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Evaluation of the impact of the efflux pump modulation process on the anti-adhesion and antibiofilm activities of analogues of natural marine compounds in a marine bacterium

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One strategy for enhancing the effect of natural marine antifouling compounds has been to combine them with coumarin-type molecules (VL2, VL17 and VL19). These molecules are known to have an inhibitory effect on the efflux pumps (EP) of a large number of Gram-negative bacteria. To this end, they have been used as adjuvants to natural marine compounds (OB1, AS194 and AS162) to enhance their antibiofilm activity. On the other hand, the combination of synthetic analogues with coumarins had a synergistic effect on adhesion and biofilm formation in the bacterial strain studied, *Pseudoalteromonas ulvae*. In this strain, coumarins increased the effect of AS194 on adhesion and biofilm. This increase was more marked with the coumarins VL17 and VL19. These results were confirmed by EC50 calculations. With regard to adhesion, the EC50 of OB1 alone showed a reduction in combination, from 31.7 to 65.3%. As for AS194 and AS162, reductions ranged respectively from 16.9 to 43.6% and from 11.6 to 30.2%. With regard to biofilm, these two compounds in combination showed a significant decrease in their baseline EC50 in P. ulvae TC14. This EC50 decrease was marked by reduction rates ranging from 69.2 to 75.3% for OB1 and from 65.4 to 77% for AS194. In both cases (adhesion or biofilm), the effect variation in AS162 remained relatively small. Similar results were observed with two other marine strains, namely Pseudoalteromonas lypolitica TC8 and Paracoccus spp. 4M6. This study shows that inhibition of efflux pumps by coumarins enhances the anti-biofilm effect of natural marine compounds.

Key words: Biofouling, biofilm, coumarin, efflux pumps.

INTRODUCTION

One of the bacterial defense mechanisms is the active export of antimicrobial substances outside the cell. This mechanism leads to the rejection of these molecules into the external environment, ensuring a low level of intracellular concentration, below the threshold of efficacy. The term "efflux pumps" has been proposed to

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describe these mechanisms (Boulant et al., 2020). Efflux pumps play a major role in the development of multidrug resistance by actively exporting a wide variety of harmful compounds out of the bacterium (Lamers et al., 2013). PEs take the form of transporters capable of expelling different classes of antibiotics out of the bacterial cell, contributing significantly to therapeutic failure in the treatment of infectious diseases. In the medical field, one of the major contributors to multidrug resistance (MDR) and pathogenicity in Gram-negative bacteria is the overexpression of efflux pumps (EPs) belonging to the Resistance-Nodulation-Division (RND) family. These transporters are capable of expelling different classes of antibiotics from the bacterial cell, contributing significantly to therapeutic failure in the treatment of infectious diseases. In this context, EPs are interesting targets for the discovery of new antimicrobials. To combat this resistance mechanism, efflux pump inhibitors (EPIs) are being developed as adjuvants to antibiotics, with the aim of restoring or enhancing their activity. The mechanism of efflux pump inhibition has long been implicated in the enhancement of antibiotic activity. In this study, the modulation of PEs was demonstrated, along with its impact on adhesion and biofilm formation in marine bacteria. Speaking of biofilm inhibition, PEs have been presented by several authors as interesting targets. In the course of his research, Koch (2019), demonstrated a link between biofilm production and PEs in *Yersinia pestis* through a mutant encoding the TolC gene located in the outer membrane. According to the work of Cattoir (2004) and Boulant et al. (2020), one of the factors limiting the reduction of the harmful effect of antimicrobial substances is the defense mechanism based on efflux pumps. Clearly, the rejection of a proportion of compound by bacterial cells via EPs could limit their effect. Then, modulation of EPs has most often been an effective method of enhancing a compound's antimicrobial activity. This modulation can be achieved through genetic mutation in EPs (Luchao et al., 2020 ; Aires, 2011). In this study, efflux pump inhibitors were used to potentiate the antibiofilm effect of marine compound. This alternative approach is increasingly used in the medical field to make antibiotics more effective. This study aims to use a rarely employed method on marine biofilm. Three natural marine compounds with antibiofilm character, namely OB1, AS194 and AS162, were studied. These compounds have each been described to have a strong individual effect on adhesion and biofilm formation in the marine bacterium *Pseudoalteromonas ulvae* TC14 (Gozoua et al., 2019). Anti-adhesion and antibiofilm effects of these compound can be enhanced by combining them with the three coumarin derivatives VL2, VL17 and VL19. Potentiation of the anti-adhesion and antibiofilm effects was assessed by screening the compounds on adhesion and biofilm, and calculating the median concentration (EC50) on biofilm formation. These experiments were repeated with two other marine bacteria (*Pseudoalteromonas lypolitica* TC8 and *Paracoccus spp.* 4M6) to assess reproducibility.

