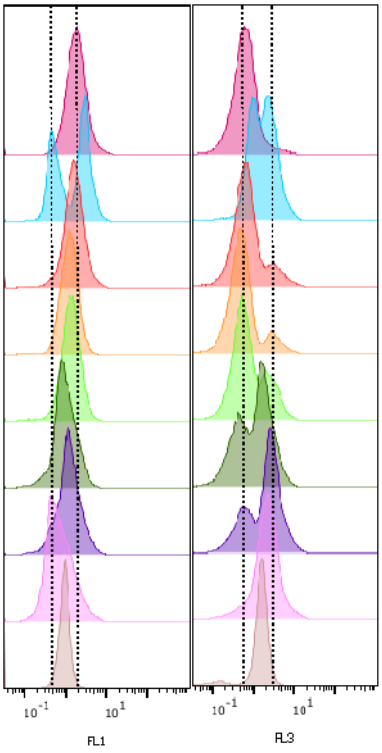


Figure 27. Viability assessment of *C. marina* cells in the presence of 30000 µg/mL of BMX-11, over time (h), using SYTO® 9+PI stains. FL1 (at left) displays viability changes assessed by emitted green fluorescence; FL3 (at right) displays viability changes assessed by emitted red fluorescence. Tnumber: sampling time in hours; LC: reference control of live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum peaks of live cells (LC) and of killed cells (KC).

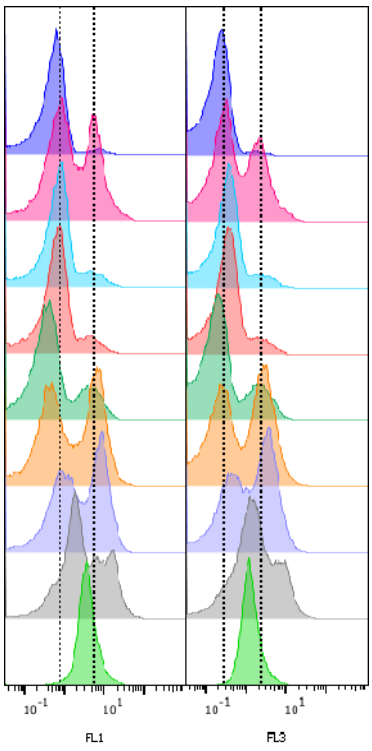


Figure 28. Viability assessment of *C. marina* cells in the presence of 30000 µg/mL of BMX-11, over time (h), using $DiBAC_4(3)$ stain. FL1 (at left) displays viability changes assessed by emitted green fluorescence; FL3 (at right) displays viability changes assessed by emitted red fluorescence. Tnumber: sampling time in hours; LC: reference control live cells; KC: reference control of killed cells. Vertical dashed lines evidence maximum peaks of live cells (LC) and of killed cells (KC).

The inclusion of SYTOX® Green stain by cells allows viability assessment by membrane permeabilization (Decker, 2001). From FL1 channel measurements (fig. 26; left), two distinct populations at T0 were seen, one of 0.15 FI modal value, representing 43.7 % of events and other of 8 FI modal value, representing 41.2 %. This data reveals that, opposite to what was supposed, at the beginning of the experiment there were more unviable (subpopulation at right) than viable cells (subpopulation at left). This suggests a rapid primary BMX-11 action against *C. marina* (maybe due to the used high BMX-11 concentration) which may then be attenuated by the bacterial stress response.

Further studies are needed since the most similar case found in literature is the one in Weide *et al.* (2017), where both Colistin-susceptible and resistant *P. aeruginosa and* Colistin-susceptible *K. pneumoniae* were killed by the concentration of 64 mg/L of a multimeric antimicrobial peptide active against Gram-negative bacteria, after just 1 hour of exposure. However, opposing to that study, *C. marina* samples showed restored viability after 1 h, with 65.7 % of cells having impermeable membrane thus were viable and with 15 % of cells having permeabilized membranes thus were unviable/dead. From 1 h, viability decreased again over time, with the viable subpopulation gradually becoming the non-viable subpopulation. Between T4 and T6 it was seen the greatest increase in membrane permeability, going from a non-permeable majority (56.5 % of impermeable at T4) to a permeable majority (51.2 % of permeable at T6), and later, at T24, almost all cells (88.7 %) were membrane permeable, thus non-viable. From FL3 channel measurements (fig. 26; right), similar responses were obtained.

The analysis of SYTO® 9+PI staining of cells also allows viability assessment by membrane permeabilization (Kort, Keijser, Caspers, Schuren, & Montijn, 2008). FL1 channel (fig. 27; left) displays emitted (green) fluorescence by SYTO® 9 stained cells, i.e. emitted fluorescence from cells both viable or unviable. From SYTO® 9 staining, as expected, no big changes in fluorescence were seen over time with exception for T0, at which 2 distinct populations were seen, one (at left) of modal value of 0.5 FI, representing 43.3 % of events, and other (at right) of modal value of 3 FI and representing 56.4 % of events. These two subpopulations were seen perhaps due to heterogeneity in terms of DNA content (Mascio, Alder, & Silverman, 2007). FL3 channel (fig. 27; right) displays emitted red fluorescence by PI stained cells, with the intense fluorescence representing the unviable cells, once PI stains cells with compromised or damaged membrane, stopping the fluorescence emission by SYTO® 9 (Quintas & Tomás, 2014). From FL3 analysis at T0, two peaks were seen, one of 1 FI of modal value and representing 46.2 % of events, and other of 2.5 FI of modal value and representing 53.7 % of events. This last peak appears to represent membrane permeable cells, which was not expected at T0, though it is in accordance with data obtained from SYTOX® Green and from $DiBAC_4(3)$ at T0. At T1 was observed a replacement of the expected viability pattern, displaying one subpopulation of 0.65 FI of modal value representing 85 % of events and a smaller subpopulation of 3 FI of modal value, representing only 13.5 % of events, thus membrane permeable subpopulation of cells. From here (T1), there has been a gradual change in membrane permeability over time, with the viable population becoming the non-viable population. Between T4 and T6 it was seen the greatest variation in membrane permeability, with the membrane impermeable subpopulation varying from representing 73.4 % of events at T4 to represent 38.6 % at T6, and with the membrane permeable subpopulation varying from representing 22.7 % at T4 to 60.6 % at T6. At T24, there was just about 9 % of membrane impermeable cells, thus viable cells (without damaged membrane).

The inclusion of $DiBAC_4(3)$ stain by cells allows viability assessment by membrane polarization, with high fluorescence intensity indicating membrane depolarization/low membrane potential (Davey & Kell, 1996; Caron, Stephens, & Badley, 1998; Winkel *et al*., 2016). From FL1 channel (fig. 28; left) two subpopulations at T0 were seen, one of 0.85 FI of modal value, representing 50 % of events, and other,

of 5.8 FI of modal value, representing 32.2 %. By comparison with KC (and with later sampling times), it was noticed that the best represented subpopulation (at left; 50 %) represents energized membranes thus viable cells and that the worse represented subpopulation (at right; 32.2 %) represents non-energized membranes thus unviable cells. This lack of viability was not expected, still, data were according to that obtained from SYTOX® Green and from SYTO® 9+PI. At T1 the expected cell viability pattern was observed, displaying much more events in respect to cells with well-energized membranes (69.3 %) than in respect to cells with non-energized membranes (10.2 %). From here, there has been a gradual change in polarization over time, with the subpopulation of cells with polarized membranes displacing to the depolarized membranes subpopulation. Between T4 and T6 it was seen the greatest variation in polarization, with the non-polarized subpopulation changing from representing 17.1 % at T4, to represent 42.4 % at T6, reaching more than half of events, however not showing a variation as great as the shown when using SYTOX® Green and/or SYTO® 9+PI. At T6 and at T8 were observed similar peaks and at T24 it was observed two peaks, one of 1.9 FI of modal value and other of approximately 12 FI of modal value, which were not exactly in the same gate as peaks of previous sampling times, however seem that represent, together (58.3 % + 36.6 %), the subpopulation with non-energized membranes, thus unviable. From FL3 channel (fig. 28; right), similar responses were obtained.

In fig. 29, fig. 30 and fig. 31 are showed over time changes in FL1 against FL3 of *C. marina* cultures at the concentration of 30000 µg/mL of BMX-11 stained with SYTOX® Green, SYTO® 9+PI and $DiBAC_4(3)$, by this order.

From the use of SYTOX® Green (fig. 29), similar results were established as described for fig. 26, being displayed 2 subpopulations soon from the T0, with the unviable one, seen in the third quadrant (Q3), representing 40.5 %, and with the viable one, seen in the fourth quadrant (Q4), representing 59.3 % of the events. Most of viability was restored at T1 (unviable/dead (Q3): 14.6 %; viable (Q4): 85.3 %), time from which non-permeable events of (Q4) begun to displace to Q3 (representing membrane permeable subpopulation). The maximum of membrane permeability counts (seen in Q3; representing 90 % of events) happened at T24.

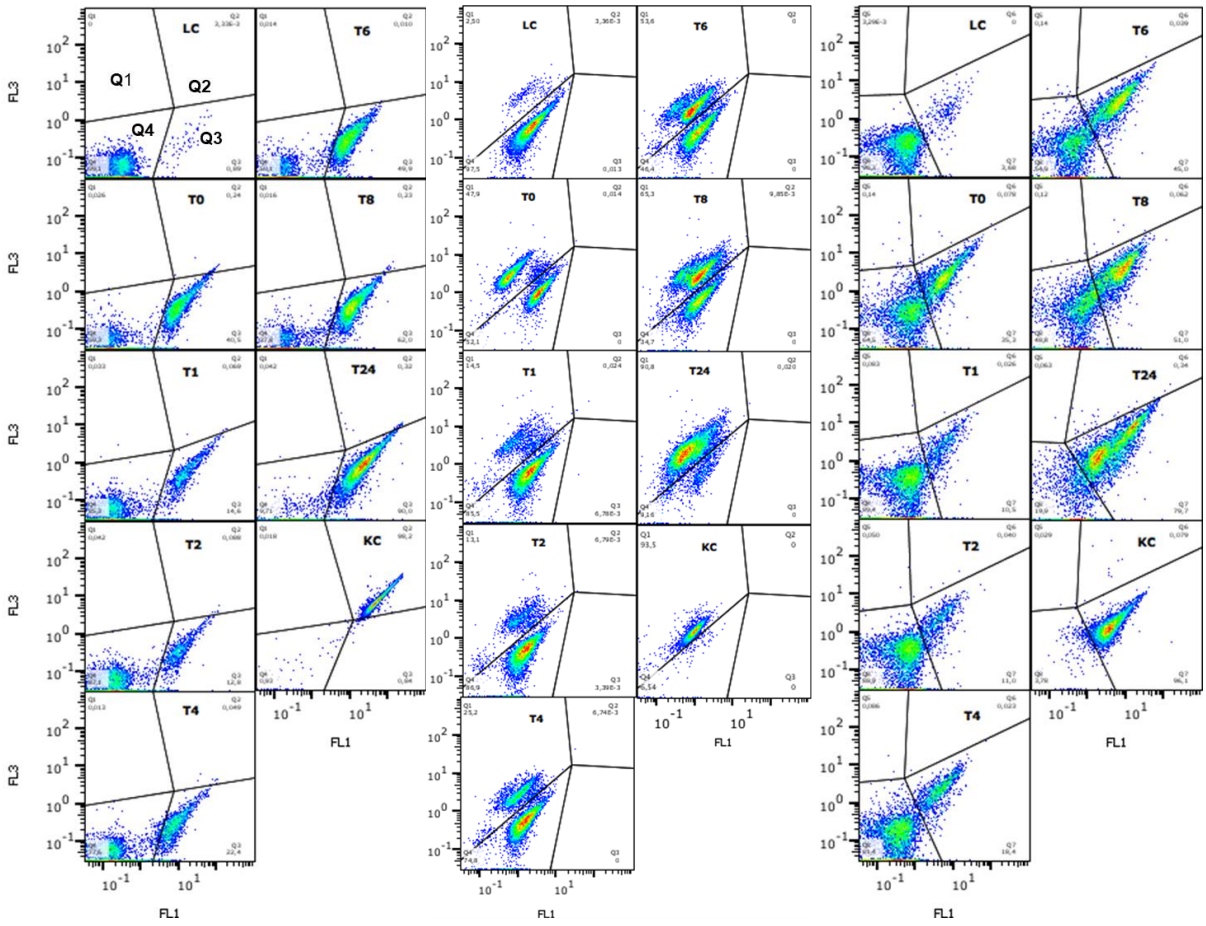


Figure 29. Pseudocolour density dot plots. Comparison of changes in *C. marina* cells in the presence of 30000 µg/mL of BMX-11 over time (h) stained with SYTOX® Green and recorded by FL1 and FL3. FL1 displays viability changes assessed from emitted green fluorescence; FL3 displays viability changes assessed from emitted red fluorescence. Tnumber: sampling time in hours; Q1, Q2, Q3 and Q4: quadrants; LC: reference control of live cells; KC: reference control of killed cells.

