

DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**IN-SILICO MODELS IN ANTIFOULING PAINT
TECHNOLOGY**



by
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September, 2022
İZMİR

IN-SILICO MODELS IN ANTIFOULING PAINT TECHNOLOGY

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In Partial Fulfillment of the Requirements for the Degree of Master of Science
In Chemistry**

**by
Cengizhan DAĞ**

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M.Sc THESIS EXAMINATION RESULT FORM

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Cengizhan DAĞ

IN SILICO MODELS IN ANTIFOULING PAINT TECHNOLOGY

ABSTRACT

The coating of inanimate surfaces by living organisms is called biofouling. Biofouling, which is a necessity of the ecosystem in the marine field, causes undesirable consequences for the marine industries. The wide living space provided by aquaculture nets to biofouling creatures, causes the invasion of the organisms to this surface. Due to the biofouling, the pores of the cage nets are closed, the total mass and the hydrodynamic friction increases and damages the cage system carriers. The net pores, which are closed due to biofouling, reduce the passage of oxygen-rich sea water into the cage and prevent the biological wastes from leaving the cage. In this study, the consistency of *in-silico* techniques was tested to evaluate the antifouling properties of coatings. The target proteins of *Balanus amphitrite*, *Hydroides elegans*, *Bugula neritina* and *Ulva lactuca* were used in molecular docking analyses. In order to coat the aquaculture nets, rosin, paraffin wax, styrene-acrylic co-polymer and lanolin were used as main binders. Econeal, diuron and irgarol were used as biocides in field experiment. According to the results of molecular docking analysis, econeal achieved the highest binding affinities to the targets proteins of *B. amphitrite*, *H. elegans*, *B. neritina* and *U. lactuca* (-7.0 kcal/mol, -6.0 kcal/mol, -9.2 kcal/mol and -6.7 kcal/mol, respectively). In field tests, it has also been observed that econeal gives the best performance compared to diuron and irgarol. In conclusion, due to the high correlation was observed, molecular docking algorithms will take traditional antifouling technology development processes to the next level.

Keywords: Aquaculture, antifouling, biofouling, *in-silico*, molecular docking

ANTIFOULING BOYA TEKNOLOJİSİNDE İN-SİLİKO MODELLER

ÖZ

Cansız yüzeylerin canlı organizmalar tarafından kaplanmasına biyofouling denir. Denizel alanda ekosistemin bir gereği olan biyofouling, denizel endüstriler için istenmeyen sonuçlara yol açmaktadır. Akuakültür ağlarının biyofouling oluşturan canlılara sağladığı geniş yaşam alanı, organizmaların bu yüzeye istilasına neden olur. Biyofouling nedeniyle kafes ağlarının gözenekleri kapanır, toplam kütle ve hidrodinamik sürtünme artar ve kafes sistem taşıyıcılarına zarar verir. Biyofouling nedeniyle kapanan ağ gözenekleri, oksijence zengin deniz suyunun kafese geçişini azaltır ve biyolojik atıkların kafesten çıkmasını engeller. Bu çalışmada, antifouling kaplamaların antifouling özelliklerini değerlendirmek için in-siliko tekniklerin tutarlılığı test edilmiştir. Moleküler doklama analizlerinde *Balanus amphitrite*, *Hydroides elegans*, *Bugula neritina* ve *Ulva lactuca*'nın hedef proteinleri kullanıldı. Kültür balıkçılığı ağlarını kaplamak için ana bağlayıcı olarak rosin, parafin mumu, stiren-akrilik kopolimer ve lanolin kullanılmıştır. Alan testlerinde biyosit olarak econea, diuron ve irgarol kullanılmıştır. Moleküler doklama analiz sonuçlarına göre econea, *B. amphitrite*, *H. elegans*, *B. neritina* ve *U. lactuca* hedef proteinlerine en yüksek bağlanma afinitesini elde etmiştir (Sırasıyla, -7.0 kcal/mol, -6.0 kcal/mol, -9.2 kcal/mol and -6.7 kcal/mol). Alan testlerinde econea'nın diuron ve irgarol ile karşılaştırıldığında en iyi performansı verdiği gözlemlenmiştir. Sonuç olarak, gözlemlenen yüksek korelasyon sayesinde moleküler doklama algoritmaları, geleneksel antifouling boya teknolojisi geliştirme süreçlerini bir sonraki seviyeye taşıyacaktır.

Anahtar kelimeler: Antifouling, akuakültür, biyofouling, in-siliko, moleküler doklama

CONTENTS

	Page
M.Sc THESIS EXAMINATION RESULT FORM	ii
ACKNOWLEDGMENTS.....	iii
ABSTRACT	iv
ÖZ.....	v
LIST OF FIGURES.....	ix
LIST OF TABLES.....	xiv
ABBREVIATIONS.....	xvi
CHAPTER 1 - INTRODUCTION	1
1.1 Aquaculture Based World Food Stock.....	1
1.2 Aquaculture in Türkiye.....	3
1.3 Marine Biological Fouling	4
1.4 Biofouling in Aquaculture	6
1.5 Main Biofouling Organisms Observed Along the Turkish Coastline	7
1.5.1 <i>Balanus amphitrite</i>	7
1.5.2 <i>Hydroides elegans</i>	8
1.5.3 <i>Bugula neritina</i>	9
1.5.4 <i>Ulva lactuca</i>	10
1.6 Adverse Effects of Biofouling on Fishing Nets	11
1.7 Historical Development of Antifouling Measures in General.....	13
1.8 Booster Biocides.....	15
1.9 Performance Testing of Antifouling Coatings.....	17
1.10What is Bioinformatics.....	20
1.10.1 The Molecular Docking.....	20

1.10.2	AutoDock Vina	23
1.10.3	Scoring Functions	24
1.10.4	Validation of the Docking Method	24
1.10.5	Wet-lab 3D Structure Analysis of Proteins	24
1.10.6	Protein Databases.....	25
1.10.7	RCSB Protein Database	27
1.10.8	Homology Modelling.....	27
1.10.9	Receptor Types - Subtypes and Activation	28
1.10.10	Conformational Change in the Attachment Region.....	29
1.11	Aims of The Thesis	30
CHAPTER 2 – MATERIALS AND METHODS		31
2.1	Materials	31
2.1.1	Field Experiment	31
2.1.2	Preparation of The Fish-Cage Net Samples.....	32
2.2	Preparation of Coatings	32
2.2.1	Preparation of Lanolin and Paraffin Wax Coatings.....	32
2.2.2	Preparation of Rosin-Based Coating	33
2.2.3	Preparation of Styrene-Acrylic Copolymer-Based Coatings.....	34
2.3	<i>In-silico</i> Molecular Docking Analysis.....	35
2.3.1	Target and Ligands	35
2.3.2	Structural Analysis and Validation of Target	35
2.3.3	Energy Minimization	36
2.3.4	Molecular Docking	36
2.3.5	Molecular Docking Software and Analysis Procedure.....	36
2.4	Tensile Strength Test on Net Samples.....	40

2.5	Biofouling Degree Assessment Criteria	41
CHAPTER 3 - RESULTS		42
3.1	<i>In-silico</i> Molecular Docking Results	42
3.1.1	<i>In-silico</i> Analysis of <i>Balanus amphitrite</i> – G Protein Coupled Receptor (GPCR)	42
3.1.2	<i>In-silico</i> Analysis of <i>Hydroides elegans</i> – Cytochrome C Oxidase Subunit 1 (COI)	51
3.1.3	<i>In-silico</i> Analysis of <i>Bugula neritina</i> – Cytochrome B (Cyt-b)	56
3.1.4	<i>In-silico</i> Analysis of <i>Ulva lactuca</i> – Photosystem II D1 (P-II-D1)	62
3.2	Field Test Results	68
3.2.1	The Field Test Result of Non-biocidal Coatings	68
3.2.2	The Field Test Result of Biocide Containing Coatings	72
3.3	The Results of Tensile Strength Test on Net Samples	75
CHAPTER 4 - DISCUSSION		77
4.1	The Field Test Results	77
4.2	The <i>In-silico</i> Results	79
CHAPTER 5 - CONCLUSION		89
REFERENCES		91

LIST OF FIGURES

	Page
Figure 1.1 The general representation of biofouling progression on submerged surfaces	5
Figure 1.2 The biofouling related <i>Balanus amphitrite</i>	8
Figure 1.3 The biofouling colony of <i>Hydroides elegans</i> (White spike like tubules are the outer shell of this organism)	9
Figure 1.4 The close-up view of <i>Bugula neritina</i>	9
Figure 1.5 The biofouling of <i>Ulva lactuca</i>	11
Figure 1.6 Development of antifouling technologies	13
Figure 1.7 The digital layout of PDB files	27
Figure 2.1 a) The overall view of the experiment field b) The sample submerged in the sea	31
Figure 2.2 The sample PE cage net in 10x10cm dimensions	32
Figure 2.3 Lanolin (a) and paraffin wax (b) are melted at 100°C	33
Figure 2.4 The preparation procedure of rosin coating	33
Figure 2.5 SAC-E + 50% PEG600 (w/w) preparation procedure	34
Figure 2.6 Sample nets coated with PEG600-free and PEG600-added SAC-E emulsions are cured in an oven at 120°C	34
Figure 2.7 The first and second filed experiment samples	35
Figure 2.8 a) Tensile strength test device, b) Single rope test sample that placed between holder clamps	40
Figure 2.9 The rope diameter evaluation via thickness gauge	41
Figure 3.1 The ramachandran plot of 3D modelled probable G coupled protein (GPCR) of <i>Balanus amphitrite</i>	43
Figure 3.2 The molecular surface of extracellular residues of G protein coupled receptor protein were visualized (A). The rest of the residues were blacked out to eliminate image clutter (B)	44
Figure 3.3 Best binding pose and related interactions of Chlorothalonil - G protein coupled receptor complex	47

Figure 3.4 Best binding pose and related interactions of Dichlofluanid - G protein coupled receptor complex	48
Figure 3.5 Best binding pose and related interactions of Diuron - G protein coupled receptor complex.....	48
Figure 3.6 Best binding pose and related interactions of Econeal - G protein coupled receptor complex.....	49
Figure 3.7 Best binding pose and related interactions of Irgarol - G protein coupled receptor complex.....	49
Figure 3.8 Best binding pose and related interactions of CMPD101 - G protein coupled receptor complex.....	50
Figure 3.9 Best binding pose and related interactions of Gallein - G protein coupled receptor complex.....	50
Figure 3.10 The ramachandran plot of 3D modelled cytochrome c oxidase subunit 1 (COI) of <i>Hydroides elegans</i>	52
Figure 3.11 The molecular surface of cytochrome c oxidase subunit 1 of <i>Hydroides elegans</i>	53
Figure 3.12 Best binding pose and related interactions of Econeal - cytochrome c oxidase subunit 1 complex	55
Figure 3.13 Best binding pose and related interactions of Diuron - cytochrome c oxidase subunit 1 complex	55
Figure 3.14 Best binding pose and related interactions of Irgarol - cytochrome c oxidase subunit 1 complex	56
Figure 3.15 The ramachandran plot of 3D modelled cytochrome b of <i>Bugula nertina</i>	58
Figure 3.16 The molecular surface of cytochrome b of <i>Bugula nertina</i>	59
Figure 3.17 Best binding pose and related interactions of Econeal - cytochrome b complex	61
Figure 3.18 Best binding pose and related interactions of Diuron - cytochrome b complex	61
Figure 3.19 Best binding pose and related interactions of Irgarol - cytochrome b complex	62

Figure 3.20 The ramachandran plot of 3D modelled photosystem II D1 (P-II-D1) of <i>Ulva lactuca</i>	64
Figure 3.21 The molecular surface of photosystem II D1 (P-II-D1) of <i>Ulva lactuca</i>	65
Figure 3.22 Best binding pose and related interactions of Econeal - photosystem II D1 complex	67
Figure 3.23 Best binding pose and related interactions of Diuron - photosystem II D1 complex	67
Figure 3.24 Best binding pose and related interactions of Irgarol - photosystem II D1 complex	68
Figure 3.25 The field results of uncoated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later.....	69
Figure 3.26 The field results of paraffin wax coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	69
Figure 3.27 The field results of styrene-acrylic copolymer coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	70
Figure 3.28 The field results of uncoated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later.....	70
Figure 3.29 The field results of uncoated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later.....	71
Figure 3.30 The field results of rosin polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later.....	71
Figure 3.31 The microscope images of the community of living organisms on biocide-free coatings, hard fouling organisms a) <i>Bugula neritina</i> , b) <i>Balanus amphitrite</i> , c) <i>Hydroides elegans</i>	72
Figure 3.32 The field results of rosin (econeal 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	73
Figure 3.33 The field results of rosin (diuron 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	73

Figure 3.34 The field results of rosin (irgarol 1051 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	73
Figure 3.35 The field results of lanolin (econeal 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	74
Figure 3.36 The field results of lanolin (diuron 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	74
Figure 3.37 The field results of lanolin (irgarol 1051 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later	75
Figure 4.1 The field test results of biocide-free coatings and uncoated control group, Columns; a) control group, b) paraffin wax coated net, c) styrene-acrylic copolymer coated net, d) styrene-acrylic copolymer – polyethylene glycol 600 cross link binder coated net, e) lanolin coated net and f) rosin coated net	78
Figure 4.2 Field test results of uncoated control group and biocidal rosin coating formulation (1-4; uncoated nets, 5-8; 3% econeal, 9-12; 3% diuron, 13-16; 3% irgarol 1051).....	78
Figure 4.3 Field test results of uncoated control group and biocidal lanolin coating formulation (1-4; uncoated nets, 5-8; 3% econeal, 9-12; 3% diuron, 13-16; 3% irgarol 1051).....	79

LIST OF TABLES

	Page
Table 1.1 Booster biocides and their open molecular structures.....	16
Table 1.2 Some of the molecular docking algorithms and web links	23
Table 3.1 Ramachandran plot statistics for the modelled G protein coupled receptor protein.....	43
Table 3.2 The molecular docking scores of all biocides to G protein coupled receptor protein of <i>Balanus amphitrite</i> . The symbol “*” is indicated the scores of known inhibitors.....	46
Table 3.3 The best scores of molecular docking analysis. The symbol “*” is indicated the best scores of known inhibitors.....	47
Table 3.4 Ramachandran plot statistics for the 3D modeled cytochrome c oxidase subunit 1 protein.	53
Table 3.5 The molecular docking scores of all biocides to cytochrome c oxidase subunit 1 of <i>Hydroides elegans</i>	54
Table 3.6 Ramachandran plot statistics for the 3D modeled cytochrome b protein	58
Table 3.7 The molecular docking scores of all biocides to cytochrome b protein of <i>Bugula neritina</i>	60
Table 3.8 Ramachandran plot statistics for the 3D modeled photosystem II D1 protein.....	64
Table 3.9 The molecular docking scores of all biocides to photosystem II D1 protein of <i>Ulva lactuca</i>	66
Table 3.10 The data related tensile strength test	76
Table 4.1 The best binding scores of target-ligand complexes.	80
Table 4.2 Best binding pose related Target-Ligand interactions and interaction types	81
Table 4.3 Biofouling scores, <i>in-silico</i> molecular docking scores and calculated correlation coefficient of <i>B. amphitrite</i> . (Evaluated biofouling score obtained from field tested biocidal-rosin coatings).	82

Table 4.4 Biofouling scores, <i>in-silico</i> molecular docking scores and calculated correlation coefficient of <i>B. amphitrite</i> . (Evaluated biofouling score obtained from field tested biocidal-lanolin coatings).....	83
Table 4.5 Biofouling scores, <i>in-silico</i> molecular docking scores and calculated correlation coefficient of <i>H. elegans</i> . (Evaluated biofouling score obtained from field tested biocidal-rosin coatings).....	83
Table 4.6 Biofouling scores, <i>in-silico</i> molecular docking scores and calculated correlation coefficient of <i>H. elegans</i> . (Evaluated biofouling score obtained from field tested biocidal-lanolin coatings).....	83
Table 4.7 Biofouling scores, <i>in-silico</i> molecular docking scores and calculated correlation coefficient of <i>B. neritina</i> . (Evaluated biofouling score obtained from field tested biocidal-rosin coatings).....	84
Table 4.8 Biofouling scores, <i>in-silico</i> molecular docking scores and calculated correlation coefficient of <i>B. neritina</i> . (Evaluated biofouling score obtained from field tested biocidal-lanolin coatings).....	84

ABBREVIATIONS

TBT	: Tributyltin
SAC-E	: Styrene acrylic copolymer emulsion
PEG 600	: Polyethylene glycol 600
XRD	:X-ray diffraction
NMR	: Nuclear magnetic resonance
EM	: Electron microscopy
GPCR	: G protein coupled receptor
COI	: Cytochrome c oxidase subunit 1
Cyt-b	: Cytochrome b
P-II-D1	: Photosystem II D1

CHAPTER 1

INTRODUCTION

1.1 Aquaculture Based World Food Stock

Aquaculture can be described as the production of aquatic organisms under optimum environmentally controlled conditions in order to increase their current production and to offer them to the benefit of people without disturbing the ecological structures and balances of water resources (Wang et al., 2017). With the rapid development of industry, environmental pollution has become one of the most important problems of today. Environmental pollution causes a decrease in marine resources and many living organisms consumed by humans as food, and as a result, breeding opportunities have been sought in order to close this gap. In recent years, fishery has gained importance due to the rapid increase in the human population and the lack of protein sources. With the increasing population in the world, the increase in the demand for living marine resources have made the aquaculture sector more attractive and it is no longer sufficient to meet this demand through fishing. Today, nearly half of the aquaculture products are produced in ocean, sea and inland waters. With the developments in recent years, the number of products that can be obtained by fishing from the sea, the decrease in large fish herds compared to the past, the disappearance of some species due to overfishing, the deterioration of the ecological balance in the seas has increased the importance of aquaculture once again (Şahin, 2011).

Aquaculture (the cultivation of aquatic plants and animals) dominates aquacultural production of food in Asia and around the world; Asian countries produce more than 91% of global aquaculture products. According to the records, 102.9 million tons of aquatic products produced in Asian countries at 2017 and overall fisheries and marine production already exceeds global capture fisheries by more than 18.32 million tons (FAO, 2019). Global production of aquaculture products reached to 110.2 million tons with finfish, mollusks, and aquatic plants. (FAO, 2018). Despite the global aquaculture's yearly rate of growth falling to an average of 5.8% from 2000 to 2016, it

is growing faster compare to other food production sectors (FAO, 2018). More precisely, over the previous few decades, marine shellfish farming has grown significantly with the production industry of Asia (production capacity more than 90% of global tonnage). Above the half of global production of mariculture products mostly made up of *Crassostrea* spp. and the *Ruditapes philippinarum* (FAO, 2018). Over the last decade finfish aquaculture has grown by more than 70%, with freshwater fish accounting for 86% of the total. Salmonids, such as *Salmo salar*, is responsible from 43% of salt-water aquaculture, according to the literature on marine biofouling in management and aquaculture of finfish (FAO, 2018). Finally, aquatic plants are gathered across the world for direct human food, medicinal and cosmetic thickening agents, animal feed and fertilizers (FAO, 2018). The primary contributor to this development has been increasing production of tropical macroalgae *Kappaphycus alvarezii* and *Eucheuma* spp. in Indonesia (FAO, 2018).

It is thought that total protein consumption, which is essential for humans, will increase depending on the increase in the population of the world (Min et al. 2016). Protein consumption is of great importance for human metabolisms since essential amino acids are not be able to synthesized. To overcome this issue, essential amino acids should be taken via protein sources daily. Since there is a decreasing trend in traditional protein sources in global level, it is very clear that a protein crisis can be experienced in very near future. European Union has recently given permission using dried *Tenebrio molitor* larva in the production of biscuits. This sample clearly show that alternative protein sources should be explored (EFSA, 2021). Türkiye which is surrounded from its three side with marine ecosystems in which aquaculture products can be stimulated. Fish farms mainly based on sea bream and sea bass are common along Turkish waters, alternative aquaculture products can also be discovered and also cultured within marine ecosystems (Rad & Şen, 2016). In all these innovative studies, the platforms are exposed to the fouling organisms and therefore, all surfaces are covered by fouling organisms. In order to get rid of negative effects of fouling organisms, anti-fouling coatings should be developed. In addition, food stocks are known to become insufficient for the human population. Therefore, it has become very

important to find effective solutions to the biofouling problem encountered in seafood farming, which constitutes a large part of the world's food stock (FAO, 2018).

1.2 Aquaculture in Türkiye

The share of farming, which had a share of 7% in 1980 in Türkiye, increased continuously in total production and increased to 40% in 2011. Another remarkable point is that although the amount of aquaculture obtained by hunting has increased by 39% in the last 30 years, it is seen that the organisms obtained from aquaculture has increased more than 13 times, making a significant contribution to the increase in total production. While the total world aquaculture production reached 170.9 million tons in 2016, 90.92 million tons (53%) of this was obtained by hunting and 80.07 million tons (47%) by aquaculture (FAO, 2016). Türkiye has an important place in the world in terms of aquaculture production, not only with hunting activities, but also with aquaculture activities. As a country surrounded by seas on three sides, Türkiye is very convenient in terms of its lakes, dams, streams, spring waters and aquaculture potential. Due to this natural structure, the importance of aquaculture activities has gradually increased in our country, which has a rich aquaculture potential in sea and inland waters. In addition, its climate zone and geographical structure make it possible to increase production in terms of aquaculture. In 2016, production by hunting in Türkiye reached 431.9 thousand tons, while aquaculture production was 240.3 thousand tons. On the other hand, if we look at the amount of aquaculture production in Türkiye in the last ten years, it is seen that the production realized through fishing has decreased by an average of 2% annually, while the aquaculture has grown by an average of 8% annually. In the last 10 years, the total amount of production in aquaculture has almost doubled (TÜİK, 2017). According to scientific researches, it is estimated that the investment in aquaculture will increase further in the coming years, the amount of cultivated organisms obtained through aquaculture will be equal to the amount of organisms obtained by hunting in 2030, and the aquaculture sector will surpass the hunting sector in the long run. One of the main reasons for this increase in aquaculture is the developments in cage systems. Because the aquaculture activities were carried out with the net cage system applied in the open seas or in the bays. In

this respect, the use of textile-based nets that have the least impact on the environment, which enables more efficient production in fish farming in cage systems, is an issue that needs attention (TÜİK, 2017).

Sea fish farms in Türkiye started production late compared to both the leading countries in the world and most European countries. Salmon production trials in the Black Sea, undertaken with the support of Norwegian experts, were not very successful at first; Towards the end of the 1980s, sea bream and sea bass farms were established in the Aegean. Although it did not reach a significant production volume until the 2000s, especially after the economic crisis in 2001, with the help of government incentives, the sector has shown a remarkable growth in trout, sea bream and sea bass production in the last 15 years. It has also been reported that aquaculture studies of alternative species such as trout, carp, eel in the inland waters of our country, and sea bream and sea bass, and alternative species such as tuna, turbot, sea bass, lark, sinagrit, fangiri, pointed cape (Akbulut, 2004).

1.3 Marine Biological Fouling

Deposition of living organisms on artificial surfaces can be described as biofouling. Marine biofouling is known as undesirable settlement of algae, bacteria, diatoms, animals and plants on any immersed surfaces in marine water. When an artificial surface such as ship's hull, oil pipe line, mariculture nets etc. is immersed in marine water, biological settlement rapidly occurs (Yebra et al. 2004; Gule et al., 2016).