Strategies for combating bacterial resistance

The fight against bacterial resistance relies on anti-resistant molecules. Anti-resistant molecules were associated as adjuvants to antibiotics (Gill et al., 2015). An adjuvant generally has no intrinsic antibacterial activity, but it enables an antibiotic to have a better action on its target when combined with it. Thus, a combinatorial approach is deemed opportune in order to potentiate the effects of antimicrobials (Sundaramoorthy et al., 2020; Jayan and Gupta 2023).

There are several classes of adjuvant compounds. These include efflux pump inhibitors and outer membrane permeabilizers (Urakawa et al., 2010; Abuzaid et al., 2012; Dias et al., 2022).

Efflux pump inhibition strategy

Overexpression of the efflux pump is an important mechanism of bacterial resistance, leading to the expulsion of antibiotics from bacterial cells. Pump inhibition is therefore a strategy that could restore the potency of current antibacterial compounds against resistant bacteria, and perhaps lead to the development of new compounds. RND efflux pumps are involved in the intrinsic resistance of many Gram-negative bacteria and, when expressed, lead to multiple drug resistance phenotypes in Enterobacteriaceae and *Pseudomonas aeruginosa*. EPs are potential sites for restoring antibiotic sensitivity (Wang et al., 2022). Possible inhibitors include phenylalanine-arginine ß-naphthylamide (PAßN), a dual permeabilizing and efflux pump inhibitor. This compound inhibits the efflux action of many RND family pumps and is capable of reducing intrinsic and mutational resistance to several antimicrobial compounds (Lamers et al., 2013; Luchao 2020). Another widely researched target is the NorA efflux pump of *Staphylococcus aureus*, which confers resistance to several antimicrobial agents, including fluoroquinolones (Kumar and Schweizer, 2005), giving rise to a multidrug resistance phenotype. Numerous compounds from different sources and classes have been tested for their ability to deactivate the NorA pump and restore antibiotic activity against resistant *S. aureus*. Research has also been carried out to develop fluoroquinolones to prevent efflux via the NorA pumps in order to improve their antimicrobial efficacy (Ince et al., 2002). Another possibility for reducing the deleterious effects of efflux pumps involves the use of antisense peptide nucleic acids, also known as ANPs. ANPs are synthetic nucleic acid homologues in which the phosphate polynucleotide backbone is replaced by a flexible pseudopeptide polymer. PNAs act as antisense

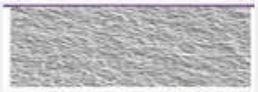
strains	TC14
Taxonomy	*Pseudoalteromonas ulvae*
Gram Type	Bacille Gram-
Morphology	Small purple colony (1,5 mm X 1.5mm)
Sampling site	Toulon military port
Surface used	fluorosilicone ELP08/55554/13
Image on VNSS Agar	
SEM Image	
References	(Briand-jaisson et al., 2014)

Figure 1. Description of the *Pseudoalteromonas ulvae* TC14 strain.

mediators, binding with high specificity to complementary DNA and RNA sequences and inhibiting gene expression and translation (Paulasova and Pellestor, 2004; Wang et al, 2022). A PNA compound was used to sensitize Campylobacter jejuni by decreasing expression of the efflux pump CmeABC, which generally confers resistance to several antimicrobials, including ciprofloxacin and erythromycin (Zhang et al., 2009).