Figure 30. Pseudocolour density dot plots. Comparison of changes in *C. marina* cells in the presence of 30000 µg/mL of BMX-11 over time (h) stained with SYTO® 9+PI and recorded by FL1 and FL3. FL1 displays viability changes assessed from emitted green fluorescence; FL3 displays viability changes assessed from emitted red fluorescence. Tnumber: sampling time in hours; Q1, Q2, Q3 and Q4: quadrants; LC: reference control of live cells; KC: reference control of killed cells.

Figure 31. Pseudocolour density dot plots. Comparison of changes in *C. marina* cells in the presence of 30000 µg/mL of BMX-11 over time (h) stained with DiBAC$_4$(3) and recorded by FL1 and FL3. FL1 displays viability changes assessed from emitted green fluorescence; FL3 displays viability changes assessed from emitted red fluorescence. Tnumber: sampling time in hours; Q1, Q2, Q3 and Q4: quadrants; LC: reference control of live cells; KC: reference control of killed cells.

From the 2D dot plot fig. 30, of FL1 in relation to FL3, that shows emitted fluorescence by cells loaded with SYTO® 9 plus PI, at T0 it is possible to notice three subpopulations: one consisting in 52.1 % of events at the fourth quadrant (Q4), representing non-permeable membrane thus viable cells, and other two subpopulations in the first quadrant (Q1), one of them quite distinct and the other not so, representing non-viable and/or injured cells. At T1, the subpopulation of non-permeable membrane cells includes most of cells (Q4=85.5 %), and the other two subpopulations almost disappeared (Q1=14.5 %). From T2, the subpopulation in respect to cells with damaged membranes reappears and has increased over time as well as the subpopulation of membrane permeable cells. The three

subpopulations were better distinguished at T6 and at T8. At T6, Q1 represented 53.6 % of events and Q4 represented 46.4 % and at T8, Q1 represented 65.3 % and Q4, 34.7 %. At T24 almost all cells (90.8 %) had permeable membranes (seen in Q1), so were injured or non-viable. Flow cytometry method is particularly useful comparing to standard culture plating methods, as (the first) allows to clearly discriminate between subpopulations of cells under stress such as ‘‘death cells”, that are not able to resume growth or the ones unable of reproduce due to damage of cellular components, called ‘‘injured cells”, that may recover or die (Sgorbati *et al*., 2001; Serio, Marsilio, & Suzzi, 2008).

The density dot plot graphics displaying emitted fluorescence from cells stained with $DiBAC_4(3)$ (fig. 31) also shows 2 subpopulations at T0, one in the third quadrant (Q3) representing 35.3 % of events and other in the fourth quadrant (Q4) representing 64.5 %. In Q3 are represented non-energized/non-polarized membranes subpopulation and in Q4 the energized/polarized membranes subpopulation. From T1 the number of energized cells (89.4 %) are much higher than the non-energized cells (10.5 %), being seen from here (T1) an increase in the subpopulation of cells with non-polarized membranes over time. Between T4 and T6 were observed the greatest variation in the polarity of membranes, being observed 81.4 % in Q4 and 18.4 % in Q3 at T4 and 54.9 % in Q4 and 45 % in Q3 at T6. At T8 it was observed 48.8 % in Q4 and 51 % in Q3 and at T24 almost none polarized cells exist (only 19.9 % in Q4). Interestingly, as observed from SYTO® 9+PI staining, it appears to exist, at T24, one subpopulation in respect to the injured cells, since two subpopulations were seen in Q3 where by comparison to KC, is known to appear the subpopulation of cells with non-energized membrane.

Although it seems that a considerable percentage of viable cells remain at T24, independently of the fluorophore in use, it should be taken in consideration that the fluorescence displayed in the graphics were emitted by cells that exist at the time of the sampling, which must be compared to the total counts of the control cultures at the same sampling time (analysis that are presented further ahead). As the total cell counts per mL had decreased between T6 and T24, (i.e. some cells may have disintegrated) and those from control didn’t, having for example 30 % of viable cells, it actually means that the accurate values would be in fact a smaller percentage in comparison to the control.

Although specific differentiation between stages of viability remains one of the greatest challenges in microbiology, as described previously by others - viable and culturable, viable but non-culturable (VBNC) dormant, non-viable and pre-lytic, and avital dead bacteria (Quintas & Tomás, 2014) - BMX-11 showed, with to doubt, having bactericidal effect against some bacteria. The staining of both *C. marina* and *E. coli* cells with SYTOX® Green and with SYTO® 9+PI, revealed BMX-11 to have bactericidal effect by at least cellular membrane permeabilization against both strains, and staining with $DiBAC_4(3)$ also showed bactericidal effect, by altering membrane polarization (although perhaps also by other ways not tested) against both strains in test. Otherwise, like other antimicrobials, BMX-11 may be exerting effect on bacterial membrane composition, hydrophobicity, charge, asymmetry and transmembrane potential (Yeaman & Yount, 2003; Yumoto *et al*., 2004). Observed BMX-11 capacity to affect cell

membrane, leading to its disruption and sometimes to the cell disintegration, leads to the hypothesis that maybe BMX-11 acts by interrupting cell wall peptidoglycan synthesis (Kaufman, 2011) (since it can result in inhibiting cell division, and subsequently to long filamentous forms, or to the formation of deficient/weak cell wall) (Mcdermott *et al*., 2003; Kaufman, 2011). In other hand, BMX-11 may also be affecting other bacterial metabolic processes as other antimicrobials do, like protein synthesis, (Mcdermott, Walker, & White, 2003); Hancock, 2005; Kaufman, 2011) nucleic acid synthesis (Mcdermott *et al*., 2003a; Mcdermott *et al*., 2003b; Hancock, 2005; Kaufman, 2011), and inhibition of other metabolic processes (e.g.: Folate synthesis (Mcdermott, Walker, & White, 2003; Kaufman, 2011) however further studies are needed.

3.6 Viabilities and Optical densities at 600 nm measurements at flow cytometry sampling times

The reliability of fluorescent techniques remains controversy when assessing different bacterial states (Quintas & Tomás, 2014). For this reason, as has been stated, measurements of bacterial viability by flow cytometry should, and were, complemented and compared with microbiological techniques like plate culture counting and OD measurements (Davey & Kell, 1996; Quintas & Tomás, 2014).

3.6.1 *E. coli* – Viability and OD_{600nm} measurements at flow cytometry sampling times and its comparison with flow cytometric results

A comparison between plate counts and OD values was done (results not shown), both increasing over time when from control cultures and decreasing over time when from test cultures. Also, OD values were transformed in CFU/mL (results not shown) by the equivalence: 1 OD=$8x10^8$ cells/mL (Myers, Curtis, & Curtis, 2013), however it seems that from a certain high number of OD, it cannot keep up with the plate counts (viability) data. This means that, when in respect to test samples, it was obtained a higher number of total cells (based on OD measurements) comparing with of viable cells (based on plate counts), but, in respect to control samples (that can reach high OD values), it was observed a number of total cells lower than the number of viable cells, what does not make sense. For that reason, OD was not considered in the following comparative analysis.

OD measurements, CFU/mL counts from plate culture, cell/mL counts from flow cytometry and comparative data are shown in the A8 annex.

Figure 32 shows a comparison between cells/mL total and viable counts obtained from flow cytometry, and viable counts (CFU/mL) obtained from plate culturing. Displayed viability values based on flow cytometry comprise the average viability using each of the three stains.

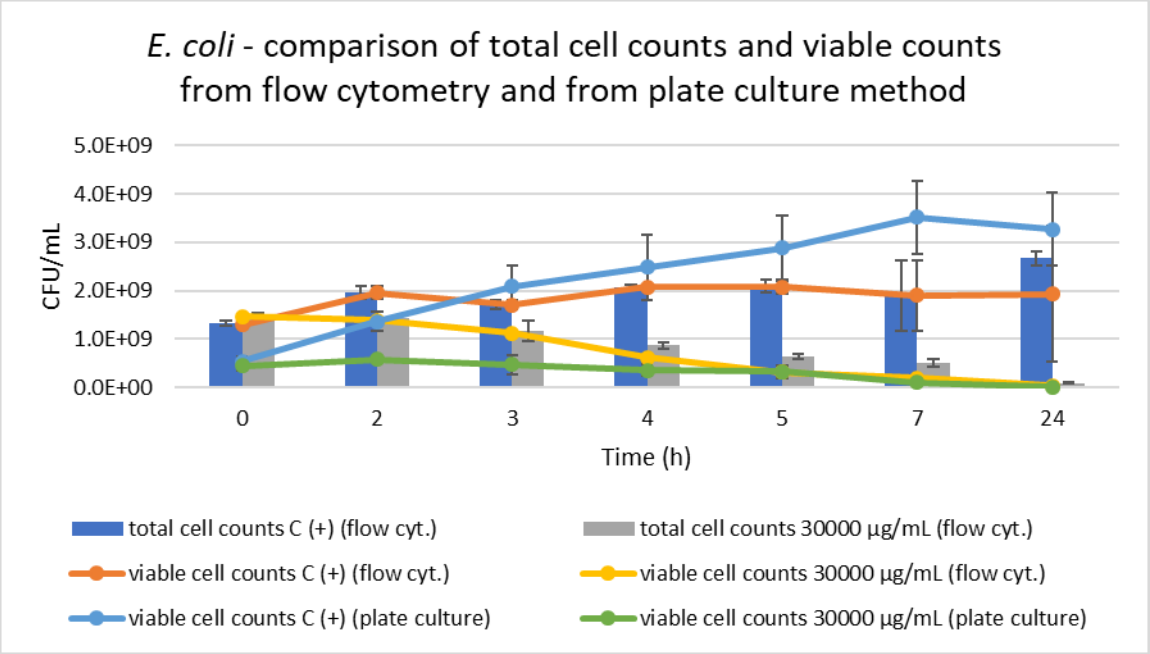


Figure 32. *E. coli*: comparison between total and viable cell counts from flow cytometry, and viable CFU counts from plate culture method, in CFU/mL against time (h), of control cultures and cultures at 30000µg/mL of BMX-11. Error bars represent standard deviations.

Total cell counts from control cultures (dark blue in fig. 32) gradually increased between 0 h and 24 h while total cell counts from test cultures (grey in fig. 32) gradually decreased during the same period.

In respect to viability, test cultures both analysed by flow cytometry (yellow in fig. 32) and by plate culturing (green in fig. 32) showed a reduction in viability over time, however, flow cytometry has displayed higher viability values than plate culture method did.

In respect to control cultures viability, from cytometric analysis (orange in fig. 32) it was observed that almost all of total cells (dark blue in fig. 32) were viable (orange in fig. 32), since the two respective bars exhibit similar sizes.

In respect to control cultures viability analysed by plate culture (light blue in fig. 32) it was observed that the experiment begun with few CFU/mL, however that number had gradually increased over time until 8 h and between 8 and 24 h it decreased a little. Contrary of the expected, viable cells from control sample had displayed the highest CFU/mL counts among all obtained. It was expected that control samples have shown more CFU/mL than test samples, however, the total CFU/mL counts should be higher than viable CFU/mL counts.

Oftentimes literature refers that fluorescent techniques have the tendency to artificially overestimate the numbers of non-vital bacteria when comparing to plate culture methods (Desjardins *et al.*, 1999; Quintas & Tomás, 2014). Viability results of control cultures (light blue and orange bars in fig. 32) from 3 h, using flow cytometry and plate culture, showed to be in concordance with that reference. Viability results obtained from test cultures (yellow and green bars in fig. 32) disagree with that reference, with

viable cells analysed by flow cytometry always displaying higher cells/mL counts than viable cells analysed by plate culture.