When the man-made surfaces are immersed in marine water, they are exposed to different physicochemical and biological stages that result in the settlement of micro- and macro- living organisms. The settlement of organic film (conditioning film) layer known as the first stage of biofouling on immersed surface. The conditioning film consists of proteins, carbohydrates and inorganic salts which readily present in the sea water (Vinagre et al., 2020). The van der Waals forces are the main factors that affect the formation of a conditioning film layer (Flemming et al., 2008). After the formation of conditioning film, the surface is invaded by living organisms. The first colonizers

are diatoms, bacteria and simple single-celled organisms. Particularly, bacteria produce extracellular polymeric substances (EPS) for easier settlement on the immersed surface. The production of EPS is caused to development of slime layer that finalized the second stage of biofouling. After the formation of slime layer, second colonizers start to cover the surface. Most known form of second colonizers are micro algae and algal spores. In the step following the secondary colonizers is the final stage. The biofouling process takes its final form when some macrofouling organisms such as molluscs, zebra mussels and tunicates settle on the surface (Flemming et al., 2008; Dafforn et al., 2011). The stages of marine biological fouling were represented at Figure 1.1.

Fouling of the natural surfaces under the sea by living organisms is a necessity of the ecosystem and it is also beneficial for the marine life. However, the fouling of man-made surfaces by living organisms results in undesirable effects. Sea transportation industry and industrial aquatic processes such as waste water treatment, water desalting and aquaculture are heavily affected by marine and freshwater biofouling. Ship's hull, aquaculture nets, oil pipelines and other under water artificial surfaces are in danger caused by biofouling (Rosenhahn et al., 2010; Yusim et al., 2017).

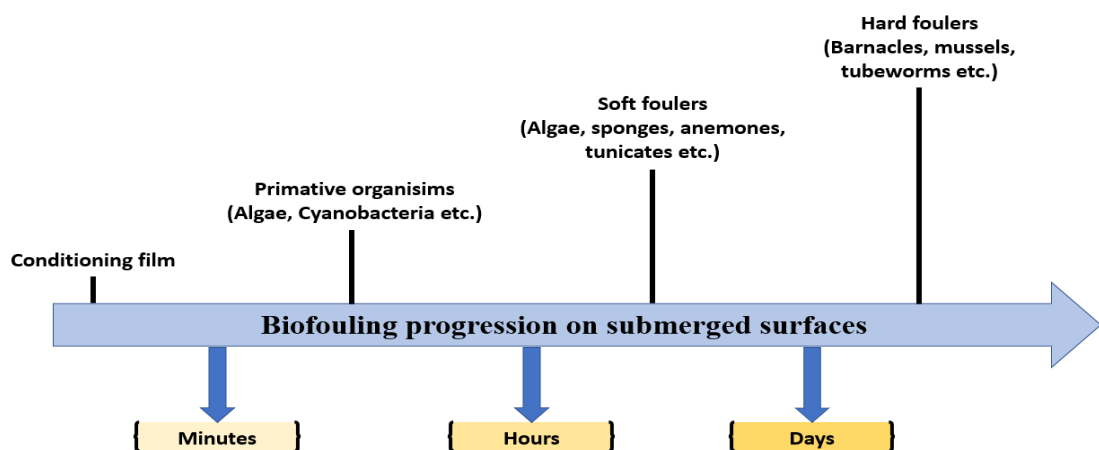


Figure 1.1 The general representation of biofouling progression on submerged surfaces

1.4 Biofouling in Aquaculture

In aquaculture, biofouling is one of the most significant obstacles to effective and long-term production (Dürr & Watson, 2010). Marine biofouling is known as unwanted deposition of aquatic organisms on man-made surfaces, and it is a global problem that affects the health of cultivated organisms such as shellfish, finfish, and seaweed. Biofouling management expenses in the mariculture business are estimated to be 5–10% of total costs. However, because farmers utilize different management methodologies and cost accounting, the expenses of marine biofouling differ significantly between aquaculture locales, species, and firms. Furthermore, many of the indirect effects have yet to be quantified, therefore the total economic expenses of marine biofouling in aquaculture are unknown, although it is seeming to be greatly underestimated (Fitridge et al. 2012). In aquaculture, fouling organisms close net openings that leads to a major increase of total weight of the cage. Biofouling also reduces the flow of the sea water with high oxygen saturation into the nets. Therefore, the oxygen supply, nutrient exchange and removal of waste products are restricting. All of these events effect the health of the cultured organisms. The fouling species invades the surface of aquaculture nets due to the wide phylogenetic variety of fouling species, huge uncolonized surface areas supplied by cage nets, and an abundance of nutrients offered by cage-based aquaculture (Bannister et al., 2019).

Although the negative effects of *Ectopleura larynx* species on fishing nets are very obvious, it is known that many different species as *Balanus amphitrite*, *Hydroides elegans*, *Ulva lactuca* and *Bugula neritina* also cause heavy biofouling in the aquaculture industry (Dobretsov et al., 2013; Baxter et al. 2012; Burden et al., 2012; Wang, 2011). Researches have been going on for many years to solve the problems encountered due to biofouling. According to the literature search, it has been determined that although the number of studies on antifouling solutions developed on ship hulls and underwater structures has increased, the number of studies on nets used for aquaculture is few.

According to the Carl et al. (2011), over the last decade *E. larynx* was discovered to be the most frequent fouling organism in the Norwegian aquaculture farming business, causing many challenges for aquaculture producers. The growth of *E. larynx* on aquaculture nets decreases water flow and nutrient exchange through nets. Therefore, the quality of water in aquaculture cages is reduced, which affects the health of cultured organisms negatively. Similarly, according to the information in the article of Carl et al (2017), it has been reported that the same problem is also observed in the Ireland marine fields and affects the finfish aquaculture industry.

According to Baxter et al. (2012), there was a significant colonization of *E. larynx* on the aquaculture net after just 1 week of immersion, with the highest growth recorded after 3 weeks of immersion. The *E. larynx* is the most visible disease-causing species in farmed fishes. The direct contact of hydroid polyps which attached onto the culture cage net with the derma of fish lead to the skin lesions on the exposed area (due to the nematocysts and stinging cells). In addition, this is not the only problem posed by the *E. larynx* but also, underwater washing of the cage nets blasts the hydroid polyps. Accordingly, the nematocysts released to the sea water and develop gill pathologies on the exposed fish.

1.5 Main Biofouling Organisms Observed Along the Turkish Coastline

The biofouling species observed on aquaculture nets as a result of the field tests completed within the scope of the thesis are explained below.

1.5.1 *Balanus amphitrite*

Cyprid is known as the larva of the *Balanus amphitrite*. *B.amphitrite* needs to stand on a solid surface while in the larval form to transform into its adult form (Carlton & Newman 2009). Therefore, any surface that does not have antifouling protection immersed in the marine ecosystem can be considered as a potential habitat for Cyprids. *Balanus* species are defined as aggressive organisms in terms of biofouling in marine environment (Bielecki et al. 2009). Known to form calcareous fouling on surfaces,

these species have detrimental effects on any surface submerged in the marine ecosystem. *B.amphitrite*, which gets a grip to the surfaces with a special cement protein, has a corrosive effect on the metallic surfaces it adheres to. It is especially difficult to clean from the surfaces of aquaculture nets, as it adheres to the surfaces very strongly. During the cleaning process, damage may occur on the net fibers for this reason (Clare & Aldred, 2009; Aldred & Clare 2008). In the field trials carried out within the scope of the thesis, the biofouling formed by the *B. amphitrite* species was observed on the surface of the aquaculture nets. The image of *B. amphitrite* is shown in Figure 1.2.



Figure 1.2 The biofouling related *Balanus amphitrite*

1.5.2 *Hydroides elegans*

The most aggressive organism in marine environment is known as *Hydroides elegans* that is a creature lives in the marine ecosystem in calcareous tubes it produced. *H. elegans*, which lives in tropical and sub-tropical climates, has been defined as a biofouling organism for all kinds of surfaces immersed in the marine water. *H. elegans* does not just choose ship hulls to attach. In addition, it has been reported in the literature that they perform biofouling on aquaculture nets in the form of colonies. *H. elegans*, which has a short larval process, fouls to surfaces with special proteins containing calcium, similar to *B. amphitrite*. For this reason, it is related to the corrosion formation on metallic surfaces and it is also harmful for aquaculture net fibers. (Qian, 1999; Seaver & Kaneshige, 2006). In the field tests carried out within

the scope of the thesis, *H. elegans* invasion was observed on aquaculture nets and carriers (Figure 1.3).



Figure 1.3 The biofouling colony of *Hydroides elegans* (White spike like tubules are the outer shell of this organism)

1.5.3 *Bugula neritina*

Bugula neritina is a species of bryozoan. Many bryozoan species have been reported as a biofouling organism in the marine ecosystem. *B. neritina* in appearance resembles a miniature tree (Figure 1.4). Many bryozoans form their own calcareous habitats in their colonies. For this reason, they are known as calcareous biofouling organisms. According to the literature, *B. neritina* forms biofouling within two to three weeks on a surface immersed in the marine ecosystem. Although its spread and development rate is slow compared to *Hydroides* and *Balanus* species, it has harmful effects on all surfaces as it forms calcareous biofouling (Piola & Johnston, 2006; Sharp et al., 2007).



Figure 1.4 The close-up view of *Bugula neritina*

1.5.4 *Ulva lactuca*

Appearance of leaves of *U. lactuca*; palm-wide, formed by lobes, the edges are smooth and slightly wavy. The leaves have thin structure. It has handles reminiscent of a very short thread in order to fix itself on an object. Its texture is nylon-like, slippery, glossy dark green or containing lighter shades of green (Figure 1.5). As the organism ages, its leaves expand and increase in number, and when observed from its initial to final form, it resembles lettuce (Cronodon, 2019). It is also known as sea lettuce in our country, and *U. lactuca*, an edible green algae species, has been reported to spread in about 117 countries around the world. The important ones among these countries are as follows; Japan, China, Israel, Malaysia, Egypt, Kenya, Tunisia, Iran, India, Bangladesh, Saudi Arabia, Vietnam, Lebanon, Denmark, France, Germany, Greece, Iceland, Netherlands, Norway, Romania, Spain, Canada, Mexico, USA. It is distributed in New Zealand, Australia, Chile, Argentina, Brazil, Peru and Uruguay (Aquamaps, 2019). In the seas of our country, there are different species of *Ulva* genus such as *U. rigida* and *U. lactuca*. These changes in species are based on differences in dynamic physical and chemical properties in the seas. Among the seas of our country, it is distributed in the Black Sea, the Aegean Sea, the Mediterranean Sea and the Marmara Sea. *U. lactuca*, known in the literature as biofouling species, has negative effects on aquaculture nets. *U. lactuca*, which can close the pores of aquaculture nets very quickly because it develops and spreads rapidly during the biofouling season when sea temperatures rise, is a creature that causes commercial losses in this sector (Tang et al., 2021). In addition to the biofouling issue, *U. lactuca*, which completed the life cycle, has unintended consequences on the coasts. Because of an increase in nitrogen, phosphate, nitrate, and sea water temperature levels in İzmir Bay, *U. lactuca* blooms in İnciraltı and Bostanlı every year in May and covers the sea surface (Kesici, 2022). In the field tests carried out within the scope of the thesis, the biofouling formed by *U. lactuca* on the aquaculture net samples were observed (Figure 1.5).



Figure 1.5 The biofouling of *Ulva lactuca* (Ulva's green leaves are just beginning to form)

1.6 Adverse Effects of Biofouling on Fishing Nets

The aquaculture nets left in the sea become living space for many living organisms in the environment when time passes. Microscopic organisms that create the biofouling to the surface of the nets are followed by larger macroscopic creatures that come to consume these primitive organisms. This layer serves as a substrate for aquatic microorganisms including bacteria and dinoflagellates. These single-celled primitive organisms subsequently form a biofilm, which allows algal spores to settle and facilitates the settling of microorganisms like mussels, a process known as biocalcification (Yebra et al., 2004; Ashraf & Edwin, 2016). The attachment of other organisms relies on the special proteins known as “extra-cellular matrix proteins” which directly affects the rate of further biofouling on submerged surfaces (Ashraf & Edwin, 2016).

Fouling is the layer formed by organisms such as crustaceans, algae, and microorganisms that grow by adhering to the surface of the ship, raft, port structures, net cages, and the underwater surfaces of fishing nets (Alyuruk et al., 2010; Karabay, 2011). The most important problem caused by the fouling hazard in mesh cages is that by increasing the surface area, the mesh net narrows the mesh opening and prevents water exchange. In this case, it becomes difficult for the metabolic wastes that may be harmful for the fish to be removed from the environment and the amount of dissolved

oxygen decreases. On the other hand, adhesion of biofouling organisms to the textile-based net cage systems used in aquaculture deform the nets and shorten the lifespan of the nets (Büyükdış et al., 2017). Fouling that occurs on cage nets begins to create an increasingly larger surface area in the meshes, leading to closure of the openings of nets. Thus, the water inlet and outlet into the cage are limited from the closed net openings, and the cage nets gradually get heavier and begin to load onto the cage assembly on the surface. In addition, as the surface area increases, the force related the water resistance on the nets increases. This results in the damage on the cage assembly and anchoring system to which the cages are attached (Edwards, 2014). It has been determined that the time dependent yield deformation on a fouled aquaculture net can be 12.5 times higher than on a clean mesh. The increase in deformation in the net causes structural fatigue of the net and resulting in mesh deformation (Fitridge et al., 2012; Edwards, 2014). It is known that between 5% and 10% of the basic expenditures of the aquaculture industry are used to eliminate the harmful effects of biofouling. It has been noted that after immersion for only a few months, biofouling can increase the weight of the nets up to two hundred times and decrease the tensile strength up to five times. The first of the harmful effects of biofouling on aquaculture nets occur a few months after the nets are immersed in the marine environment. Due to biofouling, the total mass of the net-cage system increases two hundred times, while the breaking strength of the nets decreases by five times in a few mouths (Swain & Shinjo, 2014). This increased weight and drag has historically resulted in the destruction or collapse of numerous big commercial marine aquaculture facilities (Swain & Shinjo, 2014). Biofouling also causes a decrease in the cage opening and water circulation in the cage. This situation causes a significant decrease in carrying capacity of the cage system (Dürr & Watson, 2010; Swain & Shinjo, 2014). In order to avoid these negative effects, the bioaccumulation process called biofouling should be limited as much as possible.

Since biofouling poses hazard to all surfaces used in the marine industry, researchers have been developing technologies to eliminate this problem for many years. The methods used to eliminate the biofouling problem can be called "antifouling technologies". Antifouling technologies contains all different kind of applications such

as biomimetic approaches, surface cleanings, non-toxic and toxic coatings which contain biocidal agents (Edwards et al., 2014).

1.7 Historical Development of Antifouling Measures in General

First stage includes the primitive methods to coat the surfaces with wax, tar and pitch like materials that nearly protective against biofouling organisms. The first stage in the development of antifouling technologies does not contain a scientific R&D process and it is considered as a set of works carried out by trials and errors. In the real sense, the process of developing antifouling technologies appears in the second stage as a historical sequence. Copper-based coatings, which are still in commercial use today, became a turning point in the history of antifouling technologies which started to be developed in the 1600s. When the Figure 1.6 is investigated, it is seen that the copper-based coatings are in the secondary stage in the history of antifouling technologies. Copper-based coatings have been used for a long time because their antifouling performance is very successful compared to those used in the primary stage (Jones, 2009)

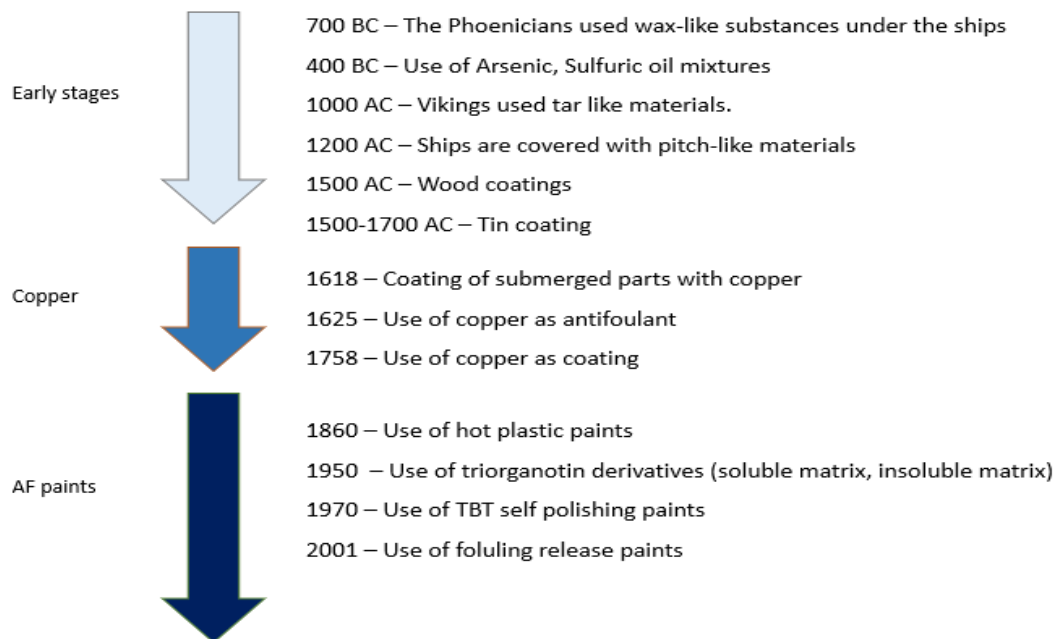


Figure 1.6 Development of antifouling technologies (Inspired from Jones, 2009)

According to the paper of Swain & Shinjo (2014), biocidal copper based antifouling paints have been utilized for over 200 years to eliminate the marine biofouling on sea transportation, aquaculture and many other industries. However, environmental concerns related to the use of copper in marine field have been attracted the attention of researchers because of the negative effects of these coatings on the marine field.

Copper and zinc metal oxides, which behave inert in many environments, have long been used as the main biocides in the production of antifouling coatings due to their anti-biofouling activities (Soroldoni et al., 2018). Although copper and zinc are essential trace elements for many different living species, the toxicity values of these two metals vary according to the dose, physicochemical parameters of seawater and the exposure time (Elia et al., 2017; Soroldoni et al., 2018). Novel papers show that, heavy metals like copper (Cu) and zinc (Zn) are being used in marine-friendly paints because of their antifouling performance (Soroldoni et al. 2018). A prior study on *Crassostrea gigas* larvae found that exposure to 10 g/L CuCl₂ had no effect, whereas exposure to greater concentrations (50 g/L) disrupted embryonic development.

Historically, last stage of antifouling paint development starts with the high temperature plastic coatings. One of the most important inventions in this period; the antifouling effects of the tributyltin (TBT) molecule discovered and it started to be used in the marine area. TBT, known as the molecule with the highest performance in the field of antifouling coatings, had a common use in the marine industry. However, in the 1970s, the harmful effects of the TBT molecule on *Crassostrea gigas* (scab development abnormality) were first detected in the Arcachon port of France. Because of the high level of water pollution, Pacific oyster production was significantly harmed, resulting in reproductive difficulties and defective shell development. These factors leads to a significant decrease in the profits of the living stock. Since January 1982, the sea oyster beds have been protected from extinction due to the regulations of the International Maritime Organization on the use of TBT.

Most antifouling paints function via discharging toxic compounds into the environment, preventing fouling organisms from being fixed. The use of such coatings

results in significant biocide emissions into the environment. It is known that the detrimental effect of tributyltin (TBT) toxicity on non-target species were first discovered in the harbor of Arcachon (France) (Alzieu, 2000). Because of the poisonous, persistent, bio accumulative, and endocrine disrupting properties of TBT, the European Union has opted to expressly include TBT compounds in its list of priority substances in water in order to manage their fate in natural systems. Furthermore, the International Maritime Organization has requested a global call for prohibiting the use of TBT containing coatings beginning January 1, 2003, and total restriction beginning January 1, 2008 (IMO, 2019). Because of the ban on the usage of TBT by the International Maritime Organization dealt a major blow to the marine transportation and aquaculture industries with the resurgence of the biofouling problem (Guardiola et al., 2012).

1.8 Booster Biocides

The need for effective antifoulants that block the target proteins and enzymes of marine organisms to prevent them from settling and developing on underwater components such as ship hulls, oil pipelines, fish cages, gears, and aquaculture nets was well recognized (Evans et al., 2000). TBT and organometallic compounds were by far the most extensively used effective compounds in antifouling coating compositions for many years, but they have been created conflicts globally since 1990 due to their destructive effects on marine ecosystems (Fent, 1996, Boxall et al. 2000). Maritime Environment Protection Committee (MEPC) and International Maritime Organization (IMO), has lately called for a restriction on TBT as a biocidal agent in the marine environment. Following the implementation, the usage of TBT in marine field was prohibited in 2008 (IMO, 2019). Booster biocides were introduced as organotin derivative options to antifouling treatments. Copper oxides and booster biocides are frequently employed as substitutes. Because of this restriction, alternative coating materials containing booster biocides have become more widely available. The most often used biocides in antifouling paints include econeal, zinc pyrithione, irgarol 1051, dichlofluanid, zineb, diuron, ziram, Sea-nine 211, copper pyrithione, chlorothalonil and TCMTB [2-(thiocyanomethylthio) benzothiazole] (Table 1.1).

Several investigations have found these biocides in European marine ecosystems due to their exploited use since 1993. These biocides have recently been found in rivers in Japan, the United States, Singapore, Australia, and Bermuda.

Table 1.1 Booster biocides and their open molecular structures

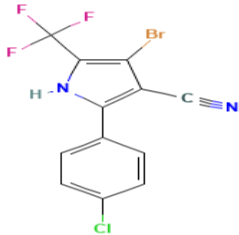
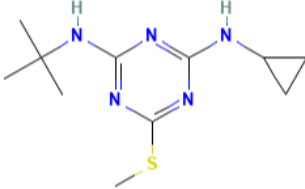
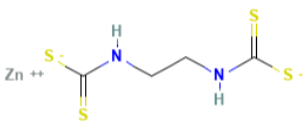
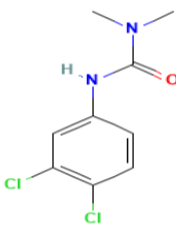
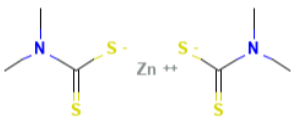
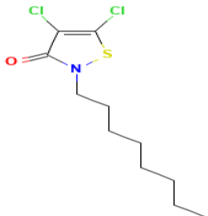
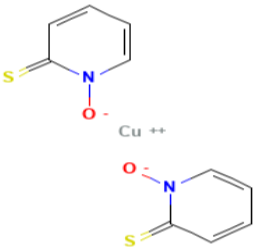
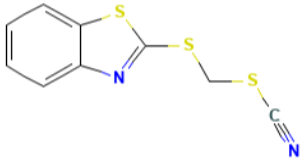
Biocide name	Chemical structure
Econea (Tralopyril) (4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitril)	
Irgarol 1051 (Cybutryne) (2-(tert-Butylamino)-4-(cyclopropylamino)-6-(methylthio)-s-triazine)	
Zineb (METIRAM) (Zinc ammoniate ethylenebis(dithiocarbamate)-poly(ethylenethiuram disulfide))	
Diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea)	

Table 1.1 Continues

Ziram (Zinc Bis(dimethyldithiocarbamate))	
Sea-Nine 211 (Kathon 930) (4,5-Dichloro-2-octylisothiazol-3(2H)-one)	
Dichlofluanid (Elvaron) (N-[dichloro(fluoro)methyl]sulfanyl-N-(dimethylsulfamoyl)aniline)	
Copper pyrithione (copper omadine) (Bis(1-hydroxy-1H-pyridine-2-thionato-O,S) copper)	
TCMTB (2-(Thiocyanatomethylthio)benzothiazole)	

1.9 Performance Testing of Antifouling Coatings

Antifouling paints are paints in the class of protective coatings designed to serve a single purpose in various ways. Among these paints, the most commonly used type is known as biocidal-antifouling paints. In biocide-based antifouling paints, the molecules that give the paint antifouling properties are toxic chemicals called booster biocides. Booster biocides show their activities by being released into sea water over

time. During the release, booster biocides pass into the tissues of the biofouling organisms that adhered to the surface, inhibit the vital enzymes of the organism and create an antifouling effect (Azemar et al., 2015; Amara et al., 2018). In traditional field tests, surfaces coated with candidate antifouling paint are immersed in the sea and left in the sea for a long time (30 – 360 days). At certain time intervals, the surface is removed from the sea and photographs are taken and the amount of biofouling is followed. As a result of the test, the paint with the least biofouling on the surface passes the test. For this reason, it is necessary to perform the performance test of each new antifouling paint (Blocher & Floerl, 2020).