Efflux pumps: Definitions and classification

Efflux pumps are either class-specific or responsible for Multidrug Resistance (MDR). They comprise several classes based on the form of energy utilization provided (Tahmina et al., 2017). Thus, proton dissipation is specific to the MFS, RND, and SMR families. The MATE family uses the sodium ion (Na+) as an energy source, while the ABC family draws its energy source from ATP hydrolysis (Vincent, 2004). In Gram-negative bacteria, efflux systems are often ternary protein complexes with a transmembrane pump, a periplasmic junction protein and an outer membrane porin. The most frequently encountered pumps are of the RND type, such as AcrB in *Escherichia coli* or MexB in *P. aeruginosa*. In Gram-positive bacteria, efflux systems consist solely of the pump. The most extensively studied are MFS pumps such as NorA or QacA in *S. aureus* and PmrA in *Streptococcus pneumoniae*. A few transporters have also been described in *mycobacteria* (Cattoir, 2004). The main efflux pump families are shown. A classification based on a soft spherical mask of the upper part of a pump is also possible (Shi et al., 2019).

MATERIALS AND METHODS

Description of the *Pseudoalteromonas ulvae* TC14 strain

The biological material used in this study consists of bacteria of marine origin, isolated from the Toulon roadstead, belonging to the collection (TC for Toulon Collection) of the MAPIEM laboratory. The main strain studied is *Pseudoalteromonas ulvae* TC14. It is described in Figure 1.

Preparation of *P. ulvae* TC14 strain

P. ulvae TC14 was first cultured in liquid VNSS at 20°C with 120 rpm agitation until the beginning of stationary phase. The cultures were then centrifuged at 6000 rpm for 10 min. The VNSS medium was then removed and replaced by ASW, to obtain a final bacterial concentration equal to 0.8 (OD600nm = 0.8). ASW keeps bacteria alive while limiting their growth, thus promoting their adhesion. Two other marine strains, *Pseudoalteromonas lypolitica* TC8 and *Paracoccus spp*. 4M6, were also tested in this study. They were used as control strains.

Presentation and structures of the coumarins tested in this study

The coumarin derivatives used in this study come from the Faculty of Pharmacy at the AIX University of Marseille, France. They are three molecules coded by the letters V and L, namely VL2, VL17, VL19. These molecules are previously known to be efflux pump inhibitors. The different structures are shown in Figure 2.