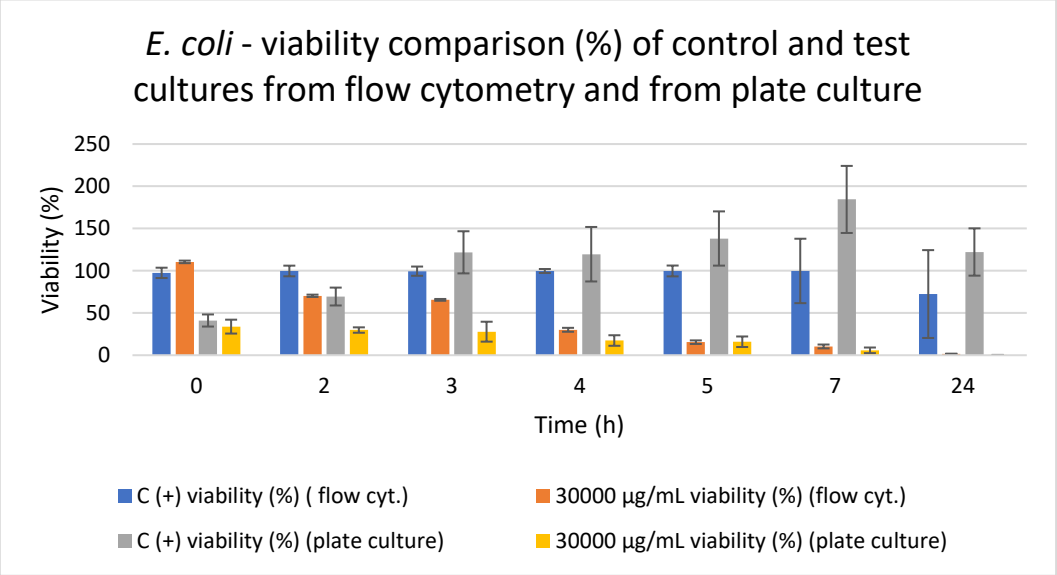


Figure 33. *E. coli*: comparison between obtained viabilities from flow cytometry and from plate culture method, in percentages, of control cultures and cultures at 30000 µg/mL of BMX-11, relatively to the control culture total counts. Error bars represent standard deviations.

In fig. 33, a comparison between *E. coli* viability data obtained using flow cytometry and using plate culture is shown. In fig. 33 can be observed that at most of sampling times, viability percentages from control cultures by plate culture method displayed higher values than viability percentages from flow cytometric method. The pattern has repeated from test cultures at least at 0, 1, 4, 8 and 24 h. Anyway, clear differences were observed between control and test cultures viability, with these last, presenting a reduction in viability soon as BMX-11 addition.

3.6.2 C. marina – Viability and OD_{600nm} measurements at flow cytometry sampling times and its comparison with flow cytometric results

As opposed to *E. coli* plating's and previous *C. marina* platings done using glass spheres, the *C. marina* plating's at flow cytometry time-points were done using a spreader with the objective of solve counting problems owing to agglomerates accumulation on the border of the petri dishes. This experimental method showed to be more efficient than the one using glass spheres, without growing colonies on the petri dishes borders, though colony count remained not reliable as desired due to the formation of uncountable agglomerates.

CFU/mL counts from plate culture method were compared with OD values (results not shown), and both increased over time if from control cultures (at first sampling times) and decreased/stabilized over time if from test cultures. Also, OD values were transformed in CFU/mL (data not shown) by the equivalence 1 OD=$8x10^8$ cells/mL (Myers, Curtis, & Curtis, 2013), and were compared with colony

counts. Related values were only showed at the first sampling times. For this reason and because *C. marina* OD values are not as reliable as desired, OD was not considered in the following analysis (fig. 34). OD measurements, CFU/mL counts from plate culture, cell/mL counts from flow cytometry and comparative data are shown in the A9 annex.

Figure 34 shows a comparison between cells/mL total and viable counts obtained from flow cytometry, and viable counts (CFU/mL) obtained from plate culturing. Displayed viability values based on flow cytometry comprise the average viability using each of the three stains.

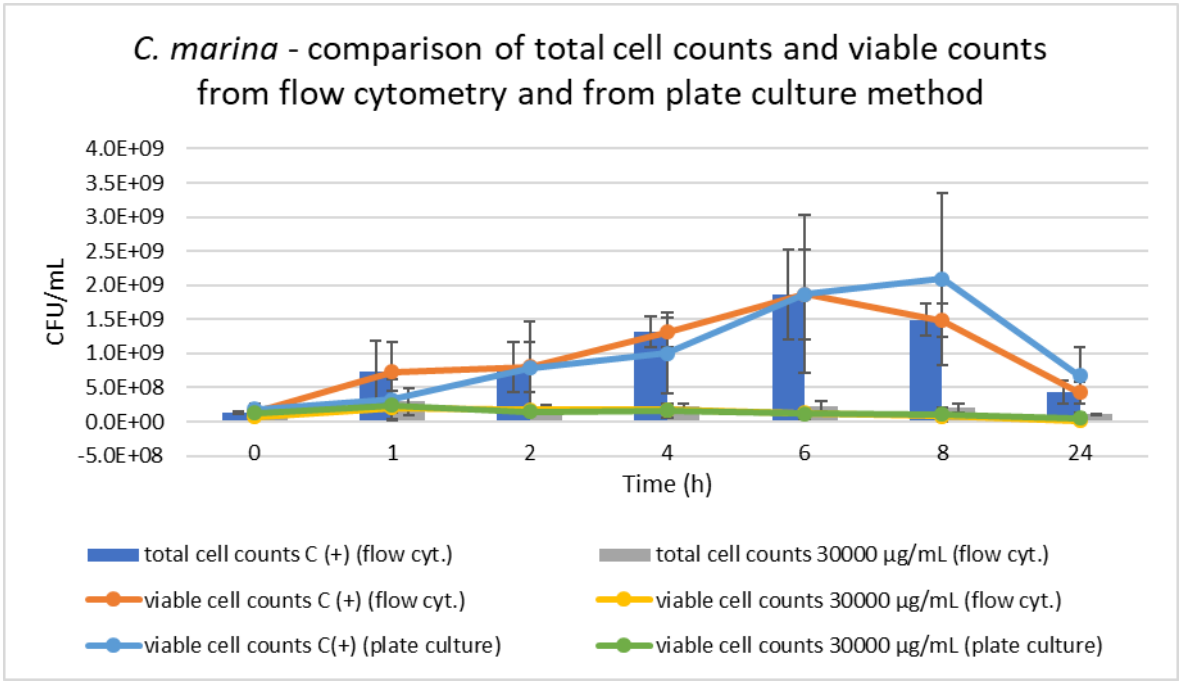


Figure 34. *C. marina*: comparison between total and viable cell counts from flow cytometry, with viable CFU counts from plate culture method, in CFU/mL against time (h), of control cultures and cultures at 30000µg/mL of BMX-11. Error bars represent standard deviations.

Total cell counts from control cultures (dark blue in fig. 34) gradually increased between 0 h and 6 h, and afterward decreased until T24, maybe due to the entrance in stationary phase, where cells aren't actively replicating, maybe due to high cell density (Mascio *et al*., 2007; Brauner, Fridman, Gefen, & Balaban, 2016) depletion of nutrients, oxygen (Faleiro M.L., 2011), changes in the pH of the media (Grant & Hung, 2014), and accumulation of toxic products (Faleiro M.L., 2011). Total cell counts from test cultures (grey in fig. 34) showed low CFU/mL at all sampling times.

In respect to test cultures viability both analysed by flow cytometry (yellow in fig. 34) and by plate culturing (green in fig. 34) showed low viable counts at all sampling times.

In respect to control cultures viability, flow cytometric analysis (orange in fig. 34) showed increasing viable CFU/mL counts from 0 to 6 h and afterward decreasing until 24 h. The control cultures viability analysed by plate culture (light blue bars in fig. 34) showed lower CFU/mL counts than those obtained

using flow cytometry from 0 to 6 hours, but afterward, it has continued to gradually increase until 8 h and decrease from 8 h.

In addition, it was observed that almost all total cells (dark blue in fig. 34) were viable (orange in fig. 34), since the two respective bars exhibit similar sizes.

In opposition to the results obtained for *E. coli* cells, all viable cell counts seem to be lower than total cell counts as expected.

Due to CFU/mL counts of *C. marina* are not as reliable as desired, the question about the tendency of fluorescent techniques to artificially overestimate the numbers of non-vital bacteria when comparing to plate culture methods (Desjardins *et al.*, 1999; Quintas & Tomás, 2014) stays open.

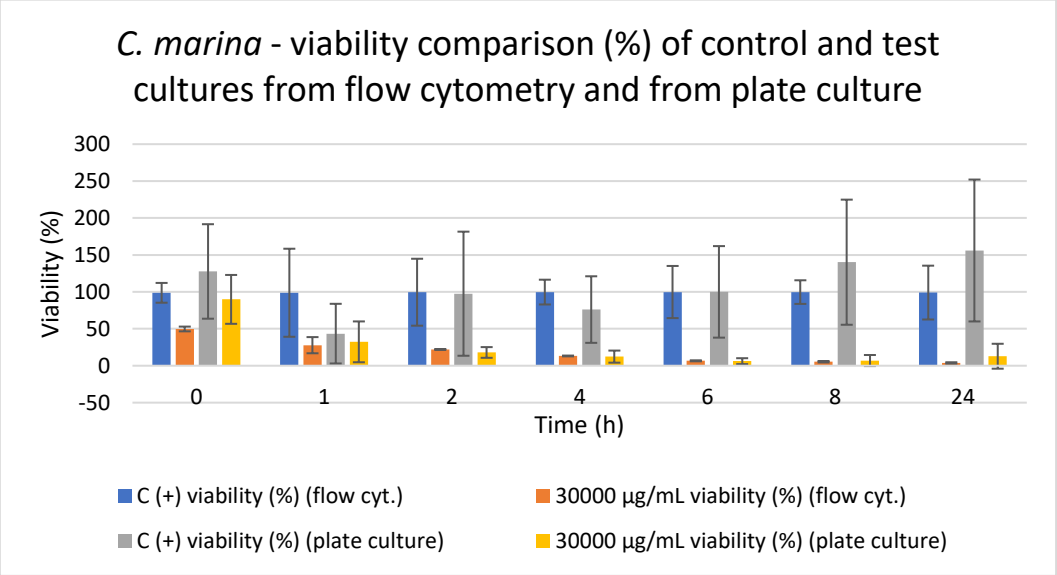


Figure 35. *C. marina*: comparison between obtained viabilities from flow cytometry and from plate culture method, in percentages, of control cultures and cultures at 30000 µg/mL of BMX-11, relatively to the control culture total counts. Error bars represent standard deviations.

In fig. 35, a comparison between *C. marina* viability data obtained using flow cytometry and using plate culture is shown.

In respect to viability of control samples, it was observed that cultures analysed by flow cytometry (dark blue bars in fig. 35) always showed above 98 % of viability, while cultures analysed by plate culture method (grey bars in fig. 35) have varied more among sampling times, showing a minimum of 43.4 % of viability at T1. Results suggests that viability counts from plate culture can have more plasticity to the results than flow cytometric analysis.

Test cultures analysed with flow cytometry (orange bars in fig. 35) ranged from 45 to 4 % of viability and test cultures from plating count method (yellow in fig. 35) ranged from 89 to 6.2 %, relatively to control cultures.

Anyway, clear differences were observed between control and test cultures viability, with these last, presenting a reduction in viability soon as BMX-11 addition.

4. Conclusion

BMX-11 is a potent antibacterial against a large set of bacterial strains. This thesis was carried out in order to better understand how this antimicrobial activity unravels and to identify its cellular targets. In this work, BMX-11 was found to be capable of inhibiting growth of stationary phase *E. coli* cultures although with less efficacy than it inhibits exponential phase *E. coli* cultures. Ciprofloxacin, which blocks DNA replication, and nafcillin and vancomycin, which perturb cell wall synthesis, are examples of antibiotics which have not bactericidal activity against stationary-phase *S. aureus* (Mascio *et al*., 2007). As opposite, gentamicin for example is bactericidal against stationary-phase cells, by disrupting the cell membrane. Daptomycin is a lipopeptide antibiotic which also has bactericidal activity against stationary phase bacteria, although its mode of action is not fully understood (Mascio *et al*., 2007). As stressed cells generally adopt a resting state in which they do not grow, but after which they can eventually resume it (or die), (Widmer *et al*., 2000; Mascio *et al*., 2007; Pletnev *et al*., 2015; Brauner *et al*., 2016) and as much antibiotics only have effect on actively growing bacteria (Mascio *et al*., 2007), it is of great importance to discover antibacterials capable to act on different growth phases, namely on growth arrested cultures/stationary growth phase cultures, a condition that generally provides increased resistance (Mascio *et al*., 2007; Kaufman, 2011; Brauner *et al*., 2016). Studies of BMX-11 bactericidal action against stationary phase cultures should be continued, including by plate culture, in the process of evaluating its potential for health related applications, as has been studied for several antibiotics in many medical papers as in (Widmer *et al*., 2000; Dajcs *et al*., 2001; Mascio *et al*., 2007; Borges *et al*., 2013; Pletnev *et al*., 2015).