The main problems encountered in traditional performance tests can be listed as: a) The toxic biocides released into the environment during the performance tests from antifouling paints, whose performances are tested with the traditional method, cause great harm to the non-target organisms living in the marine ecosystem. Micro and macro particles released from antifouling paints during the tests accumulate on the seafloor. Paint particles accumulating on the sea floor cause the continued release of toxic biocides for long periods of time (Soroldoni et al., 2017; Torres & De-la-Torre, 2021; Muller-Karanassos et al., 2021), b) Traditional performance tests are carried out for very long periods (30-365 days) (Lim et al., 2014), c) In order to test each new biocide, it is necessary to have a paint matrix that can carry the biocide. Therefore, new coatings must be prepared for the performance analysis of each new biocide. The production of new coatings creates additional costs for the product to be developed, d) The biodiversity of seas/ports in different regions is different from one another (Maghsoudlou et al., 2017; Dzhemkekova et al., 2018). For this reason, a biocide with a high efficacy in one region may lose performance in another region. The performance of biocides to be used in different regions should be tested specifically in that region. Accordingly, it is very important for the antifouling industry to develop new test methods that are faster, environmentally friendly and do not require labor intensive processes. There are different methods developed for the performance analysis of antifouling coatings in the current literature. Basically, the performance tests of antifouling paints can be examined under two main headings. The first of these is known as tests carried out in laboratory conditions, and the second is known as marine

field tests (Lim et al., 2014). Laboratory tests for the analysis of the performance of antifouling coatings are performed by artificially growing certain biofouling organisms and immersing the surfaces to be tested in this growing medium. In laboratory tests, diatoms (*Amphora* spp., *Navicula* spp. and *Nitzschia* spp.) are generally used as model micro-organisms. On the other hand, there are studies reporting that different macro-organisms such as *Amphibalanus amphitrite*, *Bugula neritina*, *Hydroides elegans* and *Ulva lactuca* are also used in laboratory tests to evaluate the antifouling performance of new coatings (Lema et al., 2019; Hadfield et al., 2021).

In field tests, basically, two types of methods are carried out. While the first method is based on leaving the surface coated with antifouling paint for short periods in the sea environment (30 – 40 days), in the second method, the surface coated with antifouling paint is kept under test for long periods of time (8 – 12 months) (Lim et al., 2014). However, it is known that laboratory tests cannot fully evaluate the performance of an antifouling coatings. For instance; the data obtained as a result of a performance test with *H. elegans* under laboratory conditions is not consistent with a marine field trial. The reason for this is that the biofilm formation required for the attachment of *H. elegans* to a surface cannot be obtained in the laboratory environment (Hadfield et al., 2021). On the other hand, although field tests fully explain the performance of antifouling coatings, it causes the release of toxic compounds that are not environmentally friendly. In addition, long test periods of 8 to 12 months are required for long-term marine field tests (Lim et al., 2014). For these reasons, it is necessary to develop new and effective performance testing methods for the quality of antifouling coatings.

In some studies, in the current literature, it has been determined that *in-silico* molecular docking analyzes are used in new biocide discovery studies (Ozupek & Cavas, 2017). In the domain of computer assisted drug development, it is well known that *in-silico* analyses are routinely employed. Structure-based drug design and ligand-based drug design are the two subcategories of computer assisted drug design. Whether or not the 3-dimensional structure of pharmaceutical targets it determines which one

to use. In the structure-based approach, small compounds that are formally and functionally suitable for the active site of therapeutic target are dealt with using de-novo ligand design and molecular docking-like technologies. The molecular docking approach, one of the structure-based analyses used in computer-aided drug design procedures, is one of the *in-silico* techniques employed in the field of antifouling coating. (Griffith et al., 2005).

1.10 What is Bioinformatics?

Bioinformatics is a chemistry-based interdisciplinary science that includes biochemistry, mathematics, and statistics. Molecular bioinformatics use "informatic approaches" including applied mathematics, computer science, and statistics, to conceive biology in terms of molecules and to better comprehend and organize the information associated with these molecules. Computer-assisted analysis is the foundation of bioinformatics research. Molecular docking, molecular modeling, and protein sequence similarity analyses are common regular investigations in this field of inquiry (Diniz & Canduri, 2017).

1.10.1 The Molecular Docking

A vital step in the search for active substances is the molecular docking technique. The molecular docking technique is based on applying computational tools to anticipate the complex structure of the ligand and the receptor. The molecular docking approach is divided into two phases. The first of these procedures is to identify the ligand conformations in the protein's active region. The second stage is to use scoring functions to evaluate such structures. The main idea behind this technique is that the binding modes of the conformation with the greatest score among the generated conformations may be empirically determined (Li et al., 2022; Zheng et al., 2022).

The design and synthesis of active chemicals and our knowledge of biological processes both benefit from molecular docking. It is the virtual modeling of the chosen chemical's binding location on the target molecule. This method is widely applied to

drug creation with computer assistance. These approaches are used to describe how chemicals interact with active compounds, drug candidates, and medicines. It is widely used to estimate affinities of small molecules for a protein target (active molecule candidates) (Morris & Lim-Wilby, 2008; Dias et al., 2008; Pangadala et al., 2017).

The region where signal transmission is triggered as a result of the molecular docking of molecule (ligand) on protein (receptor) to form a stable complex structure, is called the binding site. In order for the receptor to generate a signal response, the total energy they have when the ligand and the receptor are apart from each other must be higher than the total energy of the complex obtained by ligand-receptor coupling. This is because when the ligand and the receptor bind together, the resulting complex is lower-energy and stable. In receptor ligand interactions, many factors affect the binding state, such as desolvation energy (ΔG_{desolv}), energies due to conformational changes (ΔG_{konf}), energies due to unbound interactions (ΔG_{etk}), internal rotations (ΔG_{rot}), and ligand-receptor junction energy (ΔG_{vt}). In molecular docking studies, ligand exposures and scoring functions are determined by considering such factors. In these calculations, force field parameters used in molecular mechanic calculations are used. In addition, knowledge-based parameters or experimental data in electronic environment are also used. These are information-based parameters obtained from protein data banks or detailed experimental scoring methods such as Poisson-Boltzmann (Jain, 2006; Dias et al., 2008; Pangadala et al., 2017).

Many software is used in molecular docking method. In most of the software used, the ligand structure is considered to be flexible, while the structure of proteins is considered to be inflexible, and calculations involving all conformations of the ligand on the protein are included in the docking studies. As a result of the study, the most suitable conformation of the ligand on the protein can be selected, and the binding position of the ligand on the receptor with a 3D structure can be visualized (Fan et al., 2019).

With molecular docking studies, the parameters such as Gibbs free energy, full compatibility score, hydrogen bond length between the receptor and the ligand, and

between which atoms the bond is established are reached. By interpreting the obtained parameters, predictions can be made about the binding between the ligand and the receptor. The successful binding of the active molecules with the corresponding atoms of the receptor depends on many factors (Yuriev & Ramsland, 2015; Fan et al., 2019).

To establish the specificity of the receptors, it is essential to understand the bonds that connect the active molecule (ligand) with the receptor. Intermolecular interactions include hydrogen bonds and Van der Waals interactions mediate ligand-receptor couplings. In light of this circumstance, understanding the bonding process depends on the knowledge of the hydrogen bond parameters. Utilizing molecular docking research, the objective is to evaluate the parameters gathered in a short amount of time and predict the binding state (Agarwal & Mehrotra, 2016; Fan et al., 2019).

If molecular docking calculations are broken down into phases, the first step is to get the receptor structure to be employed in the ligand-receptor interaction from data banks such as Protein Data Bank (PDB) the structure produced by X-ray crystallography. The crystal lattice structure of a protein may be determined by shining X-rays on it. The NMR approach may also be used to identify the structure of a dissolved molecule in a water environment. However, because the NMR approach is highly difficult and needs complex solutions, X-ray crystallography is often utilized. On the Internet, there are several electronic libraries where we may get X-ray crystallography (Pinzi & Rastelli, 2019; Pangadala et al., 2017).

With the structures selected from the database, the receptor structure is prepared for the molecular docking program. In the second step, mechanical and geometric optimization of the ligand is performed with package programs such as AutoDock Tool 1.5.6, Schrödinger Maestro, Avagadro, DS visualizer and Gaussian. Thus, the structure of the ligand is prepared for molecular docking and introduced to the electronic environment (Pangadala et al., 2017; Handoko et al., 2015).

In the last step, molecular docking calculations between the ligand and the receptor are performed with various software and programs. Some of the software used for the

molecular docking process are as follows; FleXX, Auto Dock Vina, CB-Dock, and SwissDock (Table 1.2) (Yuriev & Ramsland, 2015; Pinzi & Rastelli, 2019; Liu et al., 2022).

Table 1.2 Some of the molecular docking algorithms and web links

Algorithm	Web link
FleXX	https://www.biosolveit.de/products/
AutoDock Vina	https://vina.scripps.edu
CB-Dock	http://clab.labshare.cn/cb-dock/php/index.php
SwissDock	http://www.swissdock.ch

1.10.2 AutoDock Vina

A computer-based technique called molecular docking uses 3D structures of proteins and small molecules or homology modeling from molecular dynamics simulations to anticipate the non-covalent interaction of macromolecules, or more generally, of a macromolecule (receptor) and a small molecule (ligand). Here, predicting interaction and binding affinity is the main goal. Because it is utilized to search for receptor-specific ligand-like compounds in virtual libraries to direct the active substance creation process, predicting the protein binding characteristics of small molecules is crucial (Zheng et al., 2022). In the literature, AutoDock Vina is frequently used in this area. AutoDock in docking mode, Vina's average estimations are far better than those of AutoDock4 and other programs. The PDBQT format is shared by Vina and AutoDock for input and output. Through MGLTOOLS, the PDBQT format may be read and generated interactively. The user of Vina's interface is not required to comprehend implementation specifics, modify search criteria, or be aware of the clustering outcomes (Olivera et al. 2022; Zheng et al., 2022).

1.10.3 Scoring Functions

The scoring function aims to select the best right position among all potential locations. The scoring functions simplify the computation and make assumptions rather than directly estimating the binding energies of protein-ligand complexes. Using traditional force field-based scoring methods, the binding energy relying on electrostatic and van der Waals interactions can be evaluated. Extensions to the force-field-based scoring systems are also available, including solvent, hydrogen bonding, and entropy calculations. Two of these programs are DOCK and Autodock (Meng et al., 2011).

1.10.4 Validation of the Docking Method

The major goal of the validation research is to look at the root mean square deviation (RMSD) values of the ligand bound to the protein using the docking tool to determine the degree of divergence from the original binding. According to the docking study calculations, data with an RMSD value of less than 2 are regarded suitable results. However, it has been proven that the initial result (RMSD = 0.0) acquired from molecular docking analysis is the most essential number for evaluating the overall findings (Keşkek, 2016).

1.10.5 Wet-lab 3D Structure Analysis of Proteins

In 3D structural analysis, there are three methods for gathering experimental data. The 3D structure of proteins may currently be determined experimentally using techniques including X-ray crystallography (XRD), nuclear magnetic resonance (NMR), and electron microscopy. X-rays are type of ionizing radiation; however, they have much shorter wavelengths, about 0.1 nm (the diameter of a hydrogen atom). X-rays may be utilized to explore the interatomic gaps in molecules because of their short wavelengths. The same atoms are repeatedly used to produce the crystals that make up proteins. The structure of many molecules can be determined by reflecting the X-ray beam sent to a crystal on the film, depending on whether it is refracted or passed

without refraction. In the NMR method, when the protons in the nucleus of the hydrogen atom in the analyzed sample are exposed to an electromagnetic energy of a certain frequency coming from a radio frequency source, it absorbs this energy depending on the strength of the magnetic field. Molecules of the analyzed substance are affected by the magnetic field coming on this substance in the form of radio frequency; this happens according to the positions of the nuclei of the atoms in these molecules, and therefore the frequency of absorbing the incoming energy is also affected by this situation. The signal that emerges depending on the frequency differentiation gives the NMR spectrum of the structure of the substance. Unlike X-ray crystallography, NMR requires a small concentrated protein solution placed in a strong magnetic field without the need for a crystal structure. In addition to these techniques, electron microscopy is among the experimental methods in the 3D structure analysis of proteins. However, these experimental methods have significant limitations. In order to work with the X-ray crystallography method, the sample of the protein to be studied must be pure and crystallized. For this, the studied protein must be water-soluble. However, many proteins, especially those found in the cell membrane, are not soluble in water. In addition to this, crystallizing proteins is a very laborious and long process. Although there is no need for crystallization of the protein in the NMR method, the protein sample to be studied should not be larger than 30 kDa. In addition, the information obtained in the 3D structure analysis obtained from both methods is not considered to be 100% accurate, the need for long times to carry out these methods and the exponential increase in these times in case of repeating the experiments adversely affect the studies. XRD and NMR data of proteins, whose 3D structure is determined experimentally, are uploaded to protein databases by researchers (Ruprecht & Nield, 2001; Nelson & Cox, 2005).

1.10.6 Protein Databases

Databases have been created that provide access to many biological data at once and have a very complex infrastructure related to the entry of new data into the database by users or the modification of existing data. In addition, various bioinformatic analyze methods are being developed in order to use existing biological

data on how cellular activations are regulated under normal physicochemical conditions. In this context, there are many databases to be used in the determination of the 3D structures of target proteins (Berman et al., 2000; Apweiler et al., 2004; RCSB Protein Data Bank, 2021).

Today, information stored in these databases on the computer networks operating on the Internet is stored in files, and scientists who will benefit from this information can process these files in various formats and upload them back to databases. After the primary sequences of newly identified proteins have been extracted by experimental methods, there are separate databases that are ready to examine the interactions between them. In order to process these interactions according to theoretical knowledge, large computer systems with parallel processors are required. Today, although there are many file types that collect both experimental and theoretical data, the most widely accepted are Protein Data Bank (PDB) files. These files are written in the American Standard Code for Information Interchange (ASCII) and have tags at the beginning, and the programs that process this file perform their functions according to these tags (Berman et al., 2000; Apweiler et al., 2004; RCSB Protein Data Bank, 2021).

PDB files are files that hold the polar coordinates of the atoms that make up amino acids, which are the basic building blocks of proteins. A PDB file can be thought of as 7 columns. The first column refers to the label. When processing such files, the processor behaves according to the label in the first column. The second column represents the atomic number. Since the PDB file contains the atomic order of amino acids, the second column shows the number of atoms in the processed row. The third column is the column that gives the name of the atom. In this column, the C atom is expressed as CA if α C, and CB if the side chain is not α C. The fourth column represents the three-letter abbreviation of the amino acid in which the atom is located, and the fifth column represents the single letter symbol. The sixth column expresses the amino acid number, just like the atomic number. The seventh and later columns show the coordinates of that atom in 3D space (Figure 1.7) (RCSB Protein Data Bank, 2021).

		Element	Amino Acid	Chain Name	Sequence Number				Coordinates
							X	Y	Z
ATOM	1	N	ASP	L	1		4.0606	7.303	5.186
ATOM	2	CA	ASP	L	1		4.0606	7.303	5.186
ATOM	3	C	ASP	L	1		4.0606	7.303	5.186
ATOM	4	O	ASP	L	1		4.0606	7.303	5.186
ATOM	5	CB	ASP	L	1		4.0606	7.303	5.186
ATOM	6	CG	ASP	L	1		4.0606	7.303	5.186

Element position within amino acid

Figure 1.7 The digital layout of PDB files

1.10.7 RCSB Protein Database

The Protein Database is a collection of 3D structures of biological molecules, nucleic acids, and proteins. These compounds can be found in bacteria, yeast, plants, flies, other animals, and humans, among others. A molecule's form and structure are critical in the production of active compounds. The protein database repository contains structures ranging from tiny proteins and DNA fragments to large compounds such as ribosomes (Zardecki et al., 2021; RCSB Protein Data Bank, 2021).

1.10.8 Homology Modelling

The development of high-resolution imaging techniques has enabled the precise prediction of the structure and functions of proteins. Although spectroscopic techniques can be used to determine crystal structures, given the number of systems found in nature, this is a time-consuming and expensive method. In this context, 3D protein structures obtained by using newly developed methods, computer-aided modeling techniques and increasing structure information in data banks are a good option to fill this gap. In the most general sense, proteins are formed by the sequence

of amino acids in a row. This sequence is called a primer sequence. Comparative modeling or homology modeling is a technique for constructing a model using previously known sequence and structure information. The basic idea of this modeling technique is that proteins with evolutionarily related sequences have similar functions (Vyas et al., 2012). This idea allows proteins to be classified according to their sequence. Thus, we can say that proteins in the similar order are evolutionarily related, that is, homologous or homogeneous. In the light of this information, the sequence of a protein whose crystal structure is unknown can be modeled with the help of template crystal structures to be selected. The first sequence of work done in this type of modeling is to determine the structure that will be used as a basis for the development of a known but unknown crystal structure. SWISS-MODEL, which is frequently used in the literature to homology modelling of proteins. SWISS-MODEL first compares the protein sequence given as an input in a single operation within its data library and lists the most suitable 3D protein templates for modeling. The target template selected by the user is used as a skeleton, and the primary sequence of the target protein, whose crystal structure has not yet been defined, is modeled in 3D in this way quickly and with high accuracy (Arnold et al., 2006; Bienert et al., 2017; Waterhouse et al., 2018).

1.10.9 Receptor Types - Subtypes and Activation

Receptors are defined according to the specific neurotransmitters or hormones that activate them. For example, a receptor activated by acetylcholine is known as a cholinergic receptor. In another example, the receptor activated by dopamine is called the dopaminergic receptor, or the receptor activated by adrenaline is called the adrenergic receptor. Although the receptor is a protein molecule, it is embedded in the cell membrane in the cell. The cell membrane is full of complex shapes. Among these shapes, there is a region that accepts the incoming chemical messenger with its unique shape, and this region is called the binding region. When the chemical messenger binds to the binding site, the message is received and transmitted into the cell. After the message is transmitted to the cell, the chemical messenger leaves the receptor. A point to note here between enzymes and receptors is that the chemical messenger does not give a chemical reaction with the receptor. Not all receptors are activated by the same

chemical stimulus. For example, there are differences between adrenergic receptors in the lung and adrenergic receptors in the heart. These differences are due to the sequence of amino acids. When the molecular docking analysis takes place, these should be noted if the changes are at the binding site (Weis et al., 2018). Within the scope of the thesis, G protein coupled receptor (GPCR) of *B. amphitrite*, Cytochrome c oxidase subunit 1 (COI) of *H. elegans*, Cytochrome b (Cyt-b) of *B. neritina* and Photosystem II protein D1 (P-II-D1) of *U. lactuca* were used as the targets of molecular docking analysis. According to the selected proteins, GPCR is known as receptor protein that sense inputs (neurotransmitters, hormones, physical movements) to initiate the production of cement protein (Wettschureck & Offermanns, 2005). On the other hand, COI, Cyt-b and P-II-D1 proteins have biologic activities on the synthesis of adenosine three phosphates (Uniprot, 2021).

1.10.10 Conformational Change in the Attachment Region

Shape change at the binding site occurs when a molecule inserts into the receptor. The induced localization that occurs here creates intermolecular binding interactions. As it is understood, this interaction is between the molecule and the receptor. Protein structure conformation changes due to intermolecular interactions and activates the signal transduction pathway (Nielsen et al., 2022).

1.11 Aims of The Thesis

The aim of this thesis is to create a new and rapid standard performance test method based on *in-silico* molecular modeling methods without the need for field trials to test the antifouling performance of biocides used in antifouling coatings.

The objectives of the thesis:

- Investigation of the antifouling performances of various non-biocidal binders which are used in antifouling coating formulations in Türkiye
- Preparation and the evaluation antifouling performance of biocide-based coatings.
- Investigation and determination of harmful biofouling organisms and their important proteins.
- Investigation of *in-silico* molecular docking analysis of biocides on target proteins of biofouling organisms.
- Evaluation of the compatibility of the *in-silico* and *in-vitro* results.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Field experiment

In this thesis, two different field experiments were carried out at İzmir Marina (Location coordinates: 38°24'23"K 27°04'06"D) (First field experiment date intervals: 01 – 21 August 2021 / Second field experiment date intervals: 01.- 21 September 2021) (Prior to the field trial, permission was obtained from the Ministry of Environment, Urbanization and Climate Change of the Republic of Türkiye). First trial was implemented to evaluate the antifouling performance non-biocidal coatings and find the suitable binder for the second experiment. Lanolin, paraffin wax, styrene-acrylic copolymer and rosin were used as main binders for first field experiment. In order to produce new coatings for second field experiment, lanolin and rosin binders were selected due to their non-biocidal antifouling performances and environmental compatibility. The experiment field and first submerged sample were shown at Figure 2.1. Fish-cage net was kindly provided by Nesne Elektronik (İzmir/Türkiye). The samples of first and second field experiment were shown at Figure 2.7.



Figure 2.1 a) The overall view of the experiment field b) The sample submerged in the sea

2.1.2 Preparation of the fish-cage net samples

The lanolin, paraffin wax, styrene-acrylic copolymer and rosin-based coatings were applied on to the fish-cage net samples via dip-coat method. The dimensions of polyethylene (PE) net samples were adjusted to be 10x10cm (Figure 2.2).



Figure 2.2 The sample PE cage net in 10x10cm dimensions

2.2 Preparation of coatings

2.2.1 Preparation of lanolin and paraffin wax coatings

Lanolin (50 g) and paraffin-wax (50 g) were melted separately at 100°C in beakers (Figure 2.3). After obtaining a homogeneous liquid, the biocides (econe, diuron, and irgarol 1051) were added in to the liquid binders at different batches. In order to coat the fish-cage net samples, the dip-coat method was implemented at 100°C the binder temperature. All fish-cage net samples were dipped in the coatings just one time (each fish-cage net sample was hold in the binder for 10 seconds).