Preparation of coumarin-type molecules and natural marine compounds

Preparation of test molecules and compounds

Coumarins and compounds synthesized in the laboratory and other

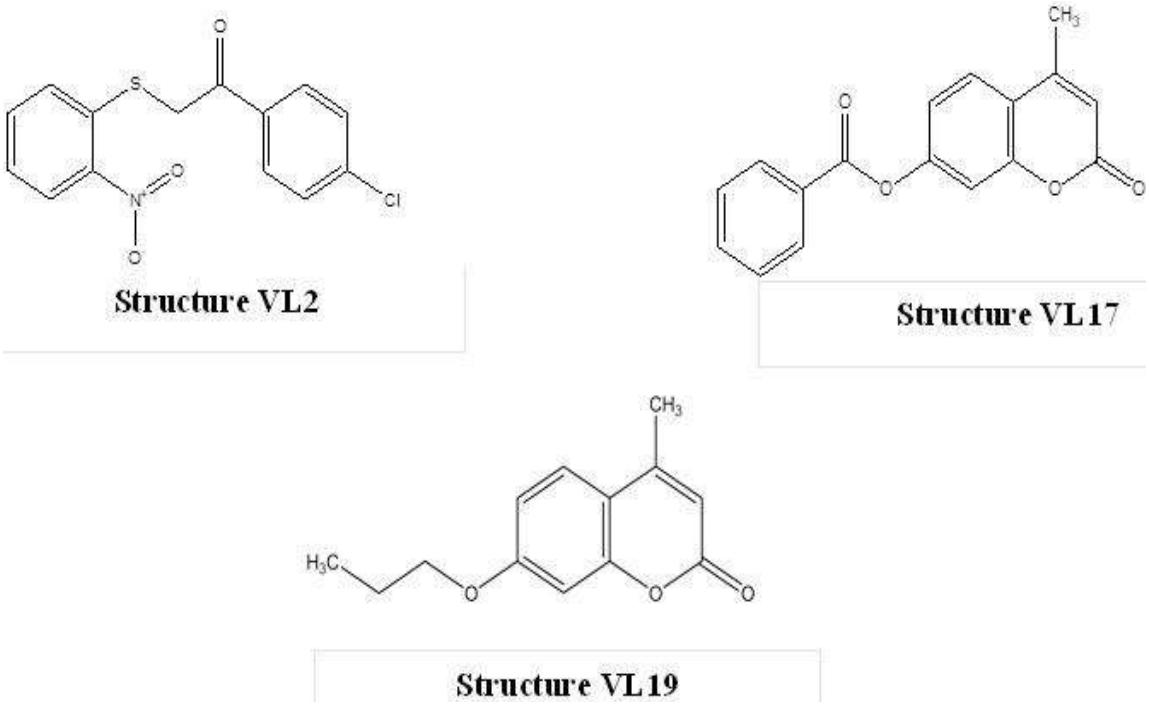


Figure 2. Schematic representations of Coumarin-type molecules.

molecules were first solubilized in DMSO to a stock concentration of 10 mM, then diluted in ASW to concentrations of 10, 50, 100, 150 and 200 µM.

Adhesion and biofilm principle and protocol

The marine bacterium *P. ulvae* TC14 has the ability to attach to natural or synthetic surfaces. These bacteria have been described to have a good adhesion capacity on polystyrene (Brian-Jaisson et al., 2014). The protocol used to perform the adhesion test was adapted from the anti-adhesion test protocol developed by Camps et al. (2011) and taken up by Aye et al. (2015). This protocol was repeated in the course of this study with the difference that the molecules tested in this case are coumarins, and the compounds synthesized in the laboratory. In some works, this technique is used to define cell migration, invasion, and adhesion strategies. It has been applied by Pijuan et al. (2019) on cancer cells. In our case, experiments were carried out in black 96-well polystyrene microplates for the adhesion test and in transparent polystyrene microplates for the biofilm test. It should be noted that during the adhesion and biofilm tests, 50 µM coumarins were added to the concentration range of marine compounds. Results were processed in TECAN, Infinit M 200 pro.

EC50 determination

EC50 is defined as the concentration capable of eliminating 50% of the effect. It correspond to the concentration of substance that elicits a response halfway between the baseline and the maximum response (Fechner et al., 2012).

EC50 value also makes it possible to avaluate an anti-biofilm compound's activity (Malouch et al., 2023). In this study, the EC50 of compounds and different combinations were determined using GraphPadPrism 5 software.

RESULTS AND DISCUSSION

Evaluation of the impact of efflux pump modulation on the anti-adhesion effect of compounds using a combinatorial approach

Adhesion tests, with combinations of antifouling compounds and coumarins, were carried out according to the adhesion protocol described by Camps et al. (2011) and taken up by Spriano et al. (2017). Coumarins previously tested alone showed no significant effect on adhesion and biofilm formation (results not reported in this study). Coumarin derivatives alone showed no antibiofilm effect here even though in the work of He et al. (2022), the coumarin derivatives tested showed anti-biofilm effects for the dispersion of advanced, pre-formed biofilms. However, the aim of this study goes beyond this aspect. Coumarin derivatives described as molecules that inhibit efflux pumps were instead tested in combination with antibiofilm compounds (OB1, AS194, AS162) with the aim of enhancing their effects, which will involve the notion of potentiation. These combination tests carried out on the adhesion of the *P. ulvae* strain showed a reinforcement of the effect of the marine compounds by the coumarins.