As a result from MIC and MBC determination methods, BMX-11 showed to be capable of inhibit growth and to be bactericidal (*i.e.* was capable to kill 99.9 % of bacteria comparing to the final inoculum (Barry *et al*., 1999) against strains of *C. marina*, *E. coli*, *E. faecalis*, *L. monocytogenes*, *S. aureus* and *V. vulnificus,* showing a strong impact on a very diverse panel of bacterial species. However, two-fold microdilution method used in this analysis, has the disadvantage of producing large differences when the tested compound is at high dilutions (Barry *et al*., 1999), which can turn interpretation of data somehow difficult. To determine with more precision the MIC and MBC values, other intermediate concentrations in the interval of concentrations here determined for MIC and MBC should be tested.

From time-kill method analysis, we can conclude that BMX-11 has a concentration-dependent bactericidal activity against all strains in test (Mascio, Alder, & Silverman, 2007). Taking this into account, and although there is no clear definition of the terms “broad-spectrum” and “narrow-spectrum” antibiotics, BMX-11 proved to be a broad spectrum antibacterial, since it has effect on a range of Gram-positive and Gram-negative organisms (Mcdermott, Walker, & White, 2003).

Flow cytometric analysis in combination with fluorescent techniques proved to be a very useful technique to evaluate the effects of BMX-11 treatment in membrane permeability and membrane polarization of *C. marina* and *E. coli* cells. BMX-11 revealed an effect in bacterial membrane permeability and polarization leading to impairment of essential processes, and finally to cellular death (Serio, Marsilio, & Suzzi, 2008). Flow cytometry allowed clear discrimination between viable intact and permeabilized cells by monitoring retention of SYTOX® Green and SYTO® 9+PI, with this last allowing

to discriminate between permeable, injured and non-permeable cells. Staining with $DiBAC_4(3)$ also allowed discrimination between energized, injured and non-energized cells, revealing physiological heterogeneity within the cell populations. As inhibition and reduction of bacterial growth is important to study BMX-11 mode of action, quantification of injured cells is of interest, providing useful information. Also, as depolarization leads to a loss of transport and other metabolic activity, and consequently to the inhibition of lipoteichoic acid and peptidoglycan biosynthesis, BMX-11 may be affecting each of these mechanisms (Mascio *et al.*, 2007). Membrane permeability and depolarization leads cells to cannot synthesise ATP or to take up nutrients (Hancock, 2005). Daptomycin for example, can enters the bacterial membrane, leading to membrane depolarization, releasing of intracellular potassium ions, and causing cell death (Mascio *et al.*, 2007). The efficiency of used stains for viability assessment in stress conditions was checked as has been prior reported for several microorganisms (Wickens *et al*., 2000; Decker, 2001; Kort *et al.*, 2008). Unfortunately, there still is controversy of fluorescence techniques reliability so, data should be complemented and compared with other molecular and microbiological techniques, making the process more laborious and time-consuming, as was clearly visible in this work (Quintas & Tomás, 2014). Flow cytometric analysis of BMX-11 action should be performed using more bacteria, like Gram-positive bacteria, and using other dyes, to better understand what cell structures/functions have been targeted by BMX-11. For example, one can study bacteria cell's DNA content, protein content, enzyme activities and membrane composition and fluidity (Nebe-von-caron, 2010). Also, molecular studies and/or simulation tools should be performed to better understand what cell functions/pathways are BMX-11 be affecting.

In conclusion, BMX-11 is a compound able to disrupt bacterial cells by affecting their membrane stability and function. However, its antibacterial mechanism of action has still not been fully understood, and it is perhaps acting in various ways and in many different cell targets, such as cell wall degradation (Serio *et al*., 2008), protein synthesis (Mcdermott *et al*., 2003; Hancock, 2005; Kaufman, 2011) nucleic acid synthesis (Mcdermott *et al*., 2003; Hancock, 2005; Kaufman, 2011) and folate synthesis (Kaufman, 2011), between others. Obtained data suggests that BMX-11 activity does not require actively growing bacteria, which is consistent with the proposed mechanism of disrupting membrane function from flow cytometric analysis (Mascio *et al.*, 2007). The activity of BMX-11 against growth arrest cells, combined with the fact that BMX-11 is a mix of compounds, leads to hypothesize that it might exert more than one mechanism of action, including activities against exponential-phase cells and against stationary-phase cells (Mascio *et al*., 2007). Further studies on BMX-11 mode of action should be performed to complement the knowledge on the specificities of its mode of action, a process that is quite complex to understand, especially when the active substance contains several potential active substances.

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Annexes

A1. Data of *E. coli* exponential phase growth curves.

	Growth control (C (+))		5000 µg/mL BMX-11		10000 µg/mL BMX-11		15000 µg/mL BMX-11		30000 µg/mL BMX-11	
Time (h)	**OD_{600nm}**	**SD**	**OD_{600nm}**	**SD**	**OD_{600nm}**	**SD**	**OD_{600nm}**	**SD**	**OD_{600nm}**	**SD**
0.00	0.039	0.011	0.035	0.000	0.035	0.000	0.040	0.012	0.045	0.000
0.75	0.071	0.024					0.073	0.022	0.079	0.018
1.50	0.270	0.031	0.300	0.012	0.292	0.000	0.223	0.041		
2.00	0.426	0.074					0.444	0.066		
2.50	0.766	0.260	1.017	0.001	1.016	0.003	0.644	0.222	0.415	0.021
3.00	1.130	0.179					1.122	0.217	0.967	0.004
3.50	1.572	0.130	1.436	0.010	1.423	0.024	1.632	0.090		
4.50	1.856	0.301	1.678	0.030	1.460	0.065	1.933	0.393	1.625	0.035
5.50	2.090	0.303	1.740	0.040	1.225	0.007	1.935	0.358	0.750	0.057
6.50	2.713	0.389	1.495	0.085	1.225	0.120	2.185	0.778	0.725	0.021
7.50	2.963	0.366	1.480	0.080	0.985	0.106	1.893	0.768	0.760	0.099
8.50	3.315	0.266					1.303	0.081	0.500	0.042
9.50	3.249	0.631	1.545	0.215	0.860	0.028	1.442	0.385	0.530	0.000
10.50	3.595	0.283	1.450	0.100	0.965	0.007	1.106	0.100	0.580	0.042
22.00	3.103	0.110					1.504	0.636		
24.00	3.000	0.184	1.396	0.048	0.967	0.143	1.016	0.426	0.573	0.035
26.00	3.120	0.196	1.380	0.065	0.942	0.121	1.016	0.034	0.522	0.014
28.00	3.047	0.057	1.337	0.038	0.935	0.108	1.001	0.064	0.554	0.011

A2. Data of *E. coli* stationary phase growth curves.

	Growth control (C (+))		15000 µg/mL BMX-11	
Time (h)	**OD_{600nm}**	**SD**	**OD_{600nm}**	**SD**
0.0	0.049	0.000	0.049	0.000
12.7	3.425	0.134	3.605	0.035
13.6	3.370	0.057	3.605	0.064
14.3	3.235	0.078	3.340	0.085
15.0	3.265	0.078	3.440	0.113
15.8	3.235	0.092	3.370	0.113
16.5	3.170	0.000	3.555	0.247
17.1	3.125	0.035	3.455	0.134
17.8	3.030	0.127	3.095	0.049
20.8	3.400	0.325	2.905	0.078
21.4	3.630	0.113	3.020	0.141
22.0	3.455	0.120	3.000	0.057
38.2	2.775	0.021	1.155	0.049
38.8	2.655	0.049	1.060	0.071
40.0	2.645	0.092	1.030	0.085
41.3	2.560	0.028	1.020	0.141

A3. and A4. OD_{600nm} data of *E. coli* and *C. marina* growth curves, respectively, in the presence of several BMX-11 concentrations, analysed on bioscreen (continuous analysis of incubation with agitation).

A3. *E. coli*

	BMX-11 30000 µg/mL		BMX-11 15000 µg/mL		BMX-11 7500 µg/mL		BMX-11 3750 µg/mL		BMX-11 1875 µg/mL		BMX-11 937 µg/mL		BMX-11 468 µg/mL		BMX-11 234 µg/mL		BMX-11 117 µg/mL		C (+) (growth control)		C (-) (negative control)	
Time (h)	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
0.00	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000
0.50	0.065	0.086	0.042	0.071	0.040	0.056	0.052	0.117	0.070	0.065	0.049	0.031	0.064	0.028	0.040	0.035	0.019	0.038	0.064	0.045	0.009	0.010
1.00	0.047	0.095	0.083	0.075	0.065	0.058	0.117	0.134	0.168	0.070	0.131	0.050	0.144	0.052	0.092	0.058	0.062	0.048	0.123	0.062	-0.056	0.015
1.51	0.061	0.090	0.076	0.060	0.061	0.086	0.109	0.099	0.189	0.106	0.172	0.049	0.184	0.056	0.132	0.060	0.086	0.048	0.151	0.059	-0.124	0.012
2.01	0.049	0.088	0.044	0.110	0.053	0.083	0.091	0.094	0.206	0.121	0.204	0.052	0.228	0.064	0.167	0.069	0.121	0.052	0.193	0.068	-0.159	0.012
2.51	0.035	0.076	0.036	0.131	0.044	0.097	0.107	0.098	0.232	0.139	0.244	0.056	0.261	0.065	0.202	0.067	0.162	0.049	0.253	0.074	-0.165	0.009
3.01	0.018	0.093	0.035	0.126	0.033	0.098	0.081	0.092	0.222	0.145	0.248	0.064	0.260	0.079	0.210	0.071	0.168	0.053	0.277	0.092	-0.208	0.010
3.51	0.031	0.110	0.039	0.131	0.032	0.098	0.084	0.109	0.233	0.162	0.261	0.071	0.276	0.076	0.238	0.057	0.192	0.047	0.315	0.085	-0.197	0.012
4.02	0.039	0.107	0.033	0.134	0.014	0.102	0.071	0.114	0.219	0.175	0.250	0.069	0.267	0.075	0.242	0.050	0.200	0.045	0.338	0.085	-0.194	0.023
4.52	0.025	0.099	0.045	0.141	0.018	0.108	0.070	0.117	0.233	0.182	0.254	0.073	0.272	0.075	0.247	0.053	0.215	0.049	0.380	0.088	-0.212	0.043
5.02	0.024	0.110	0.036	0.132	0.010	0.092	0.078	0.113	0.230	0.181	0.257	0.070	0.267	0.070	0.244	0.044	0.223	0.053	0.403	0.076	-0.183	0.069
5.52	0.008	0.133	0.016	0.130	-0.003	0.091	0.075	0.134	0.221	0.178	0.256	0.076	0.263	0.072	0.245	0.045	0.227	0.066	0.414	0.074	-0.147	0.093
6.02	-0.015	0.119	0.040	0.131	-0.008	0.087	0.078	0.136	0.235	0.177	0.261	0.075	0.262	0.078	0.238	0.041	0.227	0.066	0.438	0.061	-0.171	0.080
6.52	-0.016	0.131	0.026	0.121	-0.004	0.098	0.068	0.137	0.231	0.176	0.260	0.076	0.268	0.067	0.272	0.119	0.237	0.076	0.448	0.059	-0.151	0.078
7.03	0.001	0.132	0.013	0.117	-0.018	0.083	0.066	0.137	0.225	0.177	0.254	0.081	0.252	0.078	0.230	0.054	0.231	0.077	0.471	0.053	-0.169	0.072
7.53	-0.014	0.125	0.043	0.125	-0.017	0.090	0.062	0.156	0.217	0.182	0.245	0.087	0.252	0.074	0.223	0.053	0.223	0.080	0.487	0.052	-0.210	0.046
8.03	-0.029	0.134	0.013	0.112	-0.020	0.088	0.052	0.157	0.208	0.187	0.246	0.083	0.257	0.071	0.224	0.044	0.222	0.076	0.496	0.055	-0.147	0.072
8.53	-0.028	0.142	0.002	0.131	-0.030	0.099	0.046	0.158	0.187	0.221	0.231	0.078	0.234	0.084	0.212	0.050	0.211	0.076	0.504	0.060	-0.172	0.061
9.03	-0.021	0.136	-0.001	0.140	-0.025	0.089	0.039	0.162	0.179	0.226	0.226	0.070	0.228	0.079	0.210	0.050	0.205	0.077	0.511	0.065	-0.160	0.054
9.53	-0.027	0.139	0.001	0.165	-0.028	0.096	0.042	0.157	0.173	0.231	0.214	0.064	0.230	0.065	0.205	0.055	0.196	0.082	0.511	0.061	-0.202	0.034
10.04	-0.023	0.139	0.008	0.165	-0.036	0.110	0.043	0.156	0.169	0.233	0.202	0.058	0.228	0.064	0.197	0.052	0.187	0.079	0.517	0.052	-0.178	0.039
10.54	-0.019	0.146	-0.011	0.160	-0.037	0.098	0.046	0.162	0.170	0.236	0.207	0.058	0.228	0.061	0.199	0.047	0.188	0.083	0.520	0.051	-0.144	0.044
11.04	-0.039	0.144	-0.005	0.161	-0.053	0.103	0.046	0.168	0.155	0.239	0.199	0.063	0.229	0.069	0.197	0.046	0.178	0.080	0.524	0.050	-0.154	0.035
11.54	-0.049	0.140	0.013	0.177	-0.057	0.117	0.034	0.159	0.156	0.242	0.191	0.061	0.222	0.061	0.190	0.047	0.166	0.091	0.520	0.054	-0.175	0.036
12.04	-0.059	0.132	-0.013	0.164	-0.048	0.117	0.024	0.163	0.146	0.246	0.185	0.071	0.214	0.057	0.186	0.043	0.157	0.100	0.522	0.071	-0.124	0.051