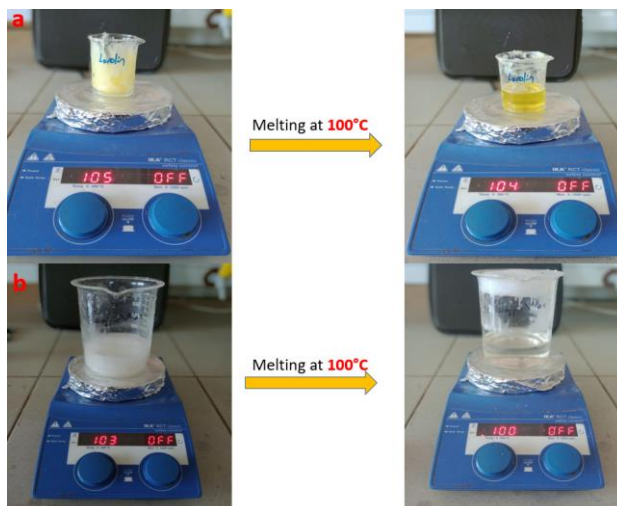


Figure 2.3 Lanolin (a) and paraffin wax (b) are melted at 100°C

2.2.2 Preparation of rosin-based coating

In order to prepare rosin-based coating, rosin was dissolved in 50 ml beaker by using xylene at 60°C temperature on the heater via stirring at a constant rate (150 rpm). After obtaining a homogeneous mixture, CaCO_3 and ZnO were added the mixture respectively. The mixture was stirred for 15 min at 60 °C to obtain the desired texture (Figure 2.4). After obtaining homogeneous texture biocides (econe, diuron and irgarol 1051) were added at different batches.

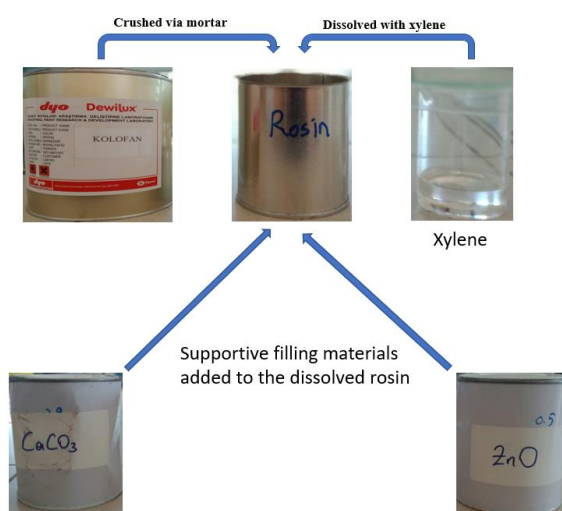


Figure 2.4 The preparation procedure of rosin coating

2.2.3 Preparation of styrene-acrylic copolymer-based coatings

In this thesis two different styrene-acrylic copolymer emulsion (SAC-E) based coatings (SAC-E + %0 PEG600 (w/w), SAC-E + %50 PEG600 (w/w)) were prepared. In order to prepare SAC-E based coatings, SAC-E was poured in 50 ml beaker and desired amount PEG600 was added under the constant stirring rate (250 rpm). PEG600, SAC-E mixtures was stirred 15 minutes (Figure 2.5). After obtaining homogeneous mixtures, all prepared coatings were coated on fish cage-net samples via the dip-coat method and coated samples were cured in the oven at 120°C (Figure 2.6).

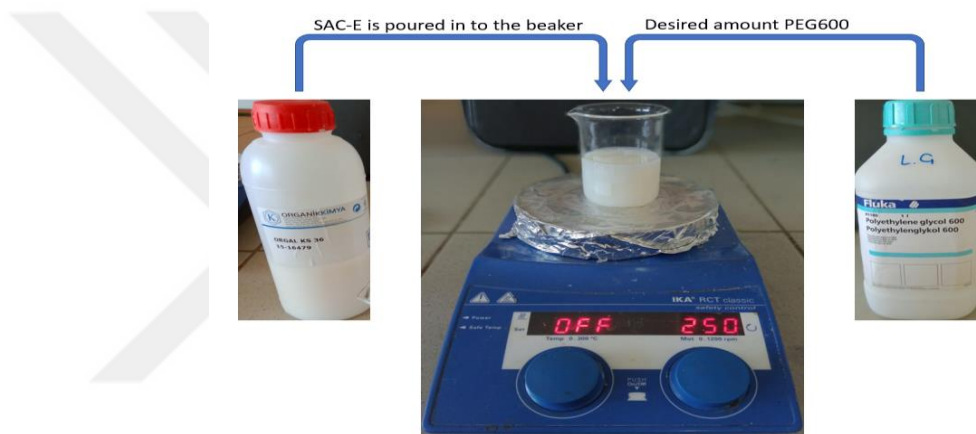


Figure 2.5 SAC-E + 50% PEG600 (w/w) preparation procedure



Figure 2.6 Sample nets coated with PEG600-free and PEG600-added SAC-E emulsions are cured in an oven at 120°C

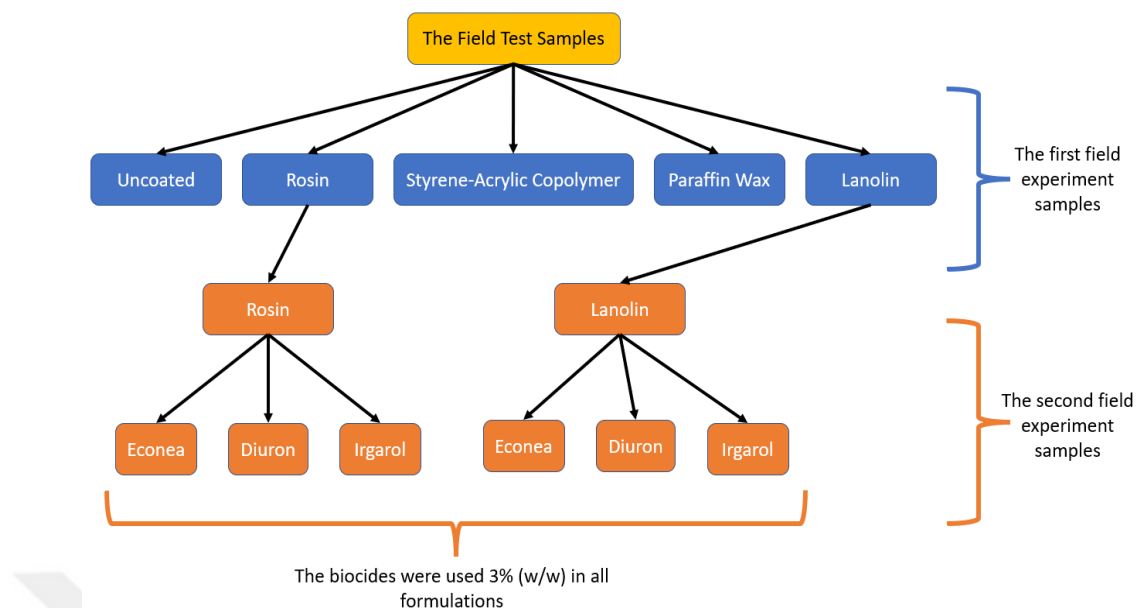


Figure 2.7 The first and second filed experiment samples

2.3 *In-silico* Molecular Docking Analysis

2.3.1 *Target and Ligands*

The primer sequences of the target proteins were obtained from Uniprot (The UniProt Consortium, 2021). SWISS-MODEL was used to digital 3D protein modelling (Guex et al. 2009; Benkert et al. 2011; Mariani et al. 2013; Bertoni et al. 2017; Bienert et al. 2017; Waterhouse et al. 2018; Studer et al. 2020; Studer et al. 2021). The sdf files of Chlorothalonil (CID:15910), Dichlofluanid (CID:14145), Diuron (CID:3120), Irgarol 1051 (CID:91590), Econea (CID:18355), CMPD-101 (CID: 11677079) and Gallein (CID: 73685) were retrieved from <https://pubchem.ncbi.nlm.nih.gov/> (Kim et al. 2019).

2.3.2 *Structural Analysis and Validation of Target*

The 3D modeled proteins were validated via Structural Analysis and Validation Server (SAVES v6.0). PROCHECK and ERRAT analysis were used to obtain the quality of modeled protein (Colovos&Yeates, 1993; Laskowski et al. 1993; Laskowski

et al. 1996). The total scores indicated that the proteins were modeled in good quality via SWISS-MODEL with the selected templates.

2.3.3 Energy Minimization

The conformational energy of the created 3D models were minimized via YASARA Energy Minimization Server (Krieger et al. 2009).

2.3.4 Molecular Docking

AutoDockVina was used to carry out *in-silico* molecular docking studies (Trott & Olson, 2010). Water molecules were removed from the target proteins before docking, and Kollman charges and polar hydrogens were added using AutoDock Tool version 1.5.6 (Sanner, 1999; Morris et al. 2009). Maestro (version 12.7 Schrödinger, LLC) was used to visualize the interactions between the target and the ligands.

2.3.5 Molecular Docking Software and Analysis Procedure

In order to carry out a molecular docking analysis, the procedure that sequenced at the bottom must be followed.

➤ Select Target Protein and Ligand

Protein and Ligand databases are searched. In particular, it is checked whether the target protein has a 3D crystal structure obtained as a result of XRD, NMR or EM. The steps sequenced below are followed for target proteins with 3D crystal structure obtained protein database. In protein databases, a three-step modeling process is required for target proteins that 3D structure has not been identified.

- ✓ RCSB - PDB (protein database)
- ✓ Enter the site “<https://www.rcsb.org>”, download the protein intermediate file as “.pdb” extension.

➤ **Three-step Modeling Process**

- ✓ Find the amino acid sequence in FASTA format of the target protein on internet databases.
 - ✓ The most reliable protein databases are given below:
 - RCSB-PDB “<https://www.rcsb.org>”
 - National Library of Medicine NCBI “<https://www.ncbi.nlm.nih.gov>”
 - UniProtKB “<https://www.uniprot.org/help/uniprotkb>”
 - ✓ Go to the site “<https://swissmodel.expasy.org>” and press the “Start Modelling” button.
 - ✓ Paste the FASTA sequence of target protein and press the “Build Model” button.
 - ✓ Select proper template (Highest coverage, lowest resolution and an oligo state with a check mark) and press build models.
 - ✓ Go to the site “<https://saves.mbi.ucla.edu>” to validate the created 3D protein structure.
- Performing an *in-silico* energy minimization procedure to minimize the conformational energy of the modeled target protein after homology modeling helps to find more accurate results in molecular docking analysis. For this reason, tools such as YASARA energy minimization server (<http://www.yasara.org/minimizationserver.php>) should be used after homology modeling.
- ✓ PubChem (Ligand database)
 - ✓ Go to the site “<https://pubchem.ncbi.nlm.nih.gov>”, download the ligand file with the “3D .sdf” extension.
- **Prepare Protein and Ligand for Molecular Docking**
- ✓ Open AutoDock Tool 1.5.6.
 - ✓ Drag the protein.pdb file onto the workspace.
 - ✓ PRESS - Edit → Delete water → Charges → Add Kollman Charges → Hydrogens → Add.

- ✓ PRESS - Grid → Macromolecules → Choose → Select protein → Press Select Molecule (File extension “.pdbqt”).

--Protein ready for molecular docking.

- ✓ Enter the “<https://cactus.nci.nih.gov/translate/>” web interface.
- ✓ Drag the ligand onto the button named as “Dosya Seç”.
- ✓ Choose PDB and 3D then press “Translate”.
- ✓ Open AutoDock Tool 1.5.6.
- ✓ Drag the ligand.pdb file to the workspace.

Ligand → Input → Choose → Select ligand → Press Select Molecule

Ligand → Output → Save as PDBQT

--Ligand is ready for molecular docking

➤ **Select the docking region of the protein (GRID)**

- ✓ Open AutoDock Tool.
- ✓ Drag the ligand.pdbqt file to the workbench.
- ✓ Drag the protein.pdbqt file onto the workbench.

Grid → Macromolecule → Choose → Protein → Press Select Molecule

Grid → Grid Box → Adjust the active site dimension

- ✓ Create a text document and set its name to "config".
- ✓ Write the text that given below into a text document named config;

receptor = protein.pdbqt

ligand = ligand.pdbqt

center_x = -30.851

center_y = 54.656

center_z = 18.000

size_x = 40

size_y = 40

size_z = 40

energy_range = 4

exhaustiveness = 8

- ✓ Save the file by entering the necessary data.

The values you see in front of “=” have been determined by AutoDockTool as default. These values are created in Step 3 and kept in the grid.txt file.

The center_x/_y/_z values vary according to the receptor studied.

--The necessary files for the dock have been prepared --

It is very important to note that, in order to use the Vina, all files prepared beforehand should be collected in a single folder

➤ Carry out the Docking

- ✓ Open the command script (cmd).
- ✓ Introduce the file location to the script.
- ✓ Write the codes given below and press enter.

“cd C:\Users\user\Desktop\Docking” → Press enter

“C:\Program Files (x86)\The Scripps Research Institute\Vina\vina.exe --receptor protein.pdbqt --ligand ligand.pdbqt --config config.txt --log log.txt --out output.pdbqt”
→ Press enter

Output.txt file was created in the folder where all files are located.

➤ Visualization of Molecular Docking Results

- ✓ Open Maestro software (v12.7).
- ✓ Drag the protein.pdbqt file to the workbench.
- ✓ Drag the output.pdbqt file to the workbench.
- ✓ Press “+” that next to the receptor than select the surface to create 3D image.

If the Exhaustiveness value was set as how many times in the 4th step, that number of docks was made and all interactions can be seen with the help of arrow keys on Maestro.

2.4 Tensile Strength Test on Net Samples

In order to evaluate the tensile strength of field experimented aquaculture nets, the device named Autograph-AG-IS was used (Figure 2.8). Before performing the tensile strength test, the net samples were cut into a single 9 cm long string. Before the test, the rope thickness was measured from 5 different points with SATRA thickness gauge (Figure 2.9) and the average thickness was calculated. 2 cm from the fore and aft parts of the net samples were placed in the main holder clamps of the device and the test was carried out as explained at the paper of Moe et al (2007).



Figure 2.8 a) Tensile strength test device, b) Single rope test sample that placed between holder clamps



Figure 2.9 The rope diameter evaluation via thickness gauge

2.5 Biofouling Degree Assessment Criteria

In order to easier calculation of the correlation coefficients between *in-silico* and field experiment the biofouling evaluation is performed. The abundance of micro and macro-organisms adhered to the surface is used to define the fouling scores. There are definitions of such fouling classification in the literature (Alyuruk, 2012; Mert et al., 2014).

Level 1. No fouling organisms on the surface

Level 2. There is microfilm layer formation occurs on artificial surface

Level 3. There is microfilm layer on the surface and macro fouling is at beginning stage

Level 4. Micro and Macro organisms observed on artificial surface

Level 5. The surface is heavily fouled by macro-organisms

CHAPTER 3

RESULTS

3.1 *In-silico* Molecular Docking Results

3.1.1 *In-silico* Analysis of *Balanus amphitrite* – G Protein Coupled Receptor (GPCR)

In this thesis, the antifouling performances of commercial biocides were evaluated via molecular docking analysis. The GPCR protein of *B. amphitrite* was chosen as molecular docking target due to the important properties on marine biofouling. The primer sequence of target protein was taken from UniProt (uniprot id: Q93127). SWISS-MODEL was used to create the 3D structure. HHblits and BLAST algorithms were utilized to find a suitable template for the initiation of homology modelling (SMTL, last update: 2021-03-03, last included PDB release: 2021-02-26). BLAST was used to compare the target sequence to the main sequence found in the SMTL. 394 templates in all were acquired. Using ProMod3, models were constructed based on the intended alignment (Studer et al., 2021). The model was cloned from the template using the saved coordinates from the template. After the cloning of template coordinates SWISS-MODEL fragment library were used to carry out the insertions, deletions and remodelling. Then, side chains were rebuilt. Finally, a force field was used to regularize the geometry of the final model. In order to do 3D modeling, Alpha2A adrenergic receptor was selected as template (template id: 6kuy.1. A). The sequence identity and resolution of template were found as 37.10 and 3.20Å, respectively. The total coverage of template was calculated to be 0.91 by SWISS-MODEL. After the 3D modeling, the created structure validated by PROCHECK and ERRAT analysis which are found on Structural Analysis and Validation Server (SAVES v6.0). According to the scores, 93.5% residues of created structure was found on most favored regions and 6.1% residues were also found on allowed regions. However, only 0.3% residue was found at disallowed region (Serine position: 271). The threshold value of this analysis is 90% for a good quality model. According to the

score, created 3D structure obtained in a good quality with the score of 93.5% (Figure 3.1 and Table 3.1).

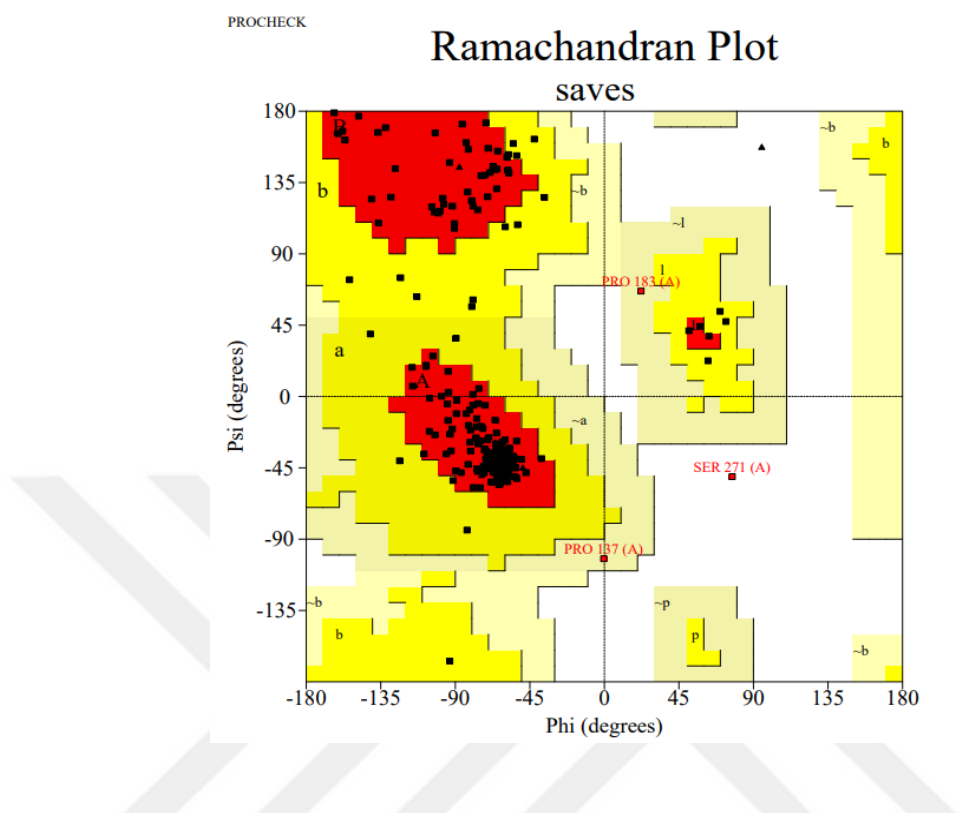


Figure 3.1 The ramachandran plot of 3D modelled probable G coupled protein (GPCR) of *Balanus amphitrite*.

Table 3.1 Ramachandran plot statistics for the modelled G protein coupled receptor

Residues	Total Number	Percentage (%)
Residues in most favoured regions	290	93.5
Residues in additional allowed regions	19	6.1
Residues in generously allowed regions	0	0.0
Residues in disallowed regions	1	0.3
Number of non-glycine and non-proline residues	310	100
Number of end-residues	2	
Number of glycine residues	9	
Number of proline residues	22	
Total number of residues	343	

The average overall quality factor score of ERRAT analysis was established to be 91% for lower resolution templates (2.5 to 3Å). The template that used in this study has 3.20 Å resolution and the ERRAT score was evaluated to be 84.384. Although the score of this analysis was obtained lower than the threshold value, the resolution value of template was also lower than the stated. Therefore, by looking at the score obtained as a result of the analysis, it can be said that the modeled structure has a good quality. Additionally, the PROCHECK score also supports this information. The conformational energy of the 3D modeled protein was minimized by YASARA energy minimization server. Initial energy and score of the modeled protein was evaluated to be -113612.0 kJ/mol and -2.75, respectively. After the minimization steps final energy and score were obtained as -179155.2 kJ/mol and -0.87. 8249 molecules of water were used at the energy minimization procedure. To conduct the molecular docking, AutoDockVina was used. AutoDockVina is known as grid docking algorithm. Before the molecular docking with AutoDockVina, the 3D space (Grid) where the analysis occur is selected. In this study, grid dimensions were selected to be center_x = 29.7244283458, center_y = 1.78939427671, center_z = 58.7687041596, size_x = 45.8700312227, size_y = 40.9236442313, size_z = 27.1115826546. The grid was located on the extracellular residues of GPCR protein. The target was selected as GPCR protein of *B. amphitrite* since it is directly related to the fouling process of cyprinid larvae. The extracellular residues (target section) of GPCR protein were visualized via Maestro. The molecular surface of target section was represented at Figure 3.2.

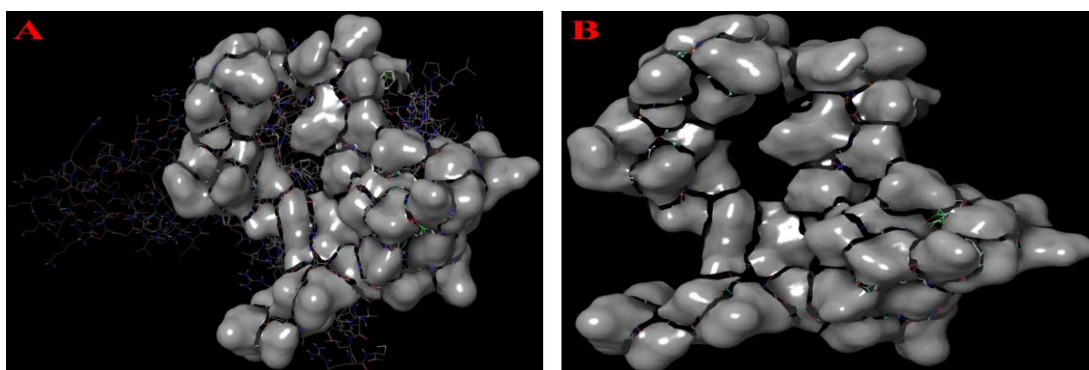


Figure 3.2 The molecular surface of extracellular residues of G protein coupled receptor protein were visualized (A). The rest of the residues were blacked out to eliminate image clutter (B)

The ligands were selected as chlorothalonil, dichlofluanid, diuron, econea and irgarol 1051 as most used commercial biocides. Gallein and CMPD-101 were selected as positive control and known inhibitors. According to our molecular docking analysis, highest binding affinities of chlorothalonil, dichlofluanid, diuron, econea and irgarol 1051 with GPCR protein of *B. amphitrite* were obtained to be -5.2 kcal/mol, -6.2 kcal/mol, -6.6 kcal/mol, -7.0 kcal/mol and -5.6 kcal/mol, respectively. All molecular docking scores of commercial biocides were given at Table 3.2. The gallein and CMPD-101 were used as known inhibitors of GPCR protein. According to the docking analysis, the highest binding scores of gallein-GPCR and CMPD-101-GPCR were found as -7.8 kcal/mol and -10.2 kcal/mol, respectively (Table 3.2). The best binding scores of all ligands were presented at Table 3.3.