Although the synergistic effect was less pronounced for OB1 from 10 µM (Figure 3), for AS194 the synergistic effect was more pronounced from 25 µM. This result seems logical, since AS194 has a higher individual effect than OB1. On the other hand, a synergistic effect was only observed for VL17 and VL19 (Figure 3B, C, E and

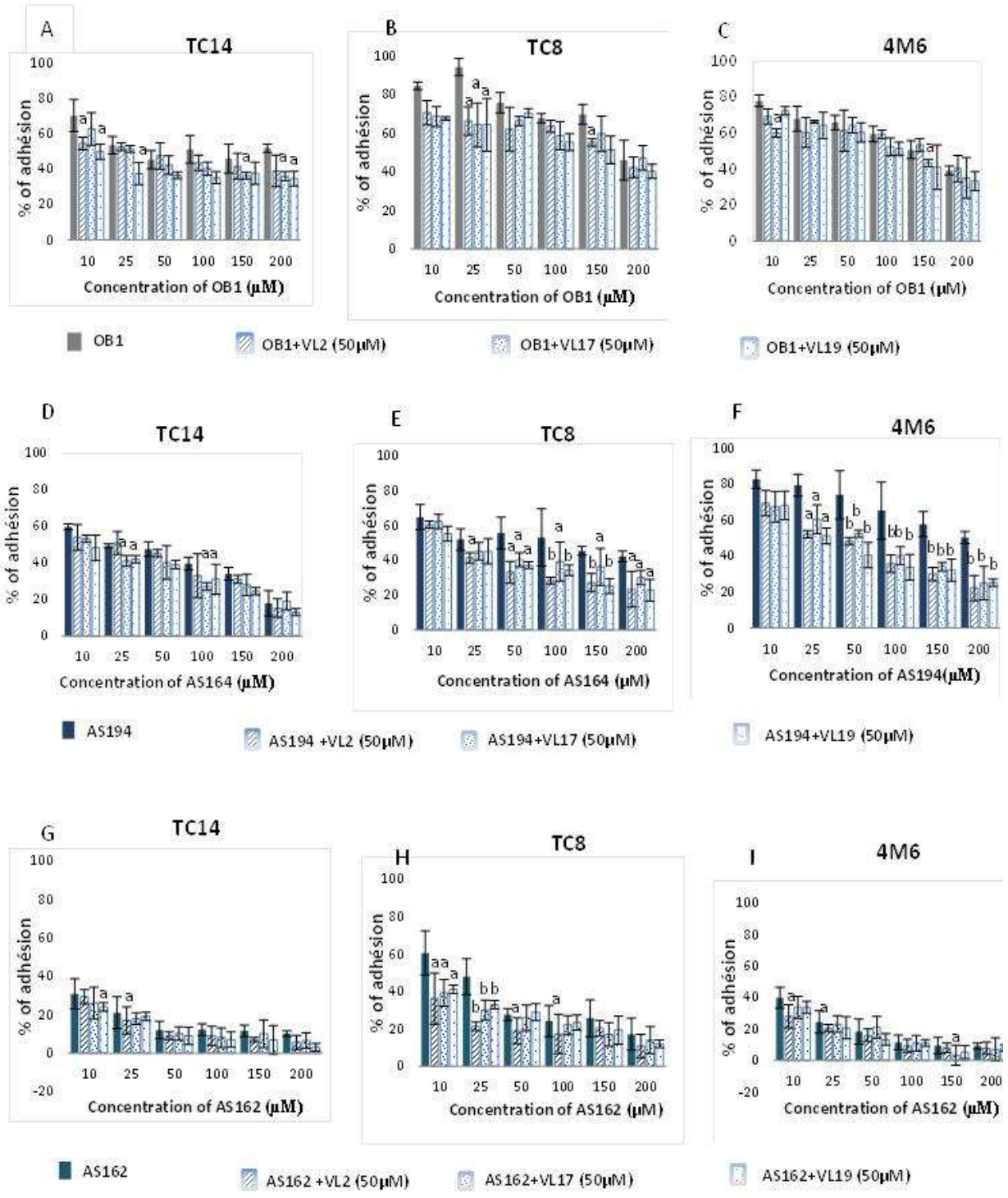


Figure 3. Effect of combining analogues and coumarins on adhesion in TC14, TC8 and 4M6. Trials were performed three times in replicates in black 96-well microplates. Assays marked with letters are significantly different (P<0.05) from the control (bacterial cultures without coumarins).