12.54	-0.064	0.133	-0.007	0.174	-0.066	0.122	0.035	0.163	0.147	0.244	0.192	0.066	0.220	0.054	0.184	0.052	0.187	0.136	0.523	0.081	-0.109	0.057
13.05	-0.045	0.130	0.003	0.160	-0.063	0.126	0.037	0.167	0.151	0.248	0.195	0.066	0.217	0.058	0.186	0.046	0.159	0.105	0.524	0.091	-0.132	0.053
13.55	-0.048	0.142	-0.021	0.155	-0.074	0.130	0.045	0.167	0.139	0.243	0.196	0.061	0.224	0.053	0.182	0.056	0.156	0.108	0.518	0.096	-0.082	0.058
14.05	-0.058	0.148	-0.019	0.188	-0.083	0.137	0.046	0.164	0.139	0.250	0.182	0.062	0.208	0.064	0.167	0.061	0.143	0.104	0.506	0.097	-0.144	0.054
14.55	-0.063	0.173	-0.028	0.185	-0.088	0.131	0.027	0.160	0.128	0.246	0.187	0.062	0.218	0.058	0.171	0.059	0.148	0.105	0.489	0.097	-0.056	0.058
15.05	-0.107	0.186	-0.043	0.178	-0.100	0.136	0.010	0.168	0.117	0.238	0.184	0.040	0.201	0.064	0.158	0.062	0.140	0.097	0.473	0.097	-0.037	0.055
15.55	-0.100	0.194	-0.027	0.188	-0.095	0.141	0.017	0.180	0.121	0.240	0.157	0.056	0.192	0.090	0.136	0.073	0.133	0.079	0.456	0.089	-0.126	0.049
16.06	-0.122	0.179	-0.034	0.189	-0.105	0.149	0.023	0.184	0.124	0.250	0.175	0.061	0.204	0.085	0.152	0.076	0.143	0.074	0.451	0.087	-0.113	0.049
17.06	-0.107	0.203	-0.051	0.194	-0.131	0.157	0.012	0.187	0.113	0.250	0.178	0.064	0.194	0.065	0.146	0.077	0.143	0.076	0.419	0.072	-0.019	0.040
17.56	-0.106	0.201	-0.043	0.202	-0.129	0.146	0.010	0.181	0.113	0.251	0.180	0.064	0.197	0.066	0.145	0.078	0.147	0.077	0.405	0.067	-0.024	0.040
18.06	-0.120	0.192	-0.043	0.206	-0.144	0.174	0.005	0.170	0.116	0.257	0.174	0.062	0.189	0.070	0.134	0.076	0.144	0.079	0.394	0.069	-0.068	0.040
18.57	-0.123	0.196	-0.049	0.201	-0.142	0.147	-0.002	0.175	0.113	0.259	0.177	0.061	0.198	0.073	0.141	0.078	0.145	0.077	0.380	0.069	-0.027	0.040
19.07	-0.114	0.209	-0.060	0.202	-0.173	0.178	-0.002	0.189	0.105	0.256	0.172	0.065	0.188	0.069	0.129	0.078	0.137	0.083	0.373	0.075	-0.014	0.034
19.57	-0.142	0.208	-0.071	0.221	-0.188	0.190	-0.012	0.183	0.090	0.274	0.179	0.044	0.183	0.072	0.125	0.080	0.134	0.084	0.365	0.076	-0.017	0.032
20.07	-0.126	0.202	-0.061	0.187	-0.187	0.194	-0.024	0.177	0.091	0.260	0.173	0.044	0.181	0.073	0.141	0.080	0.128	0.085	0.356	0.079	-0.008	0.033
20.57	-0.121	0.208	-0.092	0.188	-0.190	0.199	-0.010	0.199	0.086	0.256	0.175	0.039	0.180	0.068	0.139	0.081	0.129	0.083	0.350	0.076	-0.018	0.039
21.07	-0.122	0.205	-0.082	0.197	-0.190	0.198	-0.008	0.189	0.080	0.275	0.163	0.041	0.166	0.075	0.128	0.080	0.115	0.079	0.343	0.077	-0.072	0.045
21.58	-0.136	0.212	-0.095	0.187	-0.190	0.199	-0.013	0.186	0.079	0.277	0.162	0.040	0.170	0.075	0.129	0.081	0.123	0.083	0.340	0.077	-0.060	0.044
22.08	-0.127	0.222	-0.092	0.195	-0.185	0.198	-0.023	0.189	0.076	0.279	0.161	0.042	0.172	0.074	0.127	0.082	0.118	0.081	0.337	0.080	-0.049	0.045
22.58	-0.116	0.245	-0.103	0.192	-0.202	0.199	-0.018	0.184	0.075	0.280	0.157	0.039	0.173	0.073	0.127	0.081	0.122	0.085	0.333	0.078	-0.048	0.050
23.08	-0.116	0.245	-0.106	0.191	-0.197	0.209	-0.021	0.183	0.073	0.283	0.155	0.039	0.159	0.051	0.125	0.082	0.122	0.082	0.294	0.053	-0.059	0.054
23.58	-0.128	0.219	-0.083	0.191	-0.210	0.204	-0.028	0.178	0.082	0.285	0.154	0.059	0.163	0.055	0.133	0.083	0.133	0.077	0.314	0.032	-0.055	0.053
24.08	-0.118	0.216	-0.097	0.189	-0.190	0.195	-0.034	0.179	0.073	0.285	0.138	0.066	0.146	0.059	0.128	0.087	0.122	0.083	0.307	0.032	-0.058	0.051

A4. *C. marina*

	BMX-11 30000µg/mL		BMX-11 15000µg/mL		BMX-11 7500µg/mL		BMX-11 3750µg/mL		BMX-11 1875µg/mL		BMX-11 937µg/mL		BMX-11 468µg/mL		BMX-11 234µg/mL		BMX-11 117µg/mL		C (+) (growth control)		C (-) (negative control)	
Time (h)	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
0.00	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000
0.50	-0.008	0.063	0.024	0.039	-0.001	0.029	-0.007	0.036	-0.010	0.040	-0.014	0.039	-0.019	0.040	0.010	0.020	0.006	0.036	0.017	0.016	0.010	0.011
1.00	-0.004	0.062	0.027	0.049	0.005	0.024	0.003	0.019	-0.011	0.035	-0.015	0.026	-0.017	0.022	0.003	0.020	0.025	0.038	0.008	0.026	0.003	0.014
1.51	-0.004	0.061	0.037	0.051	0.015	0.019	0.015	0.012	-0.001	0.029	-0.012	0.026	-0.006	0.021	0.016	0.044	0.023	0.049	0.013	0.028	0.012	0.009
2.01	-0.019	0.080	0.044	0.041	0.013	0.023	0.011	0.014	0.000	0.026	-0.021	0.033	-0.009	0.022	0.006	0.044	0.009	0.049	0.005	0.028	-0.004	0.008
2.51	-0.021	0.084	0.047	0.049	0.017	0.019	0.015	0.015	0.003	0.026	-0.014	0.029	-0.012	0.021	-0.012	0.028	-0.004	0.041	0.002	0.027	0.010	0.012
3.01	-0.021	0.057	0.054	0.039	0.016	0.021	0.009	0.026	0.003	0.026	-0.017	0.036	-0.026	0.023	-0.037	0.019	-0.022	0.037	-0.032	0.021	-0.051	0.011
3.51	0.035	0.039	0.089	0.030	0.050	0.022	0.031	0.039	0.032	0.034	0.029	0.025	0.013	0.023	-0.010	0.008	0.022	0.035	0.030	0.032	0.025	0.012
4.02	-0.010	0.046	0.058	0.038	0.000	0.037	0.009	0.026	0.006	0.028	-0.035	0.052	-0.044	0.042	-0.034	0.012	-0.012	0.036	-0.012	0.031	-0.014	0.015
4.52	0.011	0.054	0.089	0.033	0.046	0.024	0.036	0.037	0.038	0.028	0.028	0.027	-0.003	0.031	-0.002	0.020	0.016	0.020	0.021	0.031	0.003	0.018
5.02	-0.023	0.053	0.062	0.044	0.013	0.028	0.012	0.029	0.004	0.031	-0.030	0.051	-0.041	0.037	-0.028	0.023	-0.006	0.031	-0.010	0.027	-0.019	0.025
5.52	0.019	0.038	0.090	0.038	0.046	0.028	0.041	0.033	0.041	0.026	0.028	0.032	0.001	0.024	0.001	0.030	0.008	0.026	0.016	0.036	0.021	0.015
6.02	-0.043	0.060	0.057	0.041	-0.003	0.042	0.007	0.029	-0.002	0.036	-0.010	0.046	-0.038	0.026	-0.007	0.043	-0.005	0.027	-0.013	0.031	-0.011	0.028
6.52	0.019	0.039	0.094	0.033	0.039	0.036	0.032	0.044	0.008	0.054	0.031	0.045	-0.011	0.026	0.016	0.048	0.034	0.033	0.015	0.041	0.041	0.019
7.03	-0.046	0.036	0.024	0.040	-0.034	0.036	-0.016	0.021	-0.020	0.029	-0.003	0.052	-0.056	0.021	-0.014	0.047	0.021	0.030	-0.015	0.032	-0.032	0.016
7.53	0.008	0.041	0.095	0.034	0.039	0.038	0.048	0.030	0.037	0.039	0.074	0.037	0.006	0.014	0.048	0.057	0.073	0.034	0.035	0.036	0.038	0.019
8.03	-0.048	0.043	0.025	0.040	-0.030	0.033	-0.023	0.028	0.012	0.047	0.066	0.052	-0.016	0.019	0.046	0.049	0.077	0.028	0.024	0.028	-0.044	0.017
8.53	-0.051	0.044	0.030	0.041	-0.025	0.030	-0.010	0.020	0.043	0.048	0.119	0.047	0.021	0.018	0.079	0.053	0.114	0.031	0.057	0.033	-0.035	0.021
9.03	-0.023	0.042	0.061	0.040	-0.004	0.041	0.010	0.032	0.104	0.046	0.182	0.045	0.071	0.018	0.127	0.056	0.156	0.036	0.093	0.036	-0.001	0.029
9.53	-0.036	0.052	0.060	0.041	0.008	0.031	0.022	0.019	0.142	0.052	0.224	0.045	0.110	0.017	0.161	0.063	0.193	0.040	0.108	0.051	-0.002	0.027
10.04	-0.042	0.036	0.030	0.037	-0.019	0.025	-0.008	0.015	0.186	0.050	0.262	0.047	0.134	0.022	0.190	0.062	0.227	0.041	0.104	0.044	-0.036	0.016
10.54	-0.027	0.046	0.063	0.044	0.010	0.033	0.031	0.015	0.247	0.050	0.325	0.055	0.186	0.027	0.230	0.065	0.262	0.047	0.151	0.054	-0.002	0.023
11.04	-0.042	0.062	0.064	0.043	0.016	0.028	0.025	0.022	0.306	0.041	0.355	0.041	0.236	0.068	0.261	0.068	0.293	0.050	0.188	0.050	-0.009	0.028
11.54	0.016	0.037	0.097	0.037	0.047	0.033	0.055	0.024	0.350	0.032	0.399	0.030	0.271	0.038	0.292	0.073	0.332	0.056	0.239	0.062	0.047	0.014
12.04	-0.032	0.048	0.064	0.043	0.012	0.033	0.026	0.020	0.394	0.033	0.381	0.047	0.265	0.036	0.314	0.075	0.358	0.058	0.256	0.061	-0.013	0.024
12.54	0.024	0.035	0.100	0.037	0.049	0.032	0.056	0.022	0.416	0.028	0.401	0.052	0.282	0.039	0.332	0.085	0.377	0.069	0.267	0.061	0.024	0.018
13.05	-0.041	0.036	0.027	0.041	-0.031	0.037	-0.008	0.025	0.425	0.043	0.342	0.034	0.254	0.036	0.329	0.082	0.385	0.063	0.268	0.072	-0.049	0.013
13.55	0.023	0.035	0.101	0.035	0.044	0.037	0.059	0.020	0.423	0.040	0.384	0.049	0.280	0.040	0.337	0.091	0.390	0.076	0.274	0.075	0.029	0.019
14.05	-0.021	0.046	0.067	0.042	0.007	0.042	0.034	0.026	0.390	0.058	0.317	0.047	0.261	0.036	0.338	0.086	0.397	0.071	0.277	0.055	-0.016	0.025