According to the best binding scores, docking poses of target-ligand complexes created via Maestro (Figure 3.3 to 3.9). All interactions of target-ligand complexes can be found on second poses of Figure 3.3 to 3.9. The molecular docking scores can reveal the antifouling performance of biocides. According to the Wettschureck & Offermanns (2005), the GPCR proteins are activated with conformational changes. The binding of molecules that different from their substrates can create different conformational changes on protein structures. As a result, their activities can be manipulated by different molecules which have high binding affinities. In our study, gallein and CMPD-101 were used as known inhibitors of GPCR protein. Their binding affinities were found to be -7.8 kcal/mol and -10.2 kcal/mol, respectively. When the binding scores of commercial biocides were compared with known inhibitors, it was obtained that the docking scores of econea was evaluated as reasonably close to the binding affinities of positive control grup. However, the scores obtained by the chlorothalonil, dichlofluanid, diuron, and irgarol 1051 remained lower even when compared to econea. The antifouling performances of commercial biocides can be sequenced as econea, diuron, dichlofluanid, irgarol 1051 and chlorothalonil according to their binding energies (Table 3.3). According to the *in-silico* results, econea could be good candidate to eradicate the marine biofouling caused by organisms which have barnacle like-GPCR proteins.

Table 3.2 The molecular docking scores of all biocides to G protein coupled receptor protein of *Balanus amphitrite*. The symbol “**” is indicated the scores of known inhibitors

Pose	Chlorothalonil	Irgarol 1051	Binding Scores (kcal/mol)				*Gallein	*CMPD-101
			Dichlofluanid	Diuron	Ecnea			
1	-5.2	-5.6	-6.2	-6.6	-7.0	-7.8	-10.2	
2	-5.2	-5.2	-5.9	-6.2	-6.9	-7.6	-9.6	
3	-5.0	-5.2	-5.6	-6.1	-6.5	-7.5	-9.5	
4	-5.0	-5.2	-5.6	-6.0	-6.4	-7.4	-9.5	
5	-5.0	-5.2	-5.5	-6.0	-6.2	-7.2	-9.5	
6	-4.8	-5.1	-5.5	-5.6	-6.2	-7.2	-9.3	
7	-4.8	-5.1	-5.2	-5.5	-6.2	-6.9	-9.2	
8	-4.8	-5.1	-5.2	-5.5	-6.1	-6.9	-9.2	
9	-4.7	-5.0	-5.1	-5.4	-5.9	-6.7	-9.1	

Table 3.3 The best scores of molecular docking analysis. The symbol “*” is indicated the best scores of known inhibitors

Complex	Score (kcal/mol)
GPCR-Chlorothalonil	-5.2
GPCR-Irgarol 1051	-5.6
GPCR-Dichlofluanid	-6.2
GPCR-Diuron	-6.6
GPCR-Econeal	-7.0
GPCR-Gallein	-7.8
GPCR-CMDP-101	-10.2

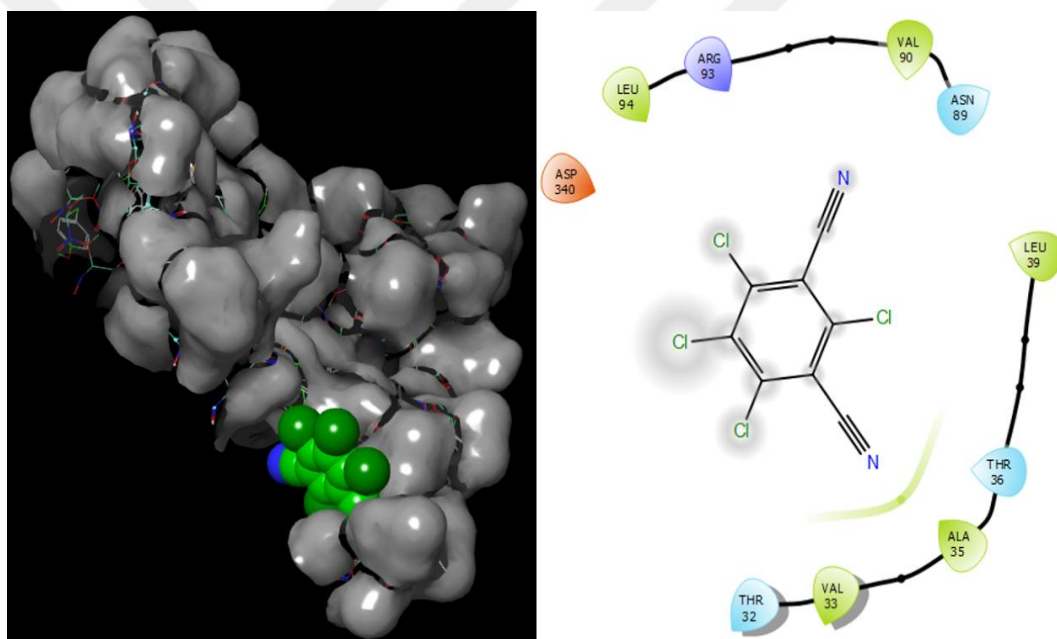


Figure 3.3 Best binding pose and related interactions of Chlorothalonil - G protein coupled receptor complex

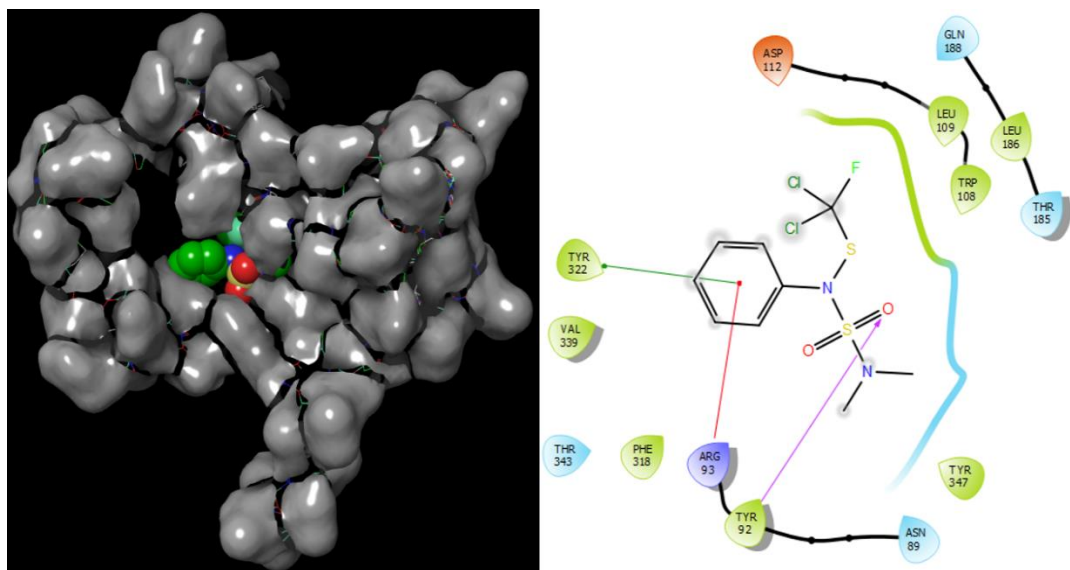


Figure 3.4 Best binding pose and related interactions of Dichlofluanid - G protein coupled receptor complex

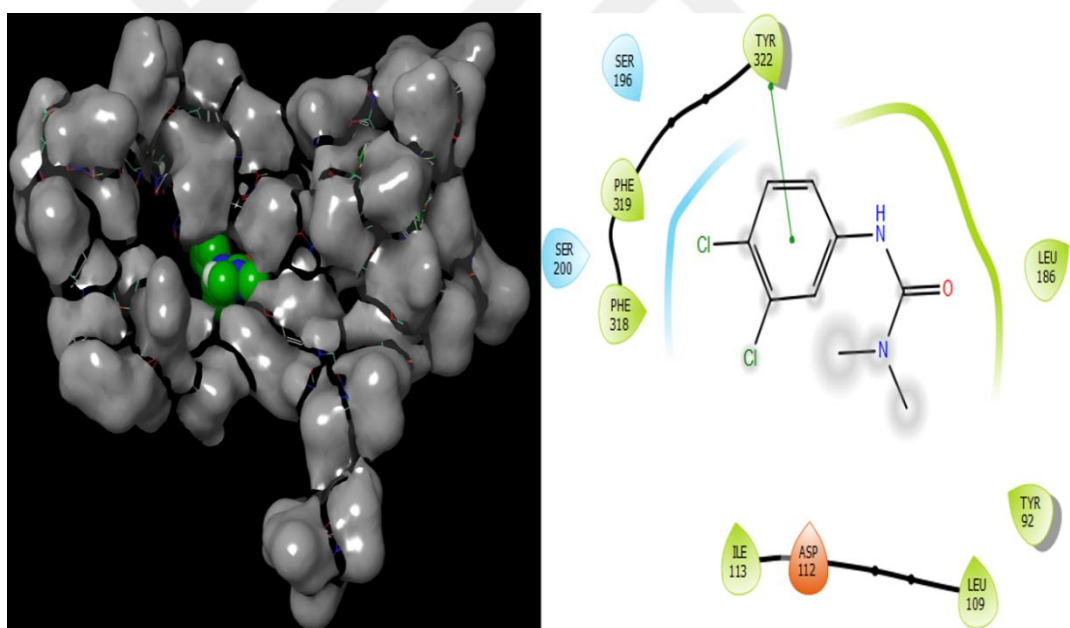


Figure 3.5 Best binding pose and related interactions of Diuron - G protein coupled receptor complex

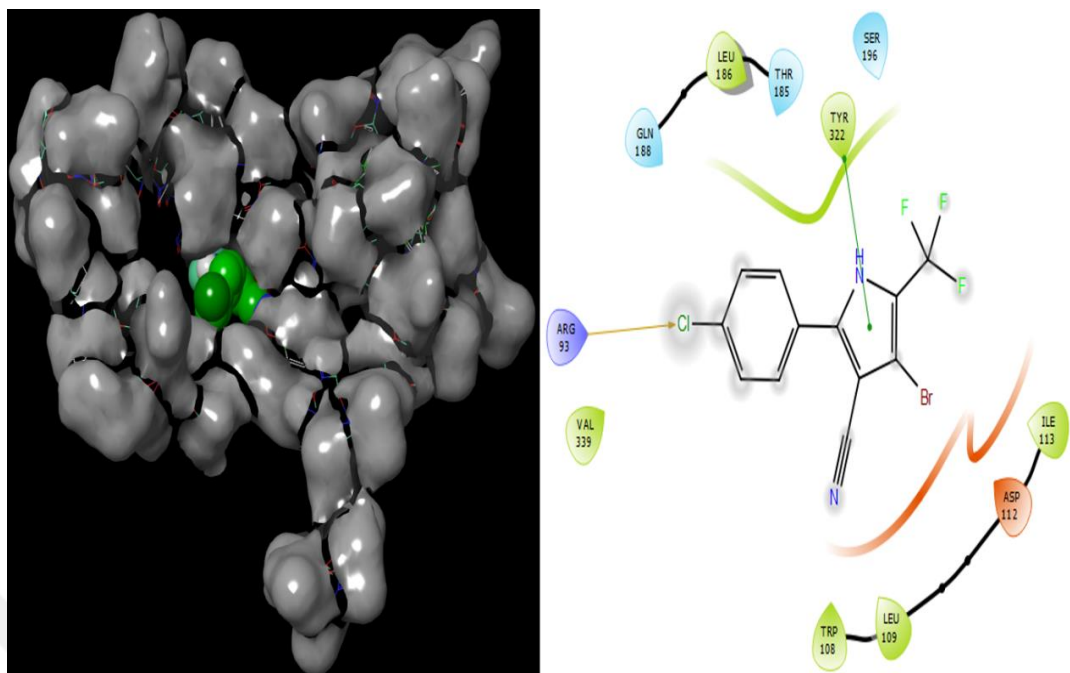


Figure 3.6 Best binding pose and related interactions of Ecomea - G protein coupled receptor complex.

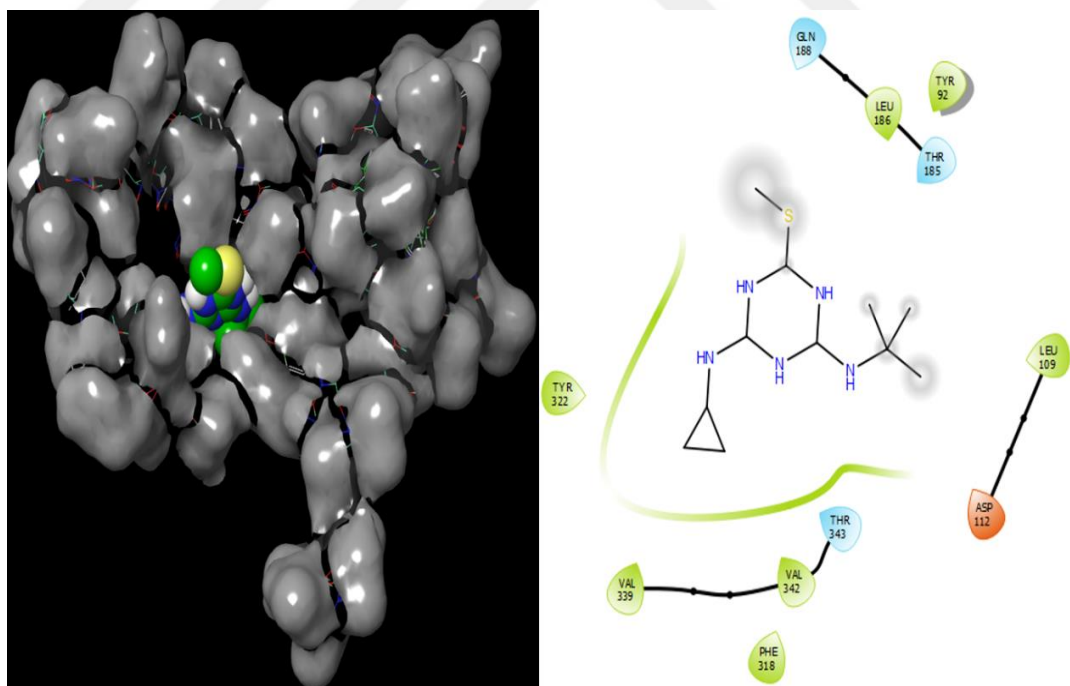


Figure 3.7 Best binding pose and related interactions of Irgarol - G protein coupled receptor complex.

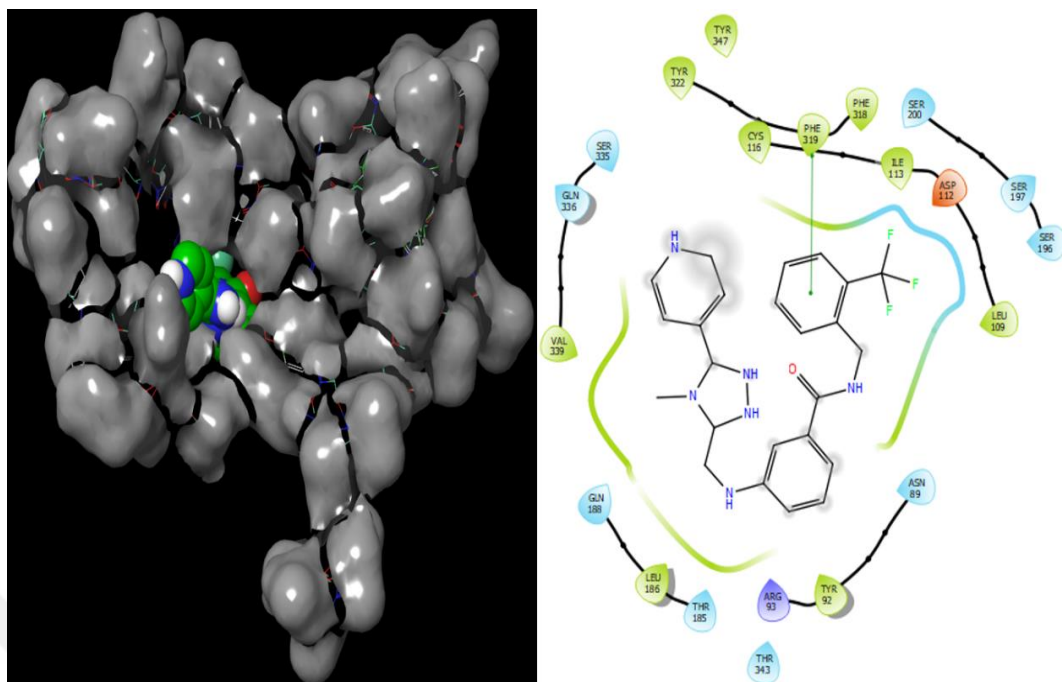


Figure 3.8 Best binding pose and related interactions of CMPD101 - G protein coupled receptor complex

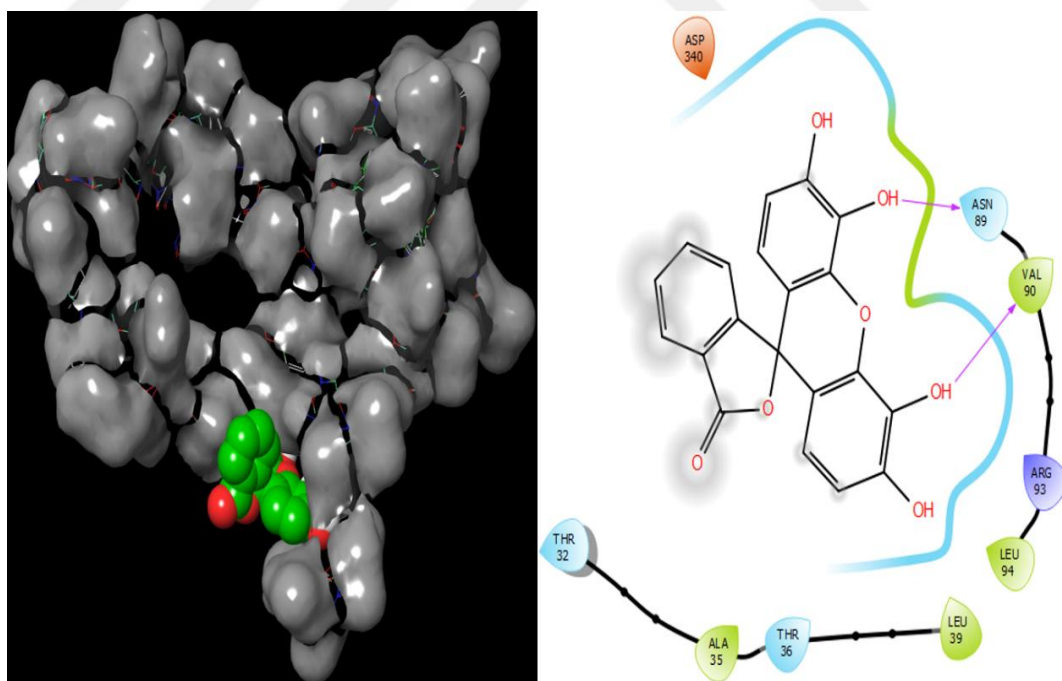


Figure 3.9 Best binding pose and related interactions of Gallein - G protein coupled receptor complex

3.1.2 *In-silico Analysis of Hydroides elegans – Cytochrome C Oxidase Subunit 1 (COI)*

To further investigate the *in-silico* methodologies antifouling coating technologies, cytochrome c oxidase subunit 1 (COI) of *H. elegans* was chosen as another target protein. In this thesis's field testing, it was discovered that *H. elegans* produced a heavy biofouling on sample cage nets (Figure 4.2 and 4.3). According to the literature, *H. elegans* is one of the species that produce calcareous biofouling, which is difficult to remove and causes considerable damage to surfaces (Nedved, 2008). As a result, it might be a useful target for preventing calcareous biofouling on submerged surfaces. In order to carry out *in-silico* analysis the primer sequence of target protein of *H. elegans* was taken from Uniprot (uniprot id: A0A191LJ12). Since there is no XRD related 3D structure of target protein in the Protein Data Base (PDB database), protein modeling was performed in digital environment using SWISS-MODEL. According to the SWISS-MODEL a total of 152 templates were obtained. Template search, target sequence examination, target-template alignment, conserved coordinate creation, remodeling the insertions and deletions, side chain reconstruction and geometry regularization were applied as performed on the GPCR protein of *B. amphitrite*. In order to 3D modelling, template was selected as III2-IV2 mitochondrial respiratory super complex from *S. cerevisiae* (template id: 6hu9.1. U). The sequence identity was found as 52.80. However, the resolution of template was unknown. The total coverage of template was calculated to be 0.99 by SWIS-MODEL. After the 3D modelling, the created structure validated by PROCHECK and ERRAT analysis. According to the scores of validation analysis, 92.9% residues of created structure was found on most favored regions and 6.1% residues were also found on allowed regions. However, only 1% residue was found at disallowed region (Serine position: 69). The threshold value of this analysis is 90% for a good quality model. According to the score, created 3D structure obtained in a good quality with the score of 92.9% (Figure 3.10) (Table 3.4) The average overall quality factor score of ERRAT analysis was established to be 91%. According to the result of ERRAT analysis, the protein was modelled in a good quality with the score of 91.07%. YASARA energy minimization server was used to reduce the conformational energy of the 3D modeled protein to its natural state. Initial energy

and score of the modeled protein was evaluated to be -164242.0 kJ/mol and -4.75, respectively. After the minimization steps final energy and score was obtained as -193164.8 kJ/mol and -0.87. 18342 molecules of water were used at the energy minimization procedure. In order to create the molecular interaction simulation between COI and biocides AutoDockVina was used. The grid dimensions were selected to be center_x = 39.5634896572, center_y = 3.8652349756, center_z = 77.6835833585, size_x = 27.4485369842, size_y = 22.7785336935, size_z = 57.3658941257. The target was selected as COI protein of *H. elegans*. COI protein was selected since it is one of the basic membrane proteins involved in the synthesis of ATP in the metabolism of *H. elegans*. The surface residues of COI protein were visualized via Maestro. The molecular surface of COI protein was represented at Figure 3.11. According to our molecular docking analysis, highest binding affinities of econea, diuron, and irgarol 1051 with COI protein of *H. elegans* were obtained to be -6.0 kcal/mol, -4.6 kcal/mol and -4.4 kcal/mol respectively. All molecular docking scores of commercial biocides were given at Table 3.5. According to the best binding scores, docking poses of target-ligand complexes created via Maestro (Figure 3.12 to 3.14). All interactions of target-ligand complexes can be found on second poses of Figure 3.12 to 3.14.

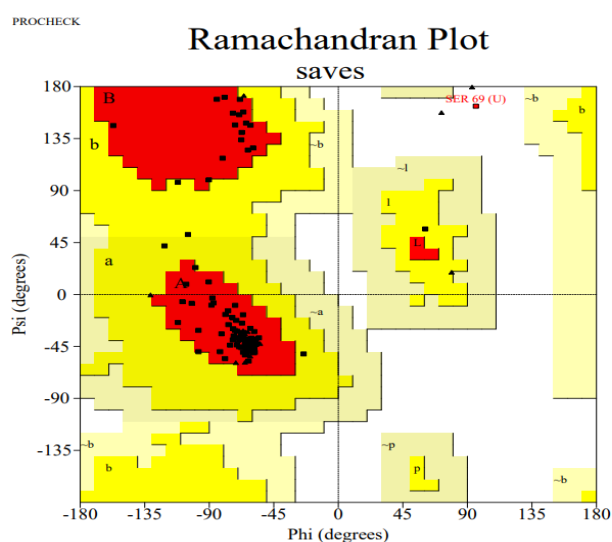


Figure 3.10 The ramachandran plot of 3D modelled cytochrome c oxidase subunit 1 (COI) of *Hydroides elegans*.