F). One of the three coumarin derivatives (VL2), in combination with the three antifouling compounds, showed no significant effect on *P. ulvae* adhesion. It has to be said that the latter was less active on the efflux pumps of this bacterium. This result was reproducible on one of the test bacteria used in this study. This was *P. lypolitica* TC8 (Figure 3H). In *Paracoccus sp*ecies 4M6, on the other hand, only slight variations in the effect of

AS162 were observed. This indicates a low level of coumarin activity in this bacterium. It should nevertheless be noted that the results observed in this chapter suggest that inhibition of efflux pumps is a pathway for potentiating the anti-adhesion effect in *P. ulvae* TC14.

Evaluation of the impact of efflux pump modulation on the biofilm effect of compounds using a combinatorial approach

Combination tests on biofilm were carried out according to the principle of Li et al. (2012) and repeat by Hawas et al. (2022). The results presented in Figure 4 showed a very marked synergistic effect of the combinations on biofilm formation in *P. ulvae* TC14 (Figures 4A, D and G) and *P. lipolytica* TC8 (Figures 4B, E and H). These results are similar to those obtained previously after the adhesion test. The adhesion stage precedes biofilm formation. Thus, a molecule with an anti-adhesive effect will have an impact on the next stage, which is biofilm formation (Rubio, 2002). In *Paracoccus spp*. 4M6 (Figures 4C, F, I), the synergistic effect of the combinations was less marked on biofilm formation, although there was a significant effect with OB1 at high concentrations (Figure 4C). In contrast, the combination of AS162 and coumarins (Figure 4I) showed a marked synergistic effect at low AS162 concentrations (10 and 25 µM).

Above 50 µM AS162, there was no synergistic effect on biofilm formation in 4M6. This shows that from 50 µM AS162 upwards, the effect of the combined molecule reaches a stationary phase. The potentiation of the bacteria's antibiofilm effect seems to be more real with relatively low concentrations of antibiofilm compounds. Above 50 µM, there seems to be a form of antagonism between the two types of compound. This is explained by the results obtained in Figure 4F, which reveal a relapse in the antibiofilm effect of compound AS194 on 4M6 when 200 µM of antibiofilm compound is reached. These results show that the quantities of antibiofilm products must be carefully adjusted to avoid antagonism.

This was explained in the work of Côté et al. (2016), who demonstrated cases of antagonism between two antibiofilm effects in which one of the effects attenuated the other in the event of concentration variation. In our case, this method of potentiating the antibiofilm effect may appear complex, as it would require rigorous monitoring of the screening data to identify the optimum concentrations. It should be noted that there are small nuances in the variation of biofilm from one bacterium to another. This clearly explains why each bacterium has a different resistance capacity. Based on what can be seen in Figure 4, 4M6 appears to be more resistant than the other two bacteria. TC14, on the other hand, shows sensitivity to the combined molecules, except that at high concentrations (200 µM), it seems to have a relatively weak effect. This aspect will be better explained in the next parts of our study dealing with EC50 values. The potentiation of the compounds' antibiofilm effect by the screening method should call on other experimental data to better appreciate the molecules' efficacy. In addition to the results obtained in Figures 3 and 4, we have calculated EC50 values, which are the concentrations likely to eliminate 50% of the effect.

Highlighting the potentiation of the anti-biofilm effect of natural compounds by determining median effective concentrations (EC50)

To better assess the potentiation of the antibiofilm effect of natural compounds (OB1, AS1984, AS162) by their combination with coumarin derivatives, EC50s were determined. The lower the median effective concentration (EC50), the more effective the compound. The synergistic effect of the combinations was very marked with both OB1 and AS194 on biofilm formation in P. ulvae TC14. In combination, these two compounds significantly reduced their baseline EC50 in P. ulvae TC14. This EC50 decrease was marked by reduction rates ranging from 69.2 to 75.3% for OB1 and from 65.4 to 77% for AS194 (Table 1). This indicates the high activity of both compounds in the presence of coumarin derivatives. This case of synergism is all the more interesting as we are dealing here with two types of compounds with different targets. On the one hand, natural antibiofilm compounds target biofilm formation, and on the other, coumarin derivatives potentially target efflux pumps. Similar results were found in the work of Ebrahimi et al. (2018). However, these authors' work was more in the medical field. They found cases of synergism between different compounds but also established an isobolographic approach to determine limiting values. Isobologram calculation could be an interesting approach in this study, but the main objective was to assess the potentiation of effects. This is a method increasingly used in the medical field, and less similar work has been done on marine biofilms.