14.55	-0.028	0.033	0.031	0.039	-0.022	0.034	0.008	0.040	0.365	0.070	0.265	0.050	0.247	0.035	0.333	0.083	0.395	0.065	0.254	0.051	-0.065	0.015
15.05	-0.016	0.044	0.060	0.049	0.007	0.040	0.039	0.046	0.337	0.073	0.272	0.057	0.253	0.033	0.335	0.084	0.391	0.062	0.254	0.065	-0.059	0.011
15.55	-0.022	0.044	0.068	0.040	0.008	0.042	0.063	0.043	0.345	0.067	0.270	0.040	0.254	0.037	0.338	0.087	0.401	0.068	0.275	0.069	0.003	0.026
16.06	-0.026	0.034	0.023	0.050	-0.021	0.035	0.065	0.058	0.307	0.072	0.231	0.059	0.235	0.034	0.335	0.084	0.396	0.062	0.263	0.064	-0.061	0.012

A5. Data of time-kill curves.

		CFU/mL					OD_{595nm}				
Species	**Time (h)**	**C (+)**	**1/2MIC**	**MIC**	**2MIC**	**MBC**	**C (+)**	**1/2MIC**	**MIC**	**2MIC**	**MBC**
	0 Mean	3.50E+04	5.00E+03	7.67E+03	2.07E+03	1.72E+03	0.26	0.26	0.26	0.26	0.26
	0 SD	2.71E+04	2.45E+03	4.63E+03	5.89E+02	4.15E+02	0.03	0.01	0.01	0.01	0.04
	1 Mean	6.20E+04	7.33E+03	4.00E+03	1.48E+03	1.40E+03	0.23	0.23	0.23	0.24	0.25
C. marina	1 SD	5.03E+04	5.61E+03	1.79E+03	4.15E+02	4.20E+02	0.02	0.03	0.03	0.03	0.02
	2 Mean	1.23E+05	9.50E+03	3.67E+03	1.57E+03	1.36E+03	0.21	0.23	0.22	0.24	0.26
	2 SD	5.72E+04	3.79E+03	3.88E+03	1.23E+03	1.47E+03	0.02	0.03	0.03	0.04	0.03
	4 Mean	5.56E+05	3.50E+03	2.33E+03	2.00E+03	3.33E+03	0.20	0.24	0.24	0.26	0.26
	4 SD	2.19E+05	3.00E+03	2.34E+03	1.79E+03	1.63E+03	0.04	0.04	0.06	0.06	0.05
	16 Mean	3.48E+07	7.93E+04	2.37E+03	1.00E+02	6.67E+01	0.24	0.24	0.24	0.26	0.28
	16 SD	1.49E+07	1.62E+04	2.75E+03	1.67E+02	1.03E+02	0.02	0.01	0.02	0.01	0.01
	24 Mean	1.44E+08	4.20E+06	8.33E+02	6.67E+01	1.00E+02	0.31	0.27	0.24	0.26	0.28
	24 SD	7.53E+07	1.11E+06	6.86E+02	1.03E+02	2.45E+02	0.04	0.04	0.02	0.01	0.01
	0 Mean	3.18E+05	3.30E+05	3.80E+05	3.43E+05	4.03E+05	0.18	0.18	0.18	0.18	0.18
	0 SD	7.55E+04	7.01E+04	7.59E+04	2.34E+04	1.07E+05	0.20	0.11	0.09	0.09	0.08
	1 Mean	2.90E+05	3.47E+05	3.47E+05	3.60E+05	3.76E+05	0.08	0.12	0.13	0.14	0.15
	1 SD	6.06E+04	2.31E+04	5.77E+04	1.00E+05	3.85E+04	0.01	0.02	0.02	0.02	0.02
E. coli	2 Mean	6.33E+05	3.60E+05	3.80E+05	2.97E+05	3.20E+05	0.12	0.17	0.18	0.19	0.19
	2 SD	8.37E+04	1.04E+05	1.12E+05	5.43E+04	1.23E+05	0.02	0.02	0.01	0.02	0.03
	4 Mean	3.08E+08	6.13E+05	5.07E+05	1.83E+05	1.70E+05	0.15	0.16	0.16	0.17	0.17
	4 SD	1.22E+08	1.03E+05	3.93E+04	1.01E+05	5.02E+04	0.02	0.01	0.01	0.02	0.02
	16 Mean	1.03E+09	7.67E+07	5.28E+04	4.67E+02	3.67E+02	0.38	0.16	0.17	0.18	0.18
	16 SD	1.54E+08	2.21E+07	3.25E+04	5.89E+02	3.88E+02	0.02	0.01	0.01	0.01	0.02
	24 Mean	6.65E+08	9.07E+07	3.83E+07	1.67E+03	1.50E+03	0.61	0.38	0.34	0.33	0.31
	24 SD	2.10E+08	2.44E+07	2.42E+07	1.73E+03	1.62E+03	0.05	0.06	0.05	0.06	0.07
	0 Mean	4.05E+05	4.93E+05	2.50E+05	1.00E+02	0.00E+00	0.12	0.12	0.12	0.12	0.12
	0 SD	9.80E+04	1.16E+05	3.29E+04	1.10E+02	0.00E+00	0.02	0.02	0.02	0.02	0.02
	1 Mean	1.09E+06	5.47E+05	2.00E+05	3.33E+01	0.00E+00	0.14	0.13	0.13	0.12	0.12
	1 SD	2.15E+05	7.55E+04	7.80E+04	8.16E+01	0.00E+00	0.03	0.03	0.03	0.01	0.01
E. faecalis	2 Mean	4.25E+06	4.60E+05	2.60E+05	0.00E+00	0.00E+00	0.14	0.13	0.12	0.12	0.12
	2 SD	1.10E+06	7.16E+04	1.17E+05	0.00E+00	0.00E+00	0.03	0.03	0.03	0.01	0.02
	4 Mean	9.58E+07	5.93E+05	2.13E+05	0.00E+00	0.00E+00	0.14	0.11	0.11	0.11	0.12
	4 SD	2.41E+07	1.42E+05	8.07E+04	0.00E+00	0.00E+00	0.01	0.00	0.00	0.00	0.02
	16 Mean	3.35E+08	2.67E+06	6.17E+04	0.00E+00	0.00E+00	0.23	0.14	0.13	0.12	0.14
	16 SD	1.91E+08	8.64E+05	2.44E+04	0.00E+00	0.00E+00	0.02	0.02	0.01	0.01	0.04
	24 Mean	4.98E+08	4.00E+06	1.36E+04	0.00E+00	0.00E+00	0.22	0.13	0.13	0.12	0.15
	24 SD	2.56E+08	7.27E+05	1.97E+03	0.00E+00	0.00E+00	0.02	0.03	0.03	0.03	0.06
	0 Mean	6.64E+05	6.28E+05	6.83E+05	7.39E+05	0.00E+00	0.15	0.15	0.15	0.15	0.15
	0 SD	2.50E+05	2.07E+05	1.39E+05	2.38E+05	0.00E+00	0.01	0.02	0.03	0.03	0.04

	1	Mean	1.55E+06	9.08E+05	6.75E+05	6.92E+05	0.00E+00	0.16	0.12	0.12	0.13	0.13
	1	SD	1.47E+06	1.66E+05	1.29E+05	1.03E+05	0.00E+00	0.01	0.01	0.00	0.01	0.01
L. monocyto-genes	2	Mean	7.08E+06	1.06E+06	7.89E+05	7.06E+05	0.00E+00	0.16	0.12	0.12	0.12	0.13
	2	SD	1.76E+06	1.76E+05	2.34E+05	2.22E+05	0.00E+00	0.01	0.00	0.00	0.00	0.01
	4	Mean	1.23E+07	1.53E+06	5.58E+05	2.58E+05	0.00E+00	0.16	0.12	0.12	0.13	0.13
	4	SD	2.85E+06	1.71E+05	2.56E+05	1.92E+03	0.00E+00	0.01	0.00	0.00	0.00	0.00
	16	Mean	1.80E+08	2.06E+06	1.03E+06	1.58E+05	0.00E+00	0.28	0.14	0.14	0.14	0.14
	16	SD	3.10E+07	5.57E+05	1.95E+05	4.68E+04	0.00E+00	0.02	0.02	0.03	0.02	0.02
	24	Mean	1.73E+08	7.11E+06	1.51E+05	1.13E+05	0.00E+00	0.28	0.17	0.16	0.16	0.14
	24	SD	2.96E+07	1.44E+06	5.60E+04	4.58E+04	0.00E+00	0.07	0.08	0.08	0.06	0.04
	0	Mean	2.38E+05	2.87E+05	2.17E+05	2.37E+05	2.63E+05	0.09	0.09	0.09	0.09	0.09
	0	SD	1.38E+05	5.32E+04	6.25E+04	9.42E+04	7.42E+04	0.00	0.00	0.00	0.00	0.00
	1	Mean	5.20E+05	5.87E+05	4.53E+05	3.27E+05	2.47E+05	0.09	0.09	0.09	0.09	0.08
	1	SD	9.61E+04	8.36E+04	1.42E+05	1.01E+05	5.16E+04	0.00	0.00	0.00	0.00	0.00
	2	Mean	7.78E+05	8.30E+05	8.97E+05	6.30E+05	2.30E+05	0.09	0.09	0.09	0.09	0.08
S. aureus	2	SD	1.29E+05	1.25E+05	1.77E+05	1.06E+05	9.36E+04	0.00	0.00	0.00	0.00	0.00
	4	Mean	3.77E+06	8.93E+05	9.90E+05	8.83E+05	1.07E+05	0.09	0.09	0.08	0.09	0.08
	4	SD	1.50E+06	4.27E+05	2.83E+05	2.17E+05	1.78E+04	0.00	0.00	0.00	0.00	0.00
	16	Mean	3.87E+08	1.80E+08	6.23E+06	3.83E+06	7.77E+04	0.26	0.23	0.18	0.20	0.15
	16	SD	2.01E+08	6.20E+07	1.58E+06	1.56E+06	1.80E+04	0.02	0.02	0.02	0.03	0.03
	24	Mean	7.07E+08	3.67E+08	3.50E+06	8.50E+05	6.10E+04	0.24	0.20	0.12	0.12	0.10
	24	SD	2.46E+08	1.01E+08	2.20E+06	1.71E+05	2.10E+04	0.05	0.06	0.05	0.05	0.03
	0	Mean	4.80E+05	5.17E+05	5.10E+05	5.43E+05	5.90E+05	0.16	0.16	0.16	0.16	0.16
	0	SD	1.13E+05	1.01E+05	1.16E+05	6.98E+04	2.33E+05	0.05	0.06	0.06	0.04	0.06
	1	Mean	6.78E+05	6.43E+05	5.53E+05	5.20E+05	5.10E+05	0.13	0.13	0.13	0.13	0.14
	1	SD	1.29E+05	1.24E+05	1.32E+05	1.04E+05	1.33E+05	0.05	0.05	0.04	0.04	0.04
	2	Mean	6.53E+06	7.47E+05	3.83E+05	3.90E+05	3.47E+05	0.11	0.11	0.10	0.11	0.10
	2	SD	1.01E+06	6.77E+04	6.50E+04	8.37E+04	1.11E+05	0.02	0.02	0.02	0.02	0.02
V. vulnificus	4	Mean	2.08E+08	4.57E+07	4.83E+04	5.70E+04	4.73E+04	0.16	0.15	0.14	0.15	0.14
	4	SD	1.27E+08	1.52E+07	1.94E+04	1.56E+04	1.67E+04	0.05	0.06	0.05	0.06	0.04
	16	Mean	5.18E+08	5.00E+08	3.67E+02	2.10E+03	3.33E+02	0.21	0.18	0.10	0.09	0.10
	16	SD	2.00E+08	1.45E+08	3.20E+02	4.66E+03	7.23E+02	0.01	0.02	0.02	0.01	0.02
	24	Mean	3.33E+08	3.07E+08	5.00E+02	3.52E+03	8.00E+01	0.21	0.19	0.10	0.10	0.10
	24	SD	7.88E+07	1.06E+08	8.46E+02	4.85E+03	1.10E+02	0.02	0.03	0.03	0.03	0.02