Table 3.4 Ramachandran plot statistics for the 3D modeled cytochrome c oxidase subunit 1 protein.

Residues	Total Number	Percentage (%)
Residues in most favoured regions	92	92.9
Residues in additional allowed regions	6	6.1
Residues in generously allowed regions	0	0.0
Residues in disallowed regions	1	1
Number of non-glycine and non-proline residues	99	100
Number of end-residues	1	
Number of glycine residues	15	
Number of proline residues	10	
Total number of residues	125	

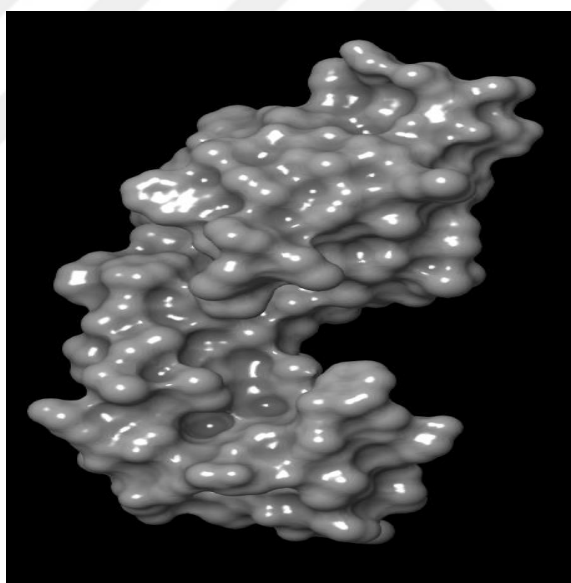


Figure 3.11 The molecular surface of cytochrome c oxidase subunit 1 of *Hydrilles elegans*

Table 3.5 The molecular docking scores of all biocides to cytochrome c oxidase subunit 1 of *Hydroides elegans*.

Poses	Binding Scores (kcal/mol)		
	Econea	Diuron	Irgarol
1	-6.0	-4.6	-4.4
2	-5.7	-4.4	-4.3
3	-5.2	-4.2	-4.2
4	-4.9	-4.2	-4.0
5	-4.4	-3.9	-3.9
6	-4.2	-3.6	-3.8
7	-4.0	-3.3	-3.5
8	-3.8	-3.2	-3.4
9	-3.6	-3.0	-3.0

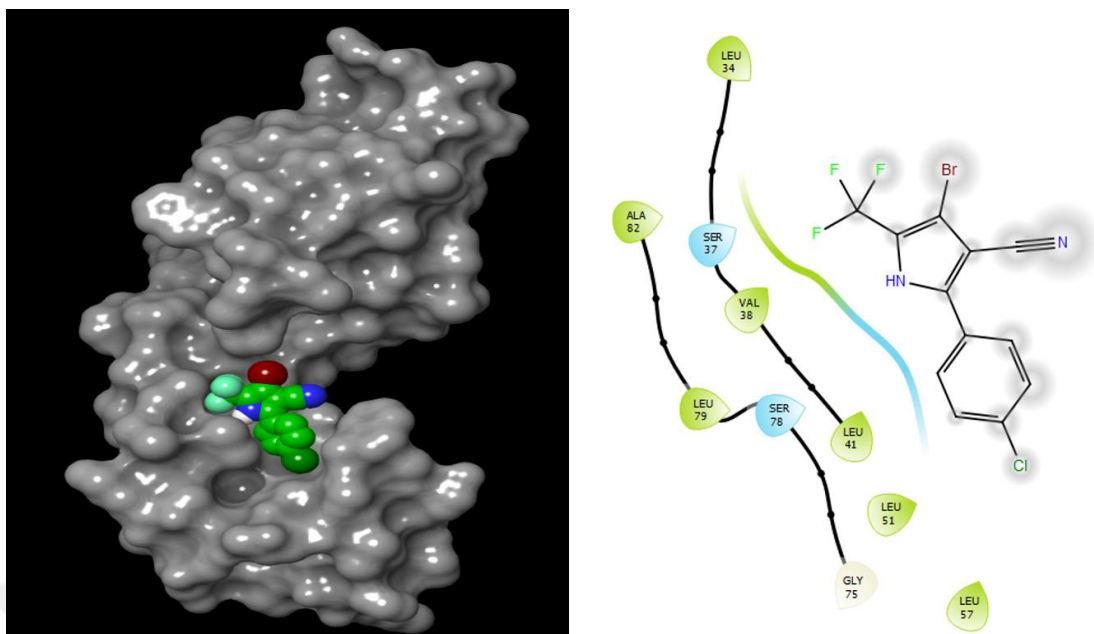


Figure 3.12 Best binding pose and related interactions of Econe - cytochrome c oxidase subunit 1 complex

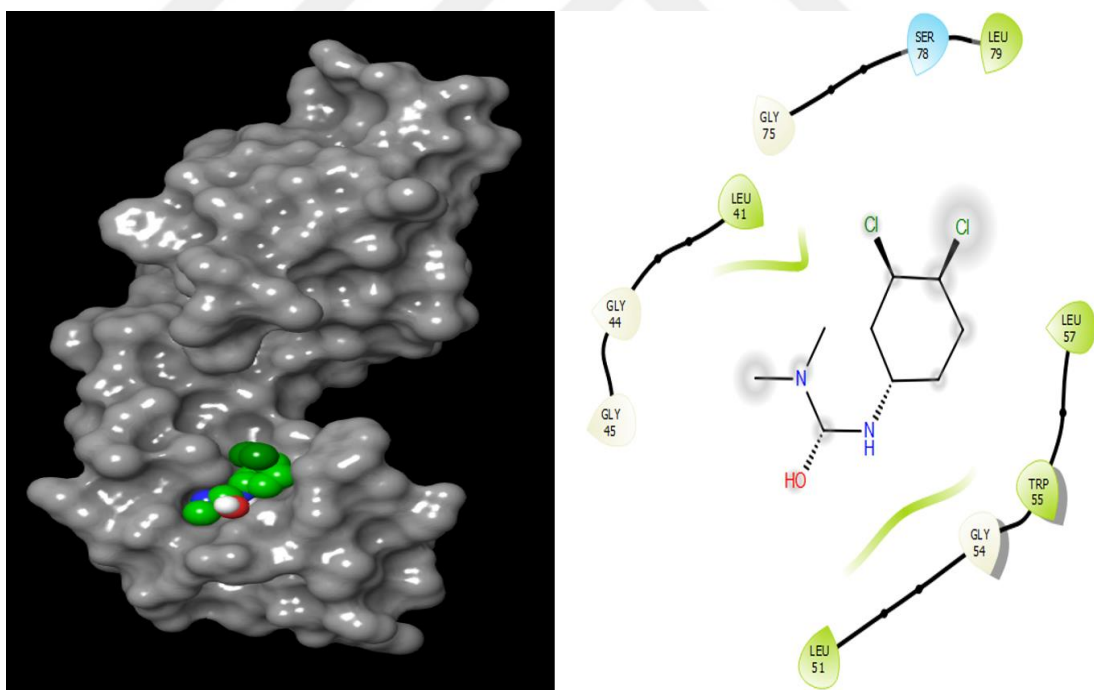


Figure 3.13 Best binding pose and related interactions of Diuron - cytochrome c oxidase subunit 1 complex

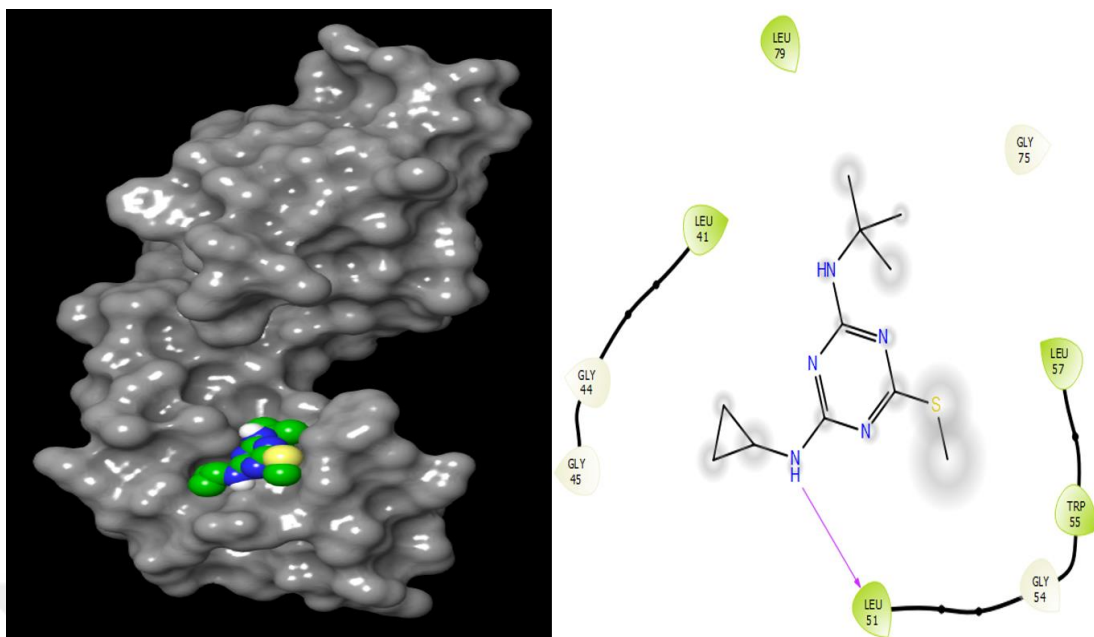


Figure 3.14 Best binding pose and related interactions of Irgarol - cytochrome c oxidase subunit 1 complex

3.1.3 *In-silico* Analysis of *Bugula neritina* – Cytochrome B (Cyt-b)

B. neritina colonized the surface of sample cage nets after the second week of trial in the field experiments described in this thesis. As a result, *B. neritina* was chosen as the third target organism for *in-silico* testing procedures. When the protein structures of *B. neritina* were examined in online data banks, no protein structure revealed by XRD or other crystallographic methods were identified. As a result, SWISS-MODEL was employed to digitally model the 3D protein structure used in *in-silico* analyses. When the UniProt database was scanned for *B. neritina*, the authoritatively approved Cytochrome b protein was found (uniprot id: A9UKA2). This protein was chosen for further analysis because it has been approved by the authorities and plays an important role in ATP synthesis in *B. neritina* metabolism. According to the SWISS-MODEL a total of 150 templates were obtained. The template search, target sequence examination, target-template alignment, conserved coordinate creation, remodeling the insertions and deletions, side chain reconstruction and geometry regularization were applied as performed on the GPCR protein of *B. amphitrite* and COI enzyme of *H. elegans*. In order to 3D modelling, template was selected as murine super complex

CIII2CIV in the assembled locked conformation (template id: 7o37.1.C). The sequence identity was found as 57.30. On the other hand, the resolution of template was unknown. The total coverage of template was calculated to be 0.99 by SWIS-MODEL. PROCHECK and ERRAT analysis were used to evaluate the validation of 3D modelled protein. According to the scores of validation analysis, 91.9% residues of created structure was found on most favored regions and 7.8% residues were also found on allowed regions. However, only 0.3% residue was found at disallowed region (Tyrosine position: 155). The threshold value of this analysis is 90% for a good quality model. According to the score, created 3D structure obtained in a good quality with the score of 91.9% (Figure 3.15 and Table 3.6). The average overall quality factor score of ERRAT analysis was established to be 91%. According to the result of ERRAT analysis, the protein was modelled in a good quality with the score of 93.48%. In order to reduce the conformational energy of 3D modelled protein YASARA energy minimization server was used. Initial energy and score of the modeled protein was evaluated to be -117856.8 kJ/mol and -9.56, respectively. After the minimization steps final energy and score was obtained as -81284.2 kJ/mol and -1.85. 21342 molecules of water were used at the energy minimization procedure. In order to evaluate the molecular docking between Cyt-b and biocides AutoDockVina was used. The grid dimensions were selected to be center_x = 43.5896246873, center_y = 76.7456983215, center_z = 48.1599874236, size_x = 61.4256987235, size_y = 14.8563214895, size_z = 13.7569842315. The target was selected as Cyt-b protein of *B. neritina*. Cyt-b protein, which has a very important missions in ATP synthesis reactions in *B. neritina* metabolism, was chosen as target because it is one of the basic enzymes necessary to maintain its vital functions. The surface residues of Cyt-b protein were visualized via Maestro (Figure 3.16). According to our molecular docking analysis, highest binding affinities of econea, diuron, and irgarol 1051 to Cyt-b protein of *B. neritina* were evaluated to be -9.0 kcal/mol, -6.2 kcal/mol and -6.8 kcal/mol, respectively. All molecular docking scores of commercial biocides were given at Table 3.7. According to the best binding scores, docking poses of target-ligand complexes created via Maestro (Figure 3.17 to 3.19). All interactions of target-ligand complexes can be found on second poses of Figure 3.17 to 3.19.

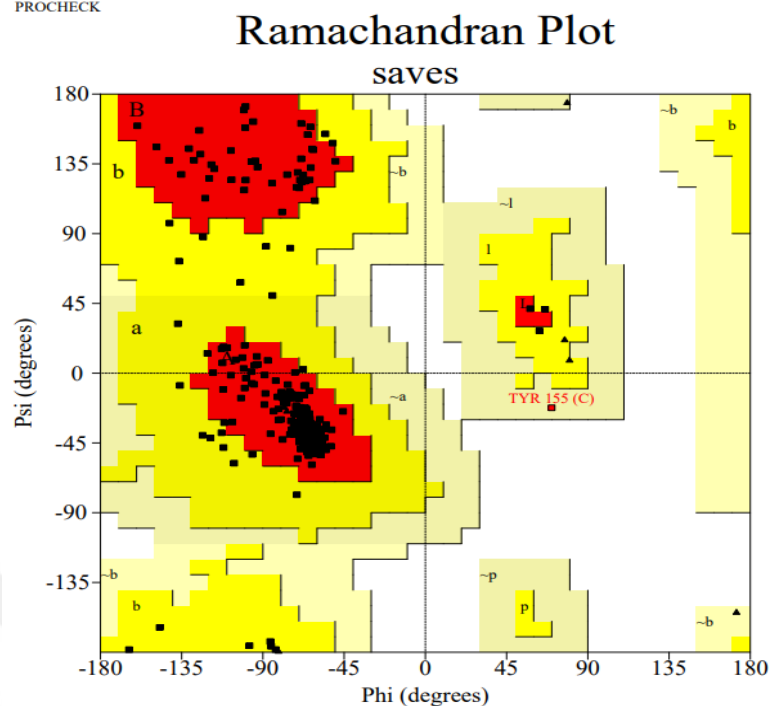


Figure 3.15 The ramachandran plot of 3D modelled cytochrome b of *Bugula nertina*

Table 3.6 Ramachandran plot statistics for the 3D modeled cytochrome b protein

Residues	Total Number	Percentage (%)
Residues in most favored regions	294	91.9
Residues in additional allowed regions	25	7.8
Residues in generously allowed regions	1	0.3
Residues in disallowed regions	0	0
Number of non-glycine and non-proline residues	320	100
Number of end-residues	2	
Number of glycine residues	21	
Number of proline residues	20	
Total number of residues	125	

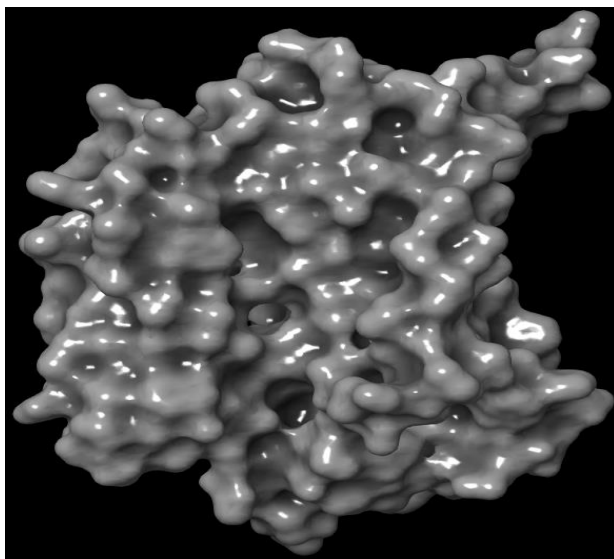


Figure 3.16 The molecular surface of cytochrome b of *Bugula nertina*

Table 3.7 The molecular docking scores of all biocides to cytochrome b protein of *Bugula neritina*.

Poses	Binding Scores (kcal/mol)			
	Econea	Diuron	Irgarol	
1	-9.2	-6.8	-6.3	
2	-9.0	-6.8	-6.2	
3	-8.2	-6.2	-6.1	
4	-7.1	-6.0	-5.6	
5	-7.1	-5.9	-4.8	
6	-6.1	-5.1	-3.6	
7	-6.1	-5.0	-3.2	
8	-5.2	-4.9	-2.2	
9	-5.0	-4.4	-2.0	

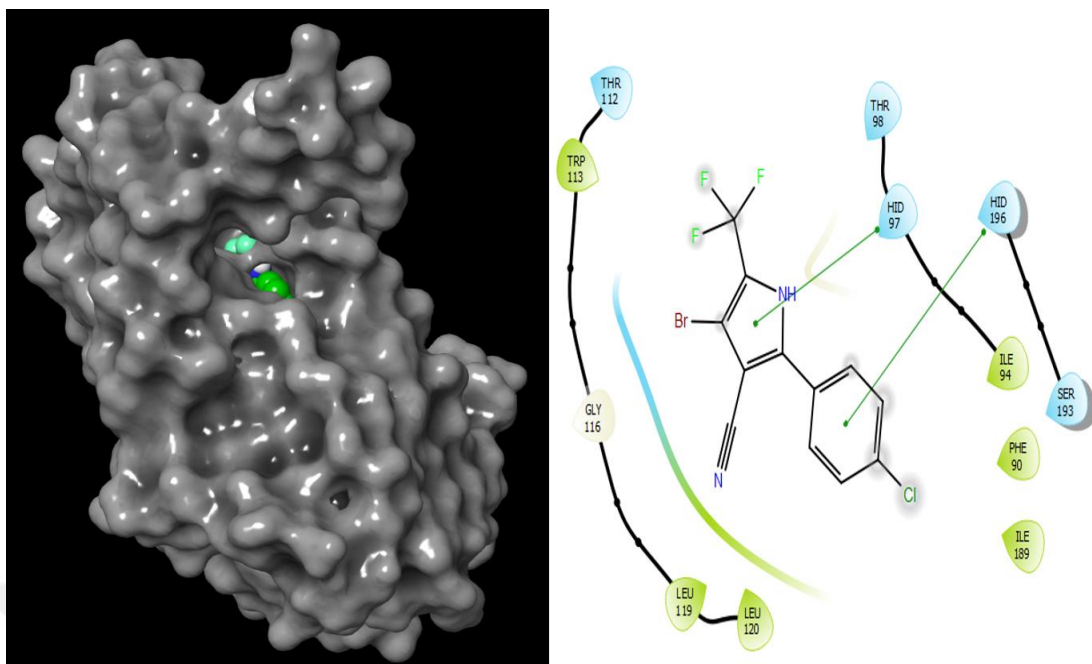


Figure 3.17 Best binding pose and related interactions of Econe - cytochrome b complex

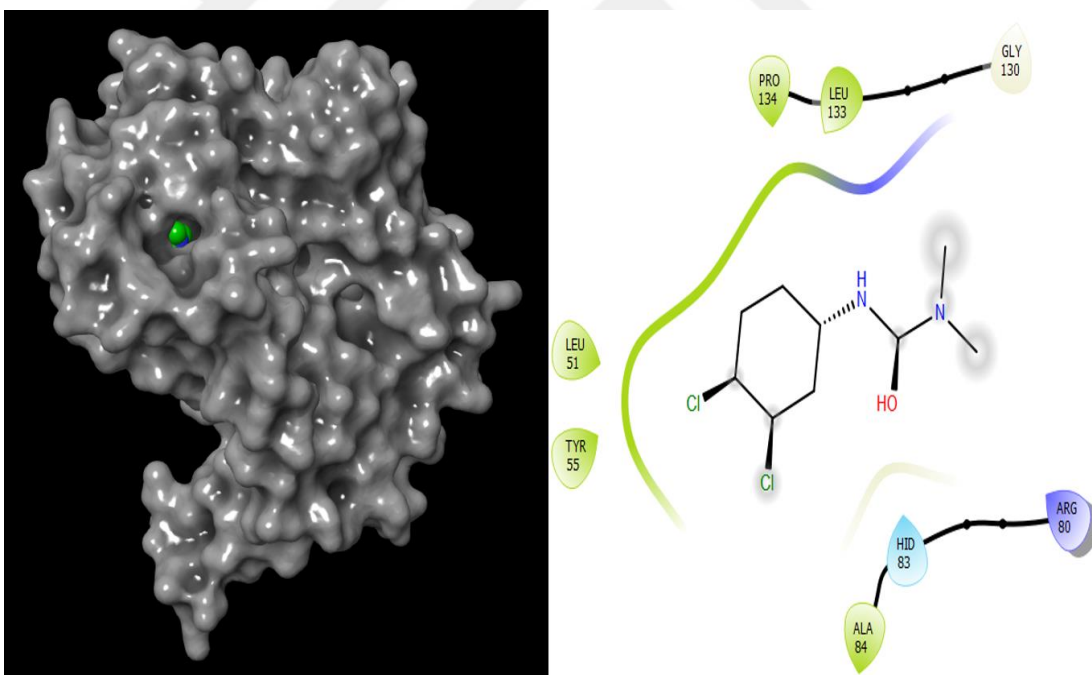


Figure 3.18 Best binding pose and related interactions of Diuron - cytochrome b complex

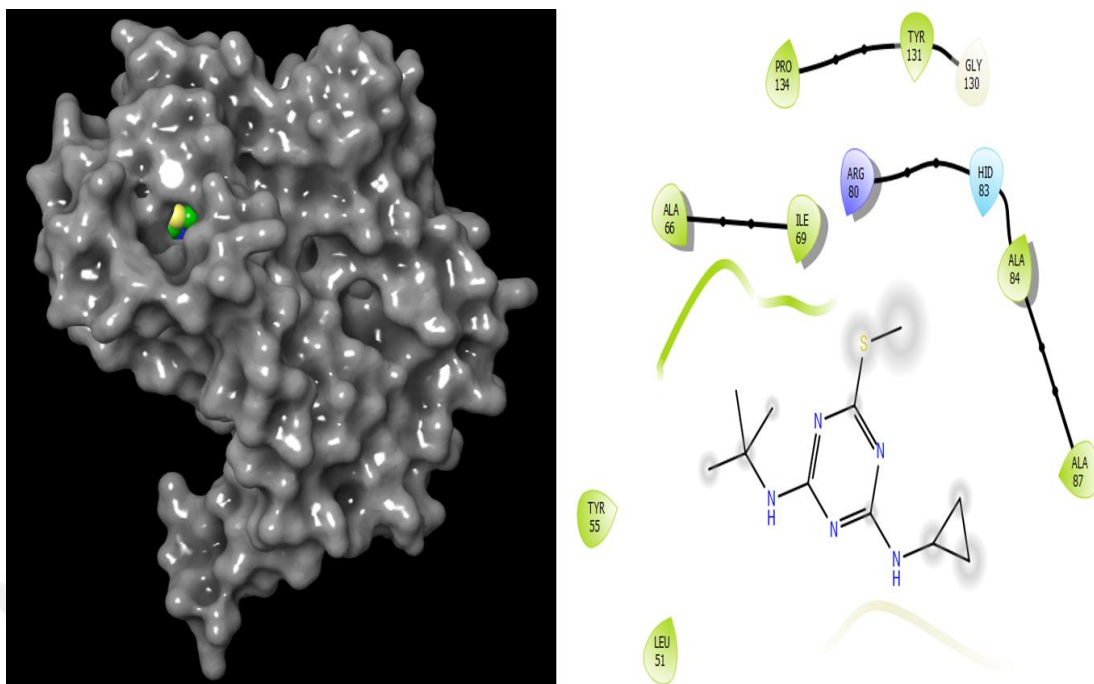


Figure 3.19 Best binding pose and related interactions of Irgarol - cytochrome b complex