Combinations with AS162 revealed a relatively low EC50 reduction in *P. ulvae* TC14. In the case of *P. lipolytica* TC8 and *Paracoccus spp.* 4M6, the synergistic effect proved highly significant, with OB1 EC50s showing a sharp reduction in both bacteria (in excess of 54%). This synergistic effect was also felt on AS162 in *P. lipolytica* TC8, where the EC50 reduction was also over 54% in the presence of each of the coumarins. In *Paracoccus spp*. 4M6, EC50s corresponding to combinations including AS194 revealed less marked synergism than those containing OB1 and AS162 (Table 1). In the previous part of our study, antagonism was noted in 4M6 at high concentrations of antibiofilm compounds. In the case of EC50s, there were no cases of antagonism, although there were cases of slight

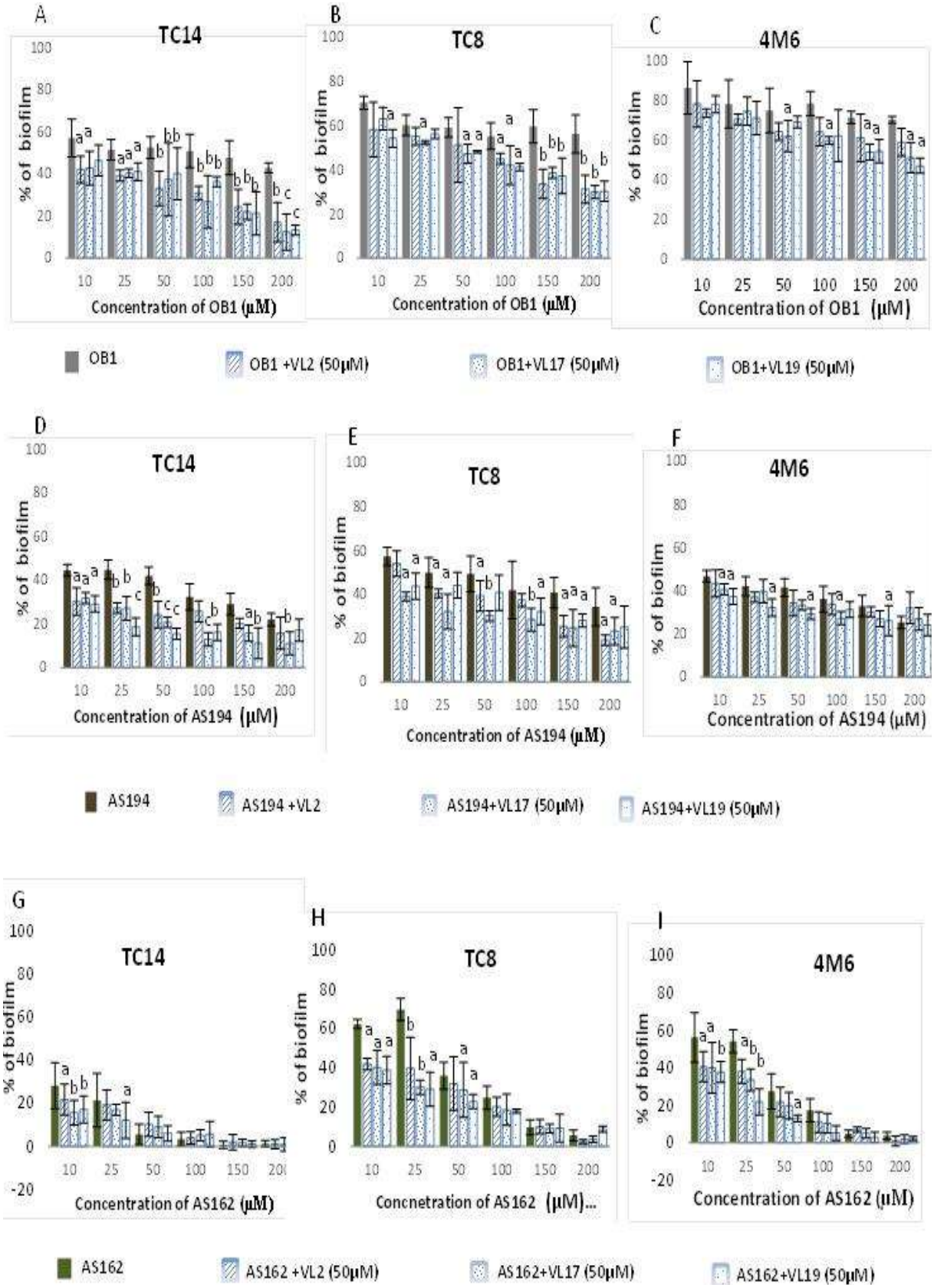


Figure 4. Effect on biofilm formation in TC14, TC8 and 4M6. Trials were performed three times in replicates in 96-well transparent microplates. Lettered assays are significantly different ($P<0.05$) from the control (bacterial cultures with analog alone).

Table 1. Determination of EC50s of combinations on strain biofilm and their reduction rates.

Strains	Compounds and combination	CE50 (µM) of biofilm formation	CE50 Reduction rate (%)
TC14	OB1	69.85±5.9	
	OB1+VL2 (50 µM)	21.5±3.1[c]	69.2
	OB1+VL17 (50 µM)	17.5±4.0[c]	75.0
	OB1+VL19 (50 µM)	17.3±3.5[c]	75.3
	AS194	24.8±3.9	
	AS194+VL2 (50 µM)	5.7±0.8[c]	77.0
	AS194+VL17 (50 µM)	7.5±1.9[c]	69.8
	AS194+VL19 (50 µM)	8.59±5.7[c]	65.4
	AS162	4.4±1.3	
	AS162+VL2 (50 µM)	2.4±1.1[b]	45.5
	AS162+VL17 (50 µM)	2.7±1.2[b]	38.6