A6. *E. coli* results analysis from flow cytometry.

A6.1. SYTOX Green

Sample	Time (h)	FSC Geometric Mean	SD	FSC Mode	SD	Q1 Freq. of Parent (%)	SD	Q2 Freq. of Parent (%)	SD	Q3 Freq. of Parent (%)	SD	Q4 Freq. of Parent (%)	SD
C (+)	**0**	1.04	0.41	1.11	0.49	0.00	0.00	0.00	0.00	0.54	0.38	99.46	0.39
C (+)	**2**	0.58	0.01	0.55	0.03	0.00	0.00	0.00	0.00	0.22	0.07	99.76	0.07
C (+)	**3**	0.54	0.01	0.50	0.02	0.00	0.00	0.00	0.00	0.92	0.49	99.08	0.49
C (+)	**4**	0.56	0.02	0.50	0.02	0.00	0.01	0.00	0.00	1.52	1.76	98.48	1.76
C (+)	**5**	0.54	0.01	0.50	0.02	0.00	0.00	0.00	0.00	3.04	3.82	96.97	3.82
C (+)	**7**	0.54	0.00	0.50	0.02	0.00	0.00	0.00	0.00	0.49	0.19	99.53	0.19
C (+)	**24**	0.53	0.01	0.48	0.02	0.00	0.00	0.00	0.00	0.40	0.24	99.59	0.23
C(+) KC	**24**	0.52	0.00	0.46	0.03	0.00	0.00	0.00	0.00	95.27	0.23	4.71	0.22
C(+) KC	**0**	0.52	0.01	0.46	0.03	0.01	0.00	0.00	0.01	63.30	1.23	36.70	1.23
Test	**0**	1.03	0.46	1.15	0.58	0.02	0.02	0.01	0.01	1.12	0.39	98.86	0.42
Test	**2**	0.95	0.03	1.07	0.07	0.03	0.04	0.00	0.01	1.71	0.26	98.26	0.28
Test	**3**	0.98	0.03	1.14	0.08	0.02	0.01	0.01	0.00	4.43	0.38	95.52	0.38
Test	**4**	0.94	0.07	1.03	0.10	0.02	0.01	0.01	0.01	25.59	2.76	74.37	2.80
Test	**5**	0.81	0.07	0.86	0.09	0.01	0.01	0.01	0.01	47.44	2.93	52.51	2.95
Test	**7**	0.77	0.04	0.78	0.08	0.01	0.00	0.01	0.01	60.94	5.22	39.02	5.23
Test	**24**	0.84	0.02	0.93	0.07	0.03	0.01	0.01	0.01	59.63	5.93	40.33	5.93
Test KC	**24**	0.70	0.02	0.70	0.03	0.01	0.01	0.00	0.00	75.17	2.40	24.80	2.38

A6.2. SYTO 9+PI

Sample	Time (h)	FSC Geometric Mean	SD	FSC Mode	SD	Q1 Freq. of Parent (%)	SD	Q2 Freq. of Parent (%)	SD	Q3 Freq. of Parent (%)	SD	Q4 Freq. of Parent (%)	SD
C (+)	**0**	0.85	0.09	0.85	0.11	0.59	0.10	0.07	0.06	0.09	0.07	99.23	0.18
C (+)	**2**	0.60	0.02	0.55	0.03	1.07	1.38	0.06	0.05	0.07	0.05	98.81	1.41
C (+)	**3**	0.55	0.01	0.49	0.02	0.82	0.33	0.05	0.01	0.05	0.03	99.09	0.34
C (+)	**4**	0.55	0.01	0.52	0.03	0.96	0.70	0.08	0.03	0.07	0.05	98.90	0.76
C (+)	**5**	0.55	0.01	0.50	0.02	1.16	0.39	0.07	0.02	0.06	0.01	98.72	0.41
C (+)	**7**	0.54	0.01	0.49	0.01	0.88	0.26	0.06	0.02	0.04	0.02	99.03	0.29
C (+)	**24**	0.56	0.03	0.50	0.03	12.31	16.82	0.07	0.03	0.01	0.01	87.61	16.83
C (+) KC	**25**	0.52	0.01	0.47	0.01	97.43	0.32	0.19	0.10	0.00	0.00	2.37	0.25
Test	**0**	0.83	0.11	0.89	0.14	1.10	0.50	0.47	0.52	0.25	0.27	98.17	1.28
Test	**2**	0.93	0.02	1.07	0.04	4.55	3.79	0.53	0.41	0.03	0.02	94.90	3.51
Test	**3**	0.98	0.03	1.10	0.08	3.03	0.50	3.60	0.78	0.12	0.03	93.26	1.09
Test	**4**	0.95	0.06	1.04	0.13	12.13	1.41	21.41	3.20	0.50	0.18	65.98	4.10
Test	**5**	0.83	0.06	0.87	0.10	37.10	5.86	19.27	1.26	0.43	0.10	43.21	5.46
Test	**7**	0.76	0.03	0.77	0.06	64.01	5.56	1.22	0.47	0.04	0.02	34.73	5.68
Test	**24**	0.84	0.03	0.90	0.07	57.93	7.51	0.66	0.23	0.11	0.08	41.32	7.28
Test KC	**25**	0.71	0.03	0.73	0.01	99.50	0.00	0.06	0.06	0.00	0.00	0.46	0.05

A6.3 $DiBAC_4(3)$

Sample	Time (h)	FSC Geometric Mean	SD	FSC Mode	SD	Q1 Freq. of Parent (%)	SD	Q2 Freq. of Parent (%)	SD	Q3 Freq. of Parent (%)	SD	Q4 Freq. of Parent (%)	SD
C (+)	**0**	0.80	0.07	0.83	0.13	0.00	0.00	0.04	0.04	0.00	2.11	97.47	2.02
C (+)	**2**	0.59	0.01	0.55	0.02	0.00	0.00	0.01	0.01	0.21	0.06	99.76	0.07
C (+)	**3**	0.54	0.01	0.48	0.02	0.01	0.01	0.04	0.02	0.43	0.34	99.52	0.33
C (+)	**4**	0.54	0.00	0.49	0.02	0.00	0.00	0.04	0.04	0.21	0.09	99.74	0.11
C (+)	**5**	0.54	0.00	0.48	0.01	0.00	0.00	0.02	0.02	0.20	0.05	99.77	0.05
C (+)	**7**	0.54	0.00	0.50	0.03	0.00	0.00	0.01	0.01	0.18	0.08	99.79	0.08
C (+)	**24**	0.54	0.00	0.50	0.02	0.01	0.01	0.06	0.06	0.38	0.15	99.57	0.15
C (+) KC	**24**	0.50	0.01	0.44	0.01	0.00	0.00	0.05	0.02	85.95	1.53	14.03	1.59
C (+) KC	**0**	0.50	0.01	0.44	0.02	0.00	0.00	0.04	0.00	84.70	0.26	15.33	0.31

Test	**0**	0.80	0.12	0.87	0.17	0.06	0.08	0.28	0.36	3.29	3.07	96.46	3.37
Test	**2**	0.95	0.06	1.10	0.10	0.02	0.03	0.23	0.33	1.96	1.10	97.80	1.45
Test	**3**	0.97	0.03	1.06	0.08	0.01	0.01	0.03	0.01	3.76	0.78	96.21	0.75
Test	**4**	0.91	0.04	1.00	0.10	0.01	0.02	0.07	0.08	23.59	5.69	76.36	5.79
Test	**5**	0.80	0.06	0.87	0.14	0.01	0.01	0.06	0.04	43.26	7.11	56.69	7.14
Test	**7**	0.73	0.02	0.69	0.09	0.01	0.01	0.03	0.02	52.66	5.23	47.33	5.21
Test	**24**	0.81	0.03	0.89	0.16	0.05	0.04	0.16	0.06	46.44	9.54	53.39	0.00
Test KC	**24**	0.61	0.01	0.57	0.03	0.00	0.00	0.10	0.02	92.63	3.74	7.31	3.79

A7. *C. marina* results analysis from flow cytometry.

A7.1. SYTOX Green

Sample	**Time (h)**	**FSC Geometric Mean**	**SD**	**FSC Mode**	**SD**	**Q1 Freq. of Parent (%)**	**SD**	**Q2 Freq. of Parent (%)**	**SD**	**Q3 Freq. of Parent (%)**	**SD**	**Q4 Freq. of Parent (%)**	**SD**
C (+)	**0**	1.06	0.10	1.03	0.13	0.00	0.00	0.03	0.03	1.14	0.31	98.83	0.30
C (+)	**1**	0.99	0.13	0.96	0.19	0.00	0.00	0.01	0.01	0.73	0.22	99.27	0.23
C (+)	**2**	0.83	0.11	0.81	0.15	0.00	0.00	0.01	0.00	0.38	0.09	99.61	0.11
C (+)	**4**	0.77	0.12	0.73	0.19	0.00	0.00	0.00	0.00	0.23	0.11	99.77	0.11
C (+)	**6**	0.71	0.07	0.65	0.10	0.00	0.00	0.01	0.02	0.13	0.03	99.84	0.05
C (+)	**8**	0.70	0.06	0.64	0.10	0.00	0.00	0.02	0.02	0.19	0.06	99.80	0.07
C (+)	**24**	0.66	0.09	0.58	0.08	0.00	0.00	0.02	0.02	0.71	0.42	99.27	0.42
KC		1.22	0.02	1.01	0.13	0.02	0.01	97.90	0.29	1.02	0.36	1.08	0.34
Test	**0**	1.17	0.12	1.07	0.27	0.04	0.02	0.19	0.09	40.66	12.86	59.10	12.86
Test	**1**	1.06	0.15	0.97	0.25	0.04	0.01	0.13	0.08	15.43	0.76	84.40	0.82
Test	**2**	1.38	0.12	1.35	0.14	0.03	0.01	0.10	0.06	14.63	5.45	85.26	5.50
Test	**4**	1.49	0.10	1.62	0.17	0.05	0.03	0.09	0.07	23.27	1.27	76.58	1.36
Test	**6**	1.51	0.15	1.99	0.14	0.05	0.04	0.04	0.03	46.41	8.37	53.49	8.35
Test	**8**	1.40	0.15	1.68	0.87	0.03	0.02	0.17	0.09	64.36	10.64	35.44	10.75
Test	**24**	0.96	0.07	0.52	0.04	0.05	0.01	0.89	0.36	84.23	4.32	14.87	4.04