3.1.4 *In-silico* Analysis of *Ulva lactuca* – Photosystem II D1 (P-II-D1)

In the field experiments described in this thesis, *U. lactuca* was found on the surface of sample cage nets coated with SAC-E-based coatings after the third week of the experiment. As a result, *U. lactuca* was chosen as the fourth target organism for *in-silico* testing procedures. When the protein structures of *U. lactuca* were examined in protein data banks, no protein structure revealed by XRD or other crystallographic techniques were identified. As a result, SWISS-MODEL was employed to digitally model the 3D protein structure used in *in-silico* analyses. The Photosystem II D1 (P-II-D1) protein was found when the UniProt database was searched for *U. lactuca* (uniprot id: A0A4D5XX70). According to the SWISS-MODEL a total of 241 templates were obtained. The template search, target sequence examination, target-template alignment, conserved coordinate creation, remodeling the insertions and deletions, side chain reconstruction and geometry regularization were applied as performed on the GPCR protein of *B. amphitrite*, COI protein of *H. elegans* and Cyt-b protein of *B. neritina*. In order to 3D modelling, template was selected as crystal structure of oxygen-evolving photosystem II from a red alga (template id: 4yuu.1). The

sequence identity was found as 87.79 and the resolution of template was 2.77Å. The total coverage of template was calculated to be 0.97 by SWIS-MODEL. PROCHECK and ERRAT analysis were used to evaluate the validation of 3D modelled protein. According to the scores of validation analysis, 90.6% residues of created structure was found on most favored regions and 8.8% residues were also found on allowed regions. However, only 0.7% residue was found at disallowed region (Asparagine position: 230 and Tyrosine position: 2). The threshold value of this analysis is 90% for a good quality model. According to the score, created 3D structure obtained in a good quality with the score of 91.9% (Figure 3.20) (Table 3.8). The average overall quality factor score of ERRAT analysis was established to be 91%. According to the result of ERRAT analysis, the protein was modelled in a good quality with the score of 97.50%. In order to reduce the conformational energy of 3D modelled protein YASARA energy minimization server was used. Initial energy and score of the modeled protein was respectively evaluated to be -147856.8 kJ/mol and -8.33. After the minimization steps final energy and score was obtained as -117128.2 kJ/mol and -3.70. 12225 molecules of water were used at the energy minimization procedure. In order to evaluate the molecular docking between P-II-D1 and biocides AutoDockVina was used. The grid dimensions were selected to be center_x = 12.5486257892, center_y = 25.4589631578, center_z = 7.4758596315, size_x = 66.4236806579, size_y = 22.4589632158, size_z = 45.5479631589. The target was selected as P-II-D1 protein of *U. lactuca* which is essential in ATP synthesis reactions in the metabolism of *U. lactuca*. The surface residues of P-II-D1 protein were visualized via Maestro (Figure 3.21). According to our molecular docking analysis, highest binding affinities of econea, diuron, and irgarol 1051 to P-II-D1 protein of *U. lactuca* were evaluated to be -6.7 kcal/mol, -5.3 kcal/mol and -5.5 kcal/mol, respectively. All molecular docking scores of commercial biocides were given at Table 3.9. According to the best binding scores, docking poses of target-ligand complexes created via Maestro (Figure 3.22 to 3.24). All interactions of target-ligand complexes can be found on second poses of Figure 3.22 to 3.24.

Ramachandran Plot

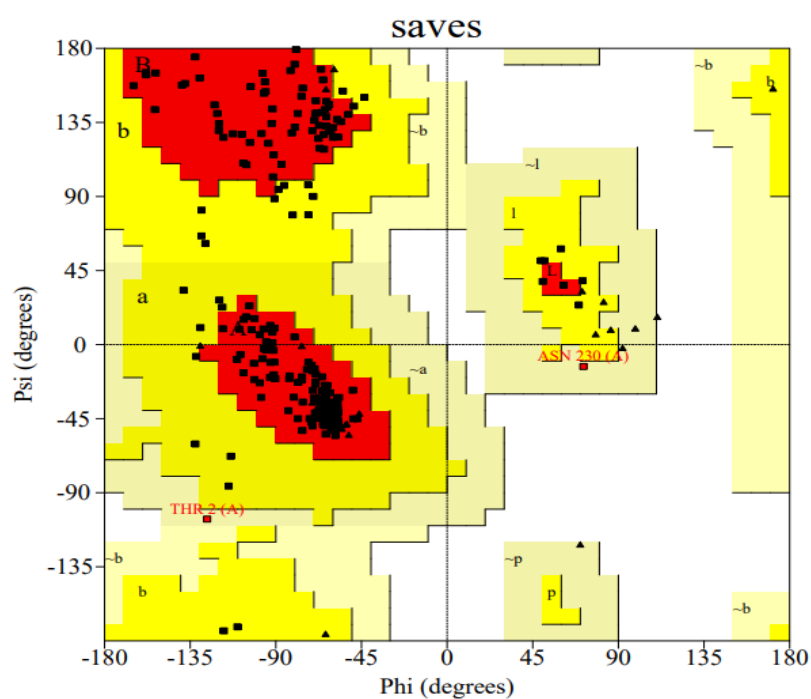


Figure 3.20. The ramachandran plot of 3D modelled photosystem II D1 (P-II-D1) of *Ulva lactuca*

Table 3.8 Ramachandran plot statistics for the 3D modeled photosystem II D1 protein

Residues	Total Number	Percentage (%)
Residues in most favored regions	269	90.6
Residues in additional allowed regions	26	8.8
Residues in generously allowed regions	2	0.7
Residues in disallowed regions	0	0.0
Number of non-glycine and non-proline residues	297	100
Number of end-residues	2	
Number of glycine residues	31	
Number of proline residues	14	
Total number of residues	344	

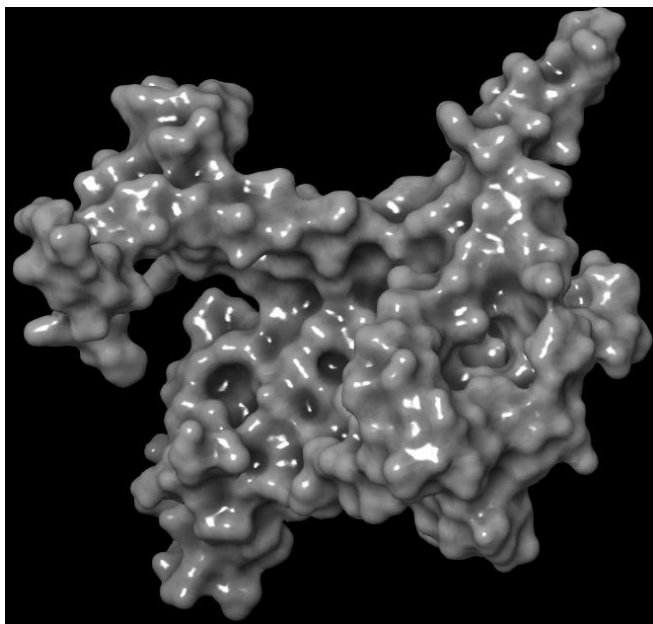


Figure 3.21 The molecular surface of photosystem II D1 (P-II-D1) of *Ulva lactuca*

Table 3.9 The molecular docking scores of all biocides to photosystem II D1 protein of *Uva lactuca*.

Poses	Binding Scores (kcal/mol)		
	Econea	Diuron	Irgarol
1	-6.7	-5.3	-5.5
2	-6.3	-4.8	-5.4
3	-6.1	-4.5	-5.1
4	-5.3	-4.4	-4.1
5	-5.0	-4.2	-4.0
6	-4.8	-4.0	-3.6
7	-4.0	-3.8	-3.4
8	-2.8	-3.6	-2.6
9	-2.0	-3.0	-2.2

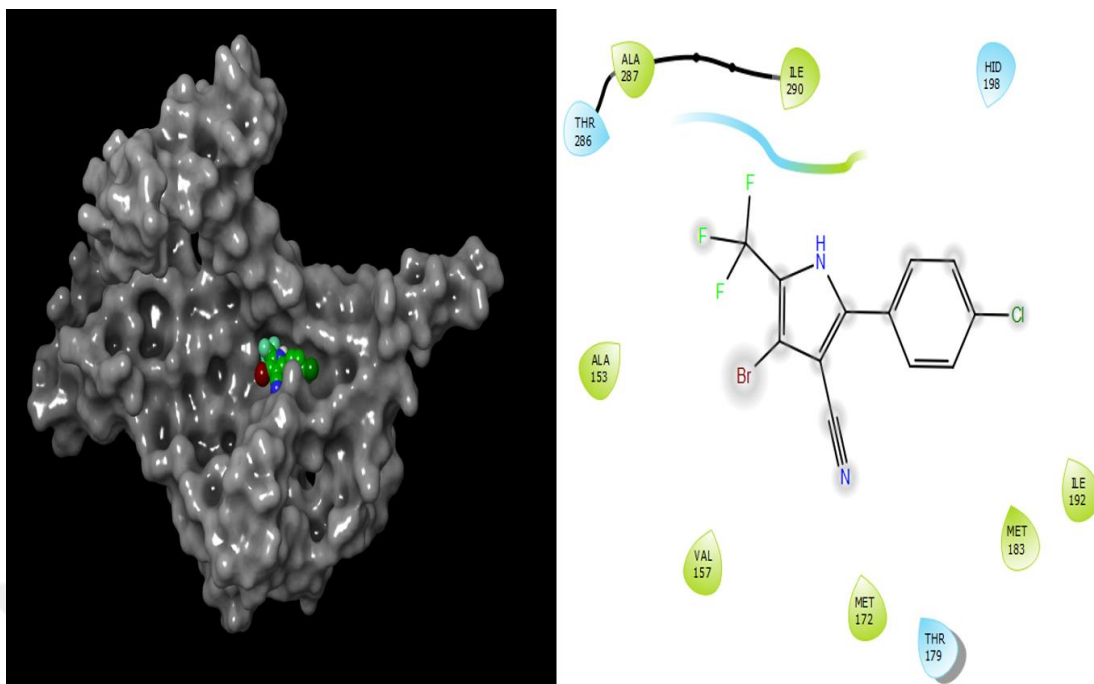


Figure 3.22 Best binding pose and related interactions of Econe - photosystem II D1 complex

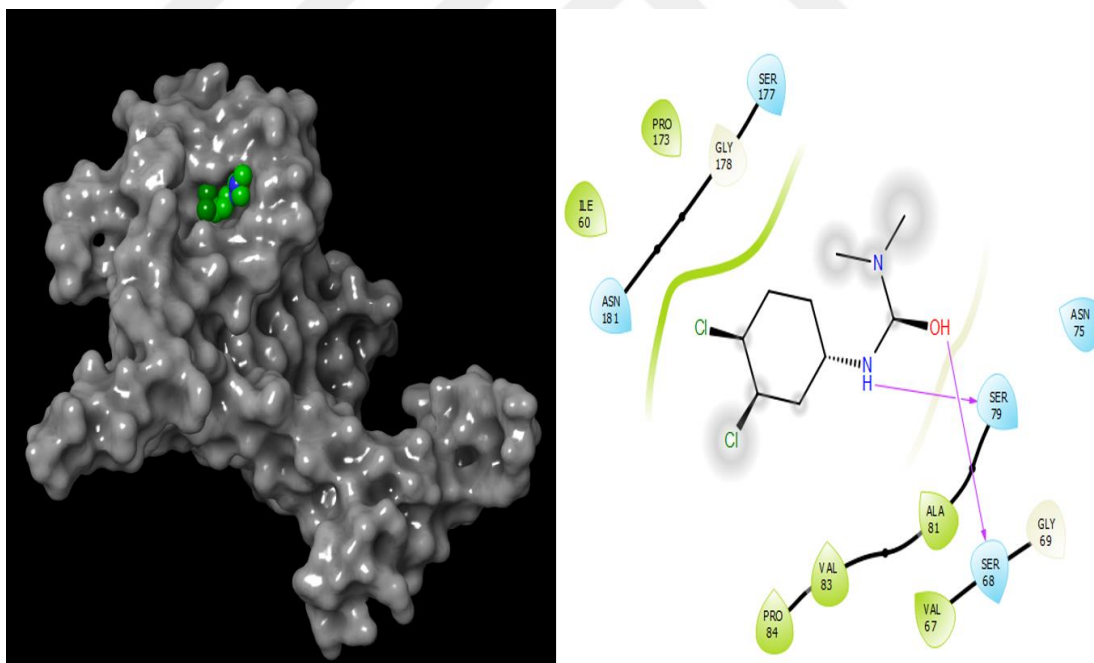


Figure 3.23 Best binding pose and related interactions of Diuron - photosystem II D1 complex

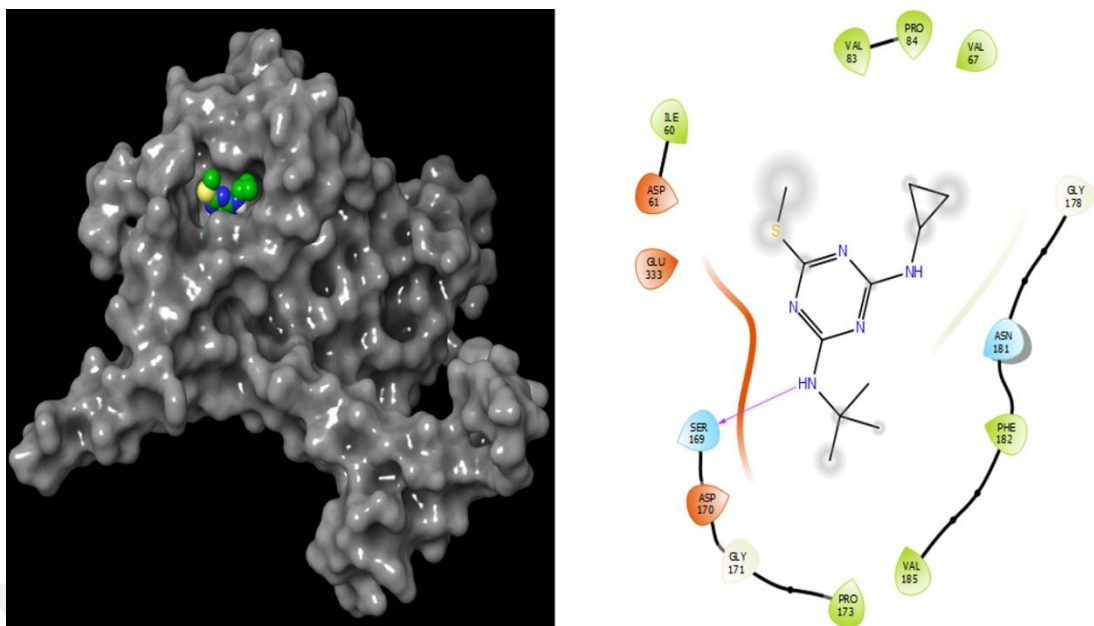


Figure 3.24 Best binding pose and related interactions of Irgarol - photosystem II D1 complex

3.2 Field Test Results

3.2.1 The Field Test Result of Non-biocidal Coatings

The biofouling progression on non-biocidal coatings are presented in Figure 3.25 to 3.30. Uncoated, paraffin wax, strene-acrylic copolymer, rosin and lanolin coated polyethylene (PE) nets were immersed in İnciraltı/İzmir (Türkiye) for the time period between 01 – 21 August 2021.

The biofouling degree assessment criteria followed as the total number of net openings is counted before and after the field experiment. The percentage is obtained by simple proportional calculation. For example; for the Figure 3.25 the total number of net openings was counted as 100 before the experiment. After three weeks, only five openings were uncolonized by fouling organisms. That means the biofouling degree was evaluated as 95 % for the first sample.

According to the Figure 3.25, uncoated nets were covered by marine fouling organisms. When the surfaces of nets are carefully examined, following fouling

organisms were identified on the surfaces: *B. neritina*, *B. amphitrite*, *H. elegans* and other soft macro fouling organisms based on tunicates. The level of coverage was detected as 95 % after three weeks of experiment.

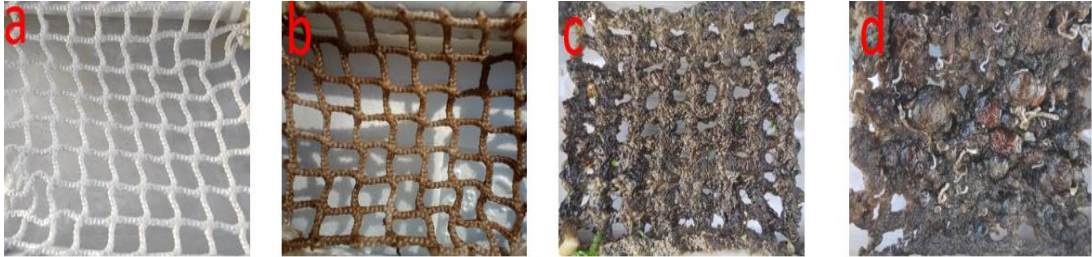


Figure 3.25 The field results of uncoated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

Paraffin wax coated nets were fouled heavily after two weeks later by marine fouling organisms. After three weeks, *B. amphitrite*, *H. elegans* and other soft macro fouling organisms were observed on the wax coatings. When the surfaces of nets are examined, the biofouling coverage was evaluated as 80 % at the end of the field test (Figure 3.26).

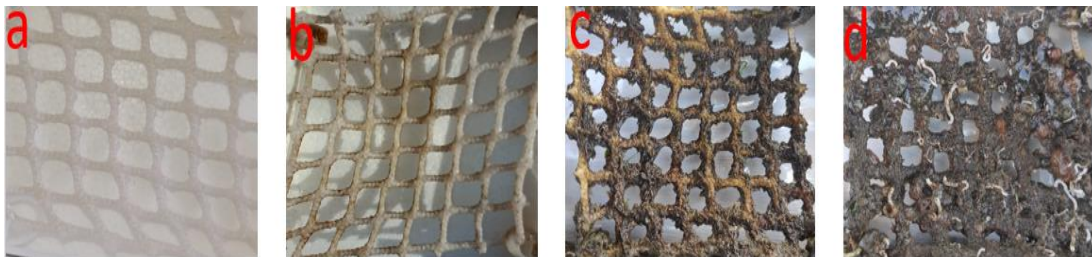


Figure 3.26 The field results of paraffin wax coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.27, styrene-acrylic copolymer coated nets were excessively fouled after two weeks later by marine fouling organisms. *B. amphitrite*, *H. elegans*, *U. lactuca* and other soft macro fouling organisms were observed on the styrene-acrylic copolymer coatings after two weeks. When the surfaces of nets are examined, the biofouling coverage was evaluated as 95 % at the end of the field test.

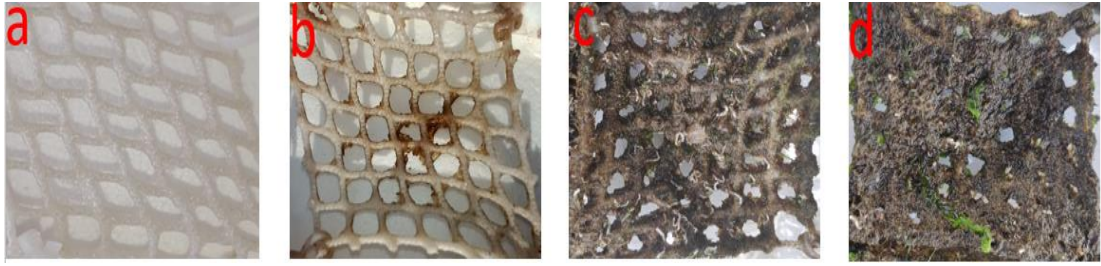


Figure 3.27 The field results of styrene-acrylic copolymer coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.28, styrene-acrylic copolymer – PEG600 crosslink coated nets were fouled after two weeks later by marine fouling organisms. *B. amphitrite*, high amount of *U. lactuca* and other soft macro fouling organisms were observed on the coatings after the three weeks. When the surfaces of nets are investigated, the biofouling coverage was evaluated as 100 % at the end of the field test.

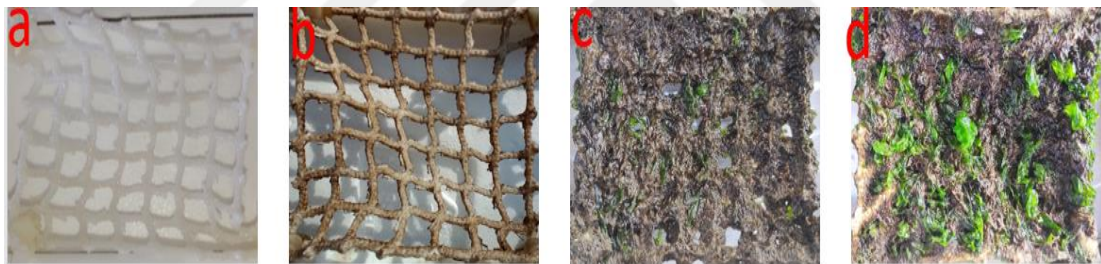


Figure 3.28 The field results of uncoated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.29, lanolin coated nets were fouled after two weeks later by marine fouling organisms. High amount of soft macro fouling organisms and a small amount of *H. elegans* were observed on the coatings after three weeks. When the surfaces of nets are examined, the biofouling coverage was evaluated as 50 % at the end of the field test.

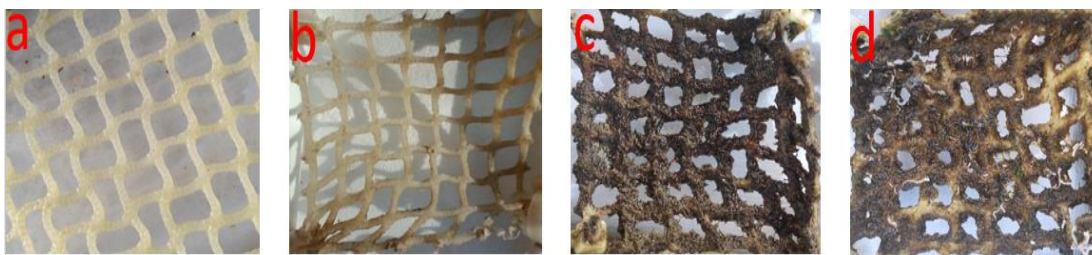


Figure 3.29 The field results of uncoated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.30, rosin coated nets were totally covered after the two weeks of immersion by marine fouling organisms. When the surfaces of nets are carefully examined, following fouling organisms were identified on the surfaces: *B. neritina*, *B. amphitrite*, *H. elegans* and other soft macro fouling organisms based on tunicates. The level of coverage was detected as 95 % after the two weeks. However, the fouling density was reduced at week three. It can be evaluated that, the reduction in fouling density directly related with self-polishing properties of rosin binder.