	AS162+VL19 (50 µM)	3.6±0.8	18.2
TC8	OB1	127.9±3.6	
	OB1+VL2 (50 µM)	47.1±2.9[c]	63.2
	OB1+ VL17 (50 µM)	48.5±2.7[c]	62.1
	OB1+ VL19 (50 µM)	50.9±3.1[c]	60.2
	AS194	48.8±5.0	
	AS194+ VL2 (50 µM)	24.0±2.9[b]	50.6
	AS194+ VL17 (50 µM)	13.8±2.5[c]	71.7
	AS194+ VL19 (50 µM)	25.4±2,4[b]	48.0
	AS162	29.37±4.0	
	AS162+ VL2 (50 µM)	9.4±2.1[c]	68.0
	AS162+ VL17 (50 µM)	10.4±1.0[c]	64.6
	AS162+ VL19 (50 µM)	13.4±1.5[c]	54.4
4M6	OB1	328.0±9.5	
	OB1+ VL2 (50 µM)	140.6±4.8[c]	57.1
	OB1+ VL17 (50 µM)	134.3±7.0[c]	59.1
	OB1+ VL19 (50 µM)	172.9±6.9[b]	47.3
	AS194	26.91±4.6	
	AS194+ VL2 (50 µM)	13.6±0.9[b]	49.4
	AS194+ VL17 (50 µM)	17.8±2.0[c]	33.8
	AS194+ VL19 (50 µM)	20.5±1.9[a]	23.8
	AS162	18.8±2.1	
	AS162+ VL2 (50 µM)	6.4±1.9[c]	66.0
	AS162+ VL17 (10 µM)	9.181±1.1998[b]	51.01
	AS162+ VL19 (5 µM)	10.34±1.77115[a]	45.2

Affected letter values (a, b and c) are significantly different from controls (natural compounds used alone).

reduction, particularly in 4M6. EC50 calculations give a good idea of the potentiation of the antibiofilm effect of natural marine compounds.

Conclusion

This study evaluated the potentiation of the anti-adhesive and antibiofilm effects of three natural marine compounds. This potentiation was made possible by combining these compound with coumarin derivatives known to be efflux pump inhibitors. It was thus possible to identify another pathway for modulating efflux pumps in marine bacteria, with the aim of enhancing the antibiofilm effect of thecompounds.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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