A7.2. SYTO 9+PI

Sample	**Time (h)**	**FSC Geometric Mean**	**SD**	**FSC Mode**	**SD**	**Q1 Freq. of Parent (%)**	**SD**	**Q2 Freq. of Parent (%)**	**SD**	**Q3 Freq. of Parent (%)**	**SD**	**Q4 Freq. of Parent (%)**	**SD**
C (+)	**0**	0.96	0.12	0.95	0.15	1.61	0.71	0.01	0.01	0.03	0.01	98.4	0.72
C (+)	**1**	1.01	0.13	0.99	0.17	7.32	8.03	0.01	0.01	0.01	0.01	92.7	8.01
C (+)	**2**	0.82	0.11	0.78	0.17	0.97	2.50	0.01	0.01	0.06	0.03	99.0	0.24
C (+)	**4**	0.80	0.12	0.78	0.18	0.81	0.17	0.00	0.00	0.02	0.02	99.2	0.18
C (+)	**6**	0.77	0.10	0.70	0.12	0.88	0.20	0.00	0.00	0.00	0.00	99.1	0.21
C (+)	**8**	0.73	0.08	0.67	0.14	1.78	1.53	0.00	0.00	0.00	0.00	98.2	1.51
C (+)	**24**	0.61	0.04	0.54	0.04	1.35	0.33	0.00	0.01	0.01	0.01	98.7	0.34
KC		0.61	0.01	0.45	0.00	93.25	0.35	0.00	0.00	0.00	0.00	6.8	0.37
Test	**0**	1.05	0.17	1.02	0.28	38.29	9.99	0.02	0.02	0.01	0.02	61.7	9.99
Test	**1**	0.99	0.15	0.94	0.22	15.62	1.87	0.01	0.01	0.00	0.00	84.4	1.87
Test	**2**	1.23	0.07	1.33	0.09	13.92	4.25	0.01	0.00	0.00	0.01	86.1	4.25
Test	**4**	1.39	0.07	1.64	0.14	24.57	2.34	0.01	0.01	0.00	0.00	75.4	2.34
Test	**6**	1.37	0.11	1.92	0.16	47.50	8.65	0.01	0.01	0.00	0.00	52.5	8.66
Test	**8**	1.32	0.14	1.52	0.89	66.93	9.12	0.01	0.01	0.00	0.01	33.1	9.12
Test	**24**	0.88	0.06	0.52	0.03	86.19	6.05	0.02	0.01	0.01	0.01	13.8	6.05

A7.3 $DiBAC_4(3)$

Sample	Time (h)	FSC Geometric Mean	SD	FSC Mode	SD	Q1 Freq. of Parent (%)	SD	Q2 Freq. of Parent (%)	SD	Q3 Freq. of Parent (%)	SD	Q4 Freq. of Parent (%)	SD
C (+)	0	0.87	0.11	0.82	0.17	0.01	0.01	0.01	0.00	2.90	0.78	97.08	0.79
C (+)	1	0.91	0.03	0.88	0.07	0.02	0.01	0.01	0.01	3.22	0.99	96.75	0.99
C (+)	2	0.75	0.10	0.66	0.14	0.01	0.01	0.01	0.01	2.37	1.47	97.61	1.46
C (+)	4	0.73	0.09	0.66	0.15	0.02	0.02	0.01	0.02	2.10	1.08	97.88	1.07
C (+)	6	0.68	0.04	0.59	0.08	0.01	0.01	0.01	0.00	2.41	1.36	97.58	1.37
C (+)	8	0.68	0.04	0.57	0.05	0.01	0.01	0.01	0.01	2.99	1.73	96.97	1.73
C (+)	24	0.63	0.06	0.54	0.06	0.02	0.02	0.01	0.02	6.86	5.25	93.10	5.24
KC		1.16	0.02	0.94	0.10	0.03	0.02	0.07	0.01	96.63	0.50	3.26	0.49
Test	0	1.00	0.10	0.81	0.13	0.15	0.07	0.07	0.03	33.20	12.50	66.58	12.45
Test	1	0.96	0.12	0.88	0.19	0.07	0.02	0.03	0.01	11.27	0.84	88.62	0.82
Test	2	1.21	0.12	1.20	0.21	0.06	0.02	0.03	0.01	10.70	2.75	89.21	2.76
Test	4	1.35	0.09	1.49	0.09	0.08	0.02	0.05	0.03	19.59	4.03	80.28	4.06
Test	6	1.41	0.05	1.80	0.12	0.12	0.06	0.09	0.06	37.44	8.98	62.36	9.05
Test	8	1.37	0.11	1.46	0.86	0.11	0.06	0.12	0.08	53.01	7.08	46.79	7.11
Test	24	1.01	0.06	0.57	0.05	0.10	0.06	0.55	0.47	80.33	7.33	19.01	7.42

A8. *E. coli* – comparison of cell counts from flow cytometry and from plate culture.

	Total cell counts/mL (from flow cytometry)			
Time (h)	C (+)	C (+) SD	30000 µg/mL BMX-11	30000 µg/mL BMX-11 SD
0	1.33E+09	6.11E+07	1.50E+09	4.58E+07
2	1.97E+09	1.25E+08	1.43E+09	4.62E+07
3	1.71E+09	9.64E+07	1.18E+09	2.08E+08
4	2.08E+09	4.93E+07	8.68E+08	5.75E+07
5	2.09E+09	1.33E+08	6.44E+08	5.82E+07
7	1.91E+09	7.29E+08	5.03E+08	8.60E+07
24	2.67E+09	1.46E+08	9.89E+07	2.32E+07

	Viable cell counts/mL (from flow cytometry)									
	C (+)		30000 µg/mL BMX-11							
Time (h)	C (+)	C (+) SD	SYTOX Green	SD	SYTO9 + PI	SD	$DiBAC_4$ (3)	SD	Average of all dyes	SD
0	1.30E+09	8.14E+07	1.48E+09	4.10E+07	1.49E+09	4.63E+07	1.45E+09	8.63E+07	1.47E+09	2.02E+07
2	1.96E+09	1.24E+08	1.40E+09	4.17E+07	1.35E+09	1.92E+07	1.40E+09	5.94E+07	1.38E+09	2.69E+07
3	1.70E+09	9.35E+07	1.13E+09	1.94E+08	1.10E+09	1.99E+08	1.14E+09	2.02E+08	1.12E+09	1.79E+07
4	2.07E+09	4.85E+07	6.46E+08	5.46E+07	5.71E+08	1.61E+07	6.60E+08	1.71E+07	6.25E+08	4.79E+07
5	2.08E+09	1.34E+08	3.30E+08	1.96E+07	2.76E+08	2.07E+07	3.62E+08	2.08E+07	3.23E+08	4.36E+07
7	1.90E+09	7.27E+08	1.99E+08	5.93E+07	1.54E+08	2.39E+07	2.41E+08	6.43E+07	1.98E+08	4.34E+07
24	1.94E+09	1.39E+09	3.89E+07	2.79E+06	3.91E+07	1.83E+06	5.11E+07	1.59E+06	4.30E+07	7.02E+06

CFU/mL counts (from plate culture method)

Time (h)	C (+)	C (+) SD	30000 µg/mL BMX-11	30000 µg/mL BMX-11 SD
0	5.48E+08	9.57E+07	4.52E+08	1.10E+08
2	1.37E+09	2.08E+08	5.87E+08	6.28E+07
3	2.08E+09	4.26E+08	4.77E+08	2.02E+08
4	2.48E+09	6.70E+08	3.62E+08	1.30E+08
5	2.88E+09	6.70E+08	3.33E+08	1.30E+08
7	3.52E+09	7.57E+08	1.11E+08	6.33E+07
24	3.27E+09	7.48E+08	8.45E+06	1.82E+06

Viability (%) in relation to each sampling time of total cell counts of C (+)

Time (h)	C (+) flow cytometry	C (+) SD	30000 µg/mL BMX-11 flow cytometry	30000 µg/mL BMX-11 SD	C (+) plate culture	C (+) SD	30000 µg/mL BMX-11 plate culture	30000 µg/mL BMX-11 SD
0	97.51	6.11	110.50	1.51	41.13	7.18	33.88	8.23
2	99.75	6.29	70.35	1.37	69.49	10.58	29.83	3.19
3	99.52	5.47	65.56	1.05	121.83	24.94	27.88	11.80
4	99.74	2.34	30.12	2.31	119.58	32.25	17.42	6.24
5	99.77	6.42	15.47	2.09	138.18	32.09	15.97	6.23
7	99.78	38.13	10.39	2.28	184.44	39.70	5.83	3.32
24	72.43	52.00	1.61	0.26	122.19	27.99	0.32	0.07

A9. *C. marina* – comparison of cell counts from flow cytometry and from plate culture.

Total cell counts/mL (from flow cytometry)

Time (h)	C (+)	C (+) SD	30000 µg/mL BMX-11	30000 µg/mL BMX-11 SD
0	1.41E+08	1.87E+07	1.14E+08	2.04E+07
1	7.42E+08	4.50E+08	2.93E+08	2.00E+08
2	8.04E+08	3.65E+08	2.03E+08	4.03E+07
4	1.32E+09	2.19E+08	2.24E+08	4.41E+07
6	1.87E+09	6.61E+08	2.18E+08	7.48E+07
8	1.49E+09	2.38E+08	2.02E+08	6.06E+07
24	4.34E+08	1.62E+08	1.07E+08	1.34E+07

Viable cell counts/mL (from flow cytometry)

	C (+)		30000 µg/mL BMX-11							
Time (h)	**C (+)**	**SD C (+)**	**SYTOX Green**	**SD**	**SYTO 9 + PI**	**SD**	**DiBAC$_4$(3)**	**SD**	**Average of all dyes**	**SD of all dyes**
0	1.39E+08	1.89E+07	6.60E+07	1.59E+07	6.96E+07	1.14E+07	7.50E+07	1.39E+07	7.02E+07	4.55E+06
1	7.33E+08	4.42E+08	2.46E+08	1.71E+08	1.13E+08	1.64E+07	2.60E+08	1.78E+08	2.06E+08	8.12E+07
2	8.01E+08	3.64E+08	1.73E+08	4.59E+07	1.76E+08	4.36E+07	1.82E+08	4.16E+07	1.77E+08	4.43E+06
4	1.31E+09	2.20E+08	1.69E+08	3.72E+07	1.70E+08	3.86E+07	1.82E+08	4.55E+07	1.74E+08	7.02E+06
6	1.86E+09	6.59E+08	1.18E+08	5.38E+07	1.17E+08	5.31E+07	1.39E+08	6.12E+07	1.24E+08	1.24E+07
8	1.49E+09	2.38E+08	7.36E+07	4.00E+07	7.05E+07	3.68E+07	9.75E+07	4.15E+07	8.05E+07	1.48E+07
24	4.29E+08	1.58E+08	1.19E+07	2.89E+06	1.48E+07	8.02E+06	2.08E+07	1.04E+07	1.58E+07	4.53E+06

CFU/mL counts (from plate culture method)

Time (h)	C (+)	C (+) SD	30000 µg/mL BMX-11	30000 µg/mL BMX-11 SD
0	1.80E+08	9.02E+07	1.27E+08	4.67E+07
1	3.22E+08	3.00E+08	2.40E+08	2.05E+08
2	7.84E+08	6.76E+08	1.44E+08	5.82E+07
4	1.00E+09	5.93E+08	1.62E+08	1.08E+08
6	1.87E+09	1.16E+09	1.16E+08	7.43E+07
8	2.09E+09	1.26E+09	1.05E+08	1.12E+08
24	6.77E+08	4.17E+08	5.58E+07	7.32E+07

Viability (%) in relation to each sampling time of total cell counts of C (+)

Time (h)	C (+) flow cytometry	C (+) SD	30000 µg/mL BMX-11 flow cytometry	30000 µg/mL BMX-11 SD	C (+) plate culture	C (+) SD	30000 µg/mL BMX-11 plate culture	30000 µg/mL BMX-11 SD
0	98.71	13.40	49.78	3.22	127.66	63.96	89.83	33.10
1	98.80	59.61	27.80	10.95	43.43	40.40	32.35	27.66
2	99.53	45.31	22.01	0.55	97.53	84.02	17.96	7.24
4	99.71	16.76	13.21	0.53	76.05	45.06	12.34	8.20
6	99.79	35.30	6.67	0.67	100.00	61.96	6.19	3.98
8	99.72	15.98	5.40	0.99	140.19	84.70	7.02	7.49
24	99.04	36.46	3.65	1.04	156.03	96.06	12.86	16.88