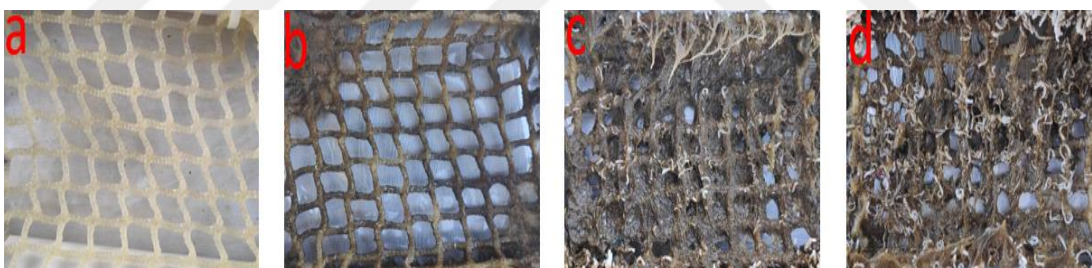


Figure 3.30 The field results of rosin polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

After the first field test with biocide-free coatings, it was found that rosin and lanolin binders have an antifouling effect. The antifouling effect obtained in the field tests is directly related to the self-polishing properties of these binders. Rosin and lanolin, which were found to be successful compared to other binders, were used in formulations containing biocides in the second field test. After the first field test, the sea organisms were collected from the surface of the net samples and the microscope images were displayed in Figure 3.31. According to Figure 3.31, various marine

organisms were found on the nets, and it was determined that *B. neritina*, *B. amphitrite* and *H. elegans* were intensively created biofouling on the net samples.

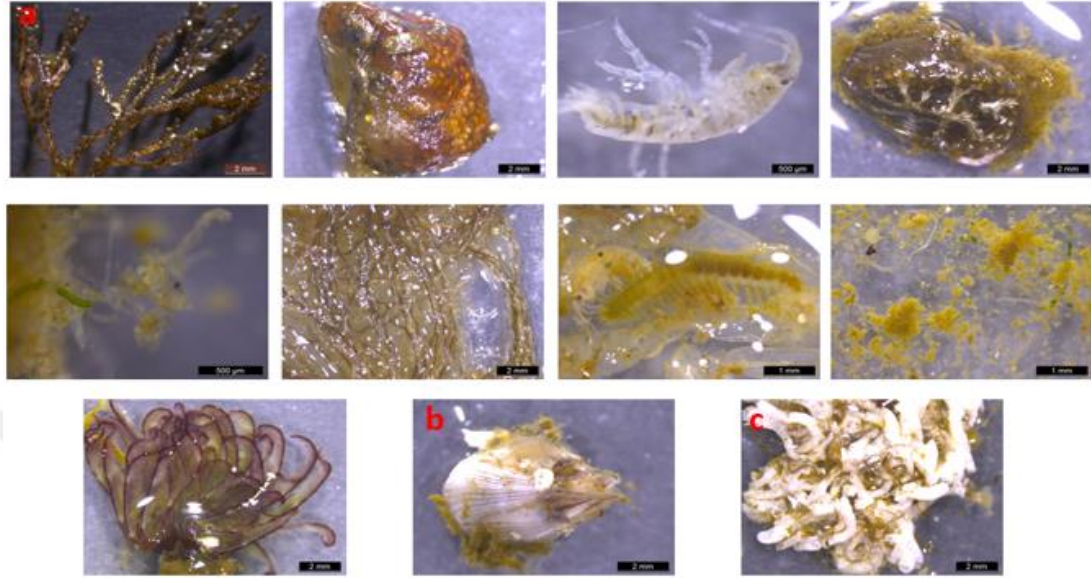


Figure 3.31 The microscope images of the community of living organisms on biocide-free coatings, hard fouling organisms a) *Bugula neritina*, b) *Balanus amphitrite*, c) *Hydroides elegans*

3.2.2 The Field Test Result of Biocide Containing Coatings

The biofouling progression on biocidal rosin and lanolin coatings are presented in Figure 3.32 to 3.37. Rosin and lanolin coated PE nets were immersed in İnciraltı/İzmir (Türkiye) for the time period of 01 – 21 September 2021. In the literature, it has been determined that the use of biocides in antifouling coating formulations does not exceed 3% (Silvia et al., 2019; Bloecher & Floerl, 2020). Therefore, in this study, Econe, diuron and irgarol 1051 were used as the main biocides at a rate of 3% (w/w). The biofouling degree determination method used on biocide-free coatings was applied in the same way.

According to the Figure 3.32, biocidal rosin coated nets were fouled after three weeks of immersion by small amount of *H. elegans*. The level of coverage was detected as lower than 1%.

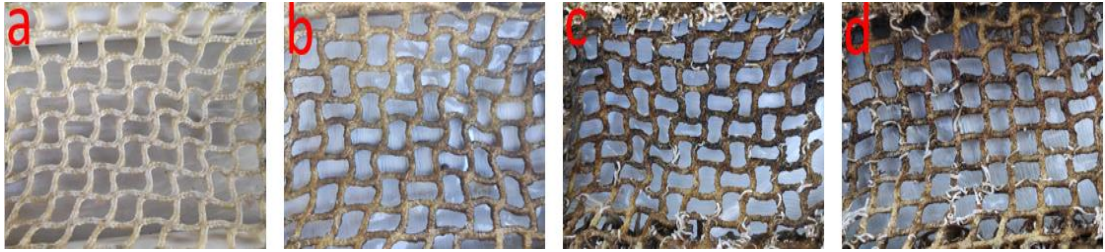


Figure 3.32 The field results of rosin (econe 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.33, 3% (w/w) diuron containing rosin coated nets were fouled after three weeks of immersion by *H. elegans* and *B. neritina*. The level of coverage was detected as 95% at the end of the experiment.

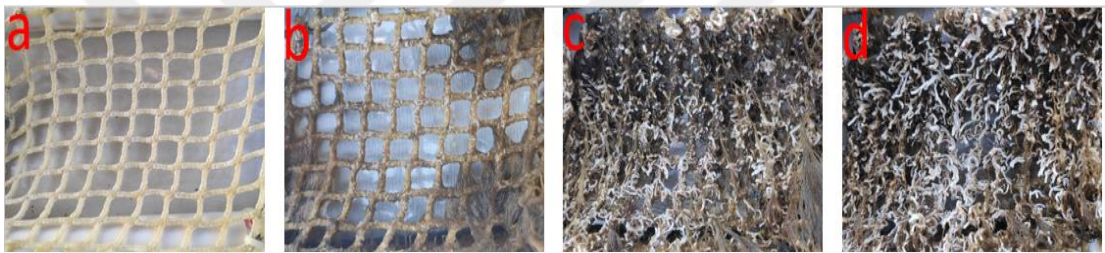


Figure 3.33 The field results of rosin (diuron 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.34, 3% (w/w) irgarol 1051 containing rosin coated nets were intensively fouled after three weeks of immersion by *H. elegans* and *B. neritina*. The level of coverage was detected as 100% at the end of the experiment.

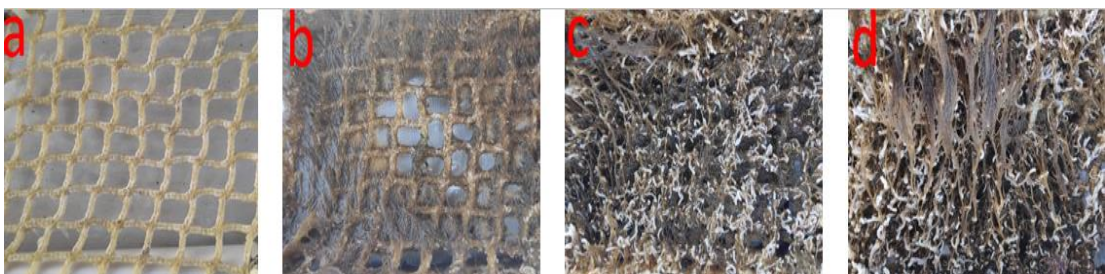


Figure 3.34 The field results of rosin (irgarol 1051 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.35, lanolin coatings containing 3% (w/w) econea coated nets were not fouled by any kind of macro fouling organisms. The level of coverage was detected as 0%.

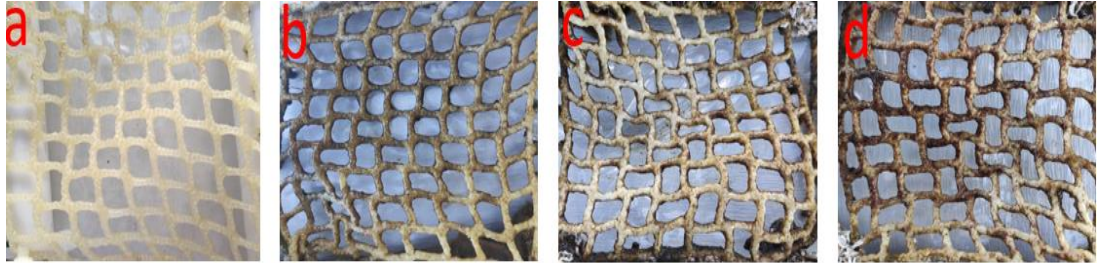


Figure 3.35 The field results of lanolin (econea 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.36, 3% (w/w) diuron containing lanolin coated nets were extensively fouled after 2 weeks of immersion by *H. elegans*, *B. neritina* and other soft macro fouling organisms. The level of coverage was evaluated as 90% at the end of the experiment.

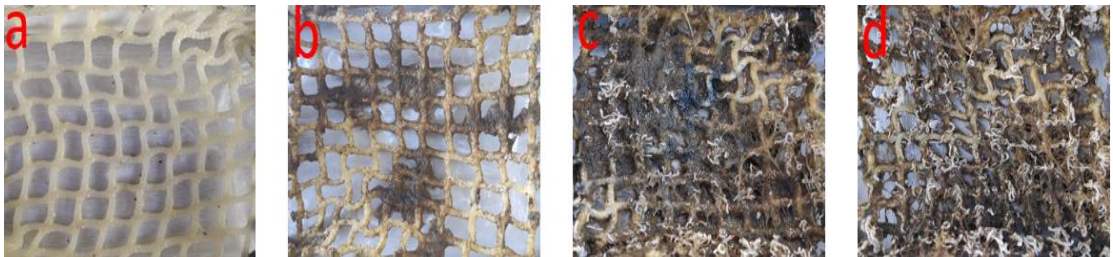


Figure 3.36 The field results of lanolin (diuron 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

According to the Figure 3.37, 3% (w/w) irgarol 1051 containing lanolin coated nets were fouled after 3 weeks of immersion by *H. elegans*, *B. neritina* and other soft macro fouling organisms. The level of coverage was evaluated as 85% at the end of the experiment.

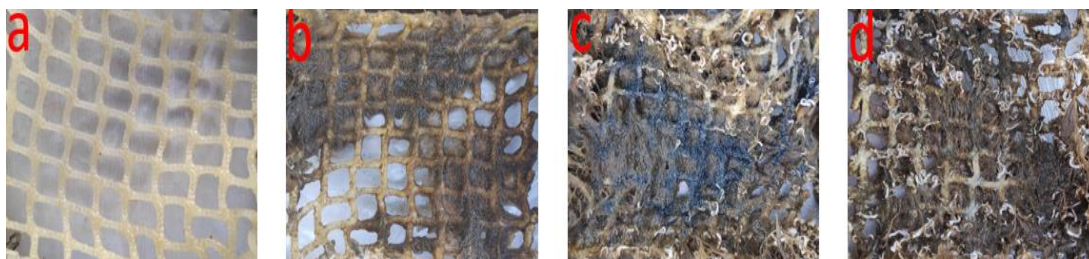


Figure 3.37 The field results of lanolin (irgarol 1051 3% (w/w)) coated polyethylene net, a) before immersion, b) 1 week later, c) 2 weeks later, d) 3 weeks later

3.3 The Results of Tensile Strength Test on Net Samples

The physical strength capacities of cage nets used in aquaculture are the most important elements for a safe and sustainable aquaculture production. Cage nets used in aquaculture are made of different fibers. High-density polyethylene and polyamide fibers are used extensively in this field because of their high physical load capacity. Aquaculture nets immersed under water are damaged over time by antifouling coating, salty sea water, UV rays, and biofouling organisms. Therefore, the measurement of physical load capacities at certain time intervals has a high importance on the determination of the lifetime of the aquaculture nets. In particular, antifouling paints coated on aquaculture nets directly affect the physical properties of the net fibers. It is vital to determine how the newly produced or untested coatings on the nets affect the net fibers (Moe et al., 2007; Liu et al., 2020).

In this thesis, the physical properties of rosin, lanolin and uncoated net samples were investigated. The test was repeated two times for all different samples. All data related the experiment is shown at Table 3.10. According to the results, nearly there is no difference between uncoated and rosin coated nets by means of tensile strength. However, when the rupture force of lanolin coated nets observed lower than the uncoated and rosin coated nets.

Table 3.10 The data related tensile strength test

Samples	Mean Thickness of Sample Net Ropes (mm)	Rupture Force (N)	Rupture Force by area (N/mm²)
Uncoated	2.56±0.07	256.4	10.0
	2.64±0.04	276.2	10.5
Rosin coated	3.00±0.05	261.2	8.7
	3.07±0.08	248.8	8.1
Lanolin coated	2.64±0.07	154.0	5.7
	2.70±0.09	164.2	6.0

CHAPTER 4

DISCUSSION

4.1 The Field Test Results

The results of the field tests are shown in Figure 3.25 to 3.37. Figure 4.1 includes the field test results of non-biocidal binders and uncoated control group. According to the results, the surface of the control group nets was 100% covered in the 2nd week. On the other hand, antifouling properties were observed in non-biocidal rosin coated nets compared to the control group. The self-polishing properties of rosin is directly related to this result (Figure 4.1).

According to Figure 4.1, self-polishing related antifouling effects are also found in lanolin coatings. It is seen in Figure 4.1 that the coatings showing the self-polishing properties are affected by the climatic conditions. During the 3th week of the field test, stormy weather was observed. For this reason, it was found that the biofouling organisms on the lanolin coating were separated from the surface.

The field test results of biocidal coatings are shown in Figure 4.2 and 4.3. While Figure 4.2 includes the results of rosin-based biocidal coatings, Figure 4.3 shows the results of lanolin-based biocidal coatings.

According to Figure 4.2 and 4.3, econea was evaluated as the best biocide for antifouling coatings. As a result of the field test, the antifouling performance ranking of the biocides was determined to be econea, diuron, and irgarol 1051, respectively.

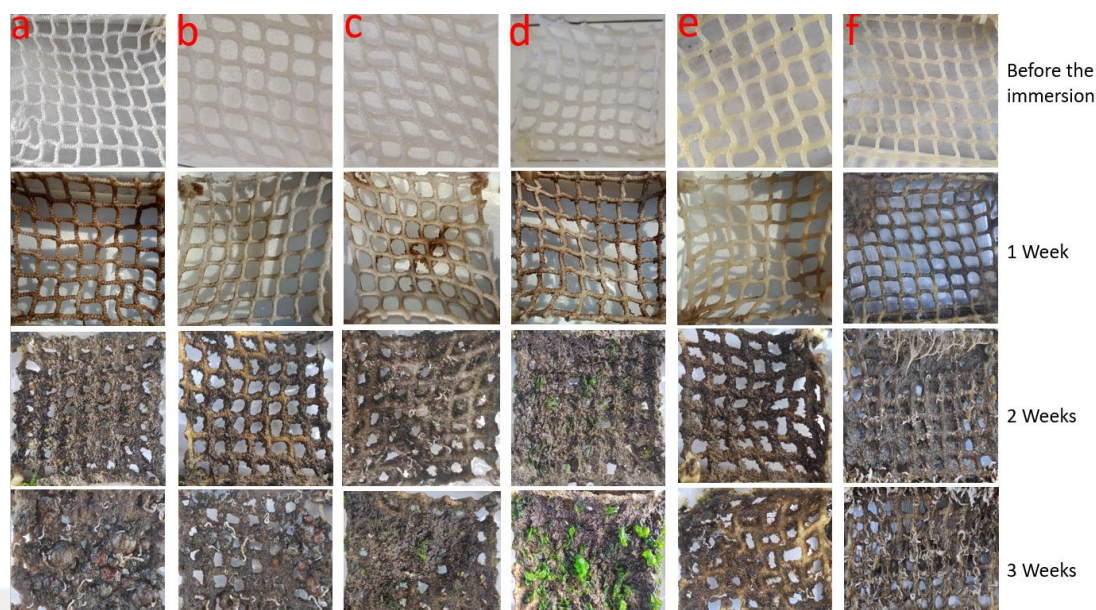


Figure 4.1 The field test results of biocide-free coatings and uncoated control group, Columns; a) control group, b) paraffin wax coated net, c) styrene-acrylic copolymer coated net, d) styrene-acrylic copolymer – polyethylene glycol 600 cross link binder coated net, e) lanolin coated net and f) rosin coated net

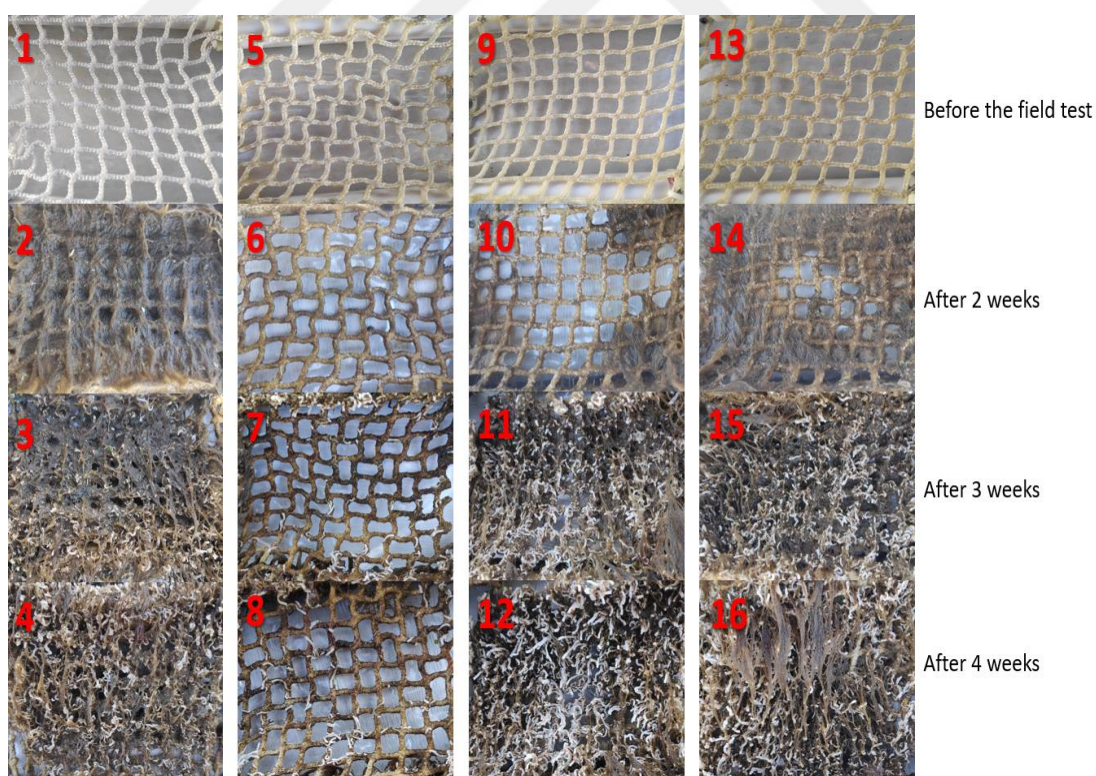


Figure 4.2 Field test results of uncoated control group and biocidal rosin coating formulation (1-4; uncoated nets, 5-8; 3% econea, 9-12; 3% diuron, 13-16; 3% irgarol 1051)

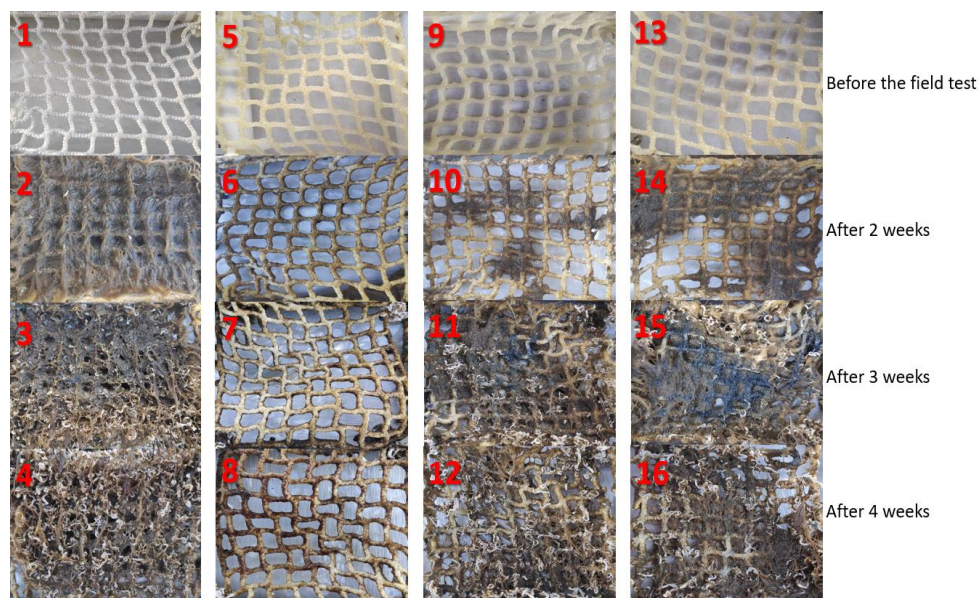


Figure 4.3 Field test results of uncoated control group and biocidal lanolin coating formulation (1-4; uncoated nets, 5-8; 3% econea, 9-12; 3% diuron, 13-16; 3% irgarol 1051)

4.2 The *In-silico* Results

According to the results, econea, diuron, and irgarol 1051 were evaluated for the inhibition of *B. amphitrite* GPCR protein, *H. elegans* COI protein, *B. neritina* Cyt-b protein and *U. lactuca* P-II-D1 protein. In the *in-silico* analyses performed in this thesis, binding energies of -7.0 kcal/mol, -6.0 kcal/mol, -9.2 kcal/mol and -6.3 kcal/mol were obtained for the GPCR-econea, COI-econea, Cyt-b-econea and P-II-D1-econea complexes, the binding energies of GPCR-diuron, COI-diuron, Cyt-b-diuron and P-II-D1-diuron complexes were found as -6.0 kcal/mol, -4.6 kcal/mol, -6.8 kcal/mol and -5.3 kcal/mol respectively. On the other hand, binding energies of -5.6 kcal/mol, -4.4 kcal/mol, -6.3 kcal/mol and -5.5 kcal/mol were obtained for the GPCR-irgarol, COI-irgarol, Cyt-b-irgarol and P-II-D1-irgarol complexes respectively. All information related best molecular docking scores are given in Table 4.1. According to the best binding scores of molecular docking analysis, econea was evaluated as the most effective biocidal agent to inhibit the important proteins of biofouling organisms studied in this thesis. As a result of the *in-silico* analysis, diuron and irgarol were placed in the second and third places respectively. After the molecular docking analysis, the interaction between target proteins and ligands were also evaluated.

According to the results, the molecule that interacted most with the GPCR protein of *B. amphitrite* was CMPD-101, while the molecule with the least interaction was observed as Chlorothaonil. On the other hand, econea had the highest interaction with the COI protein of *H. elegans*, while the least interaction was obtained in the COI-Irgarol complex. However, diuron is the biocide that interacts the most with the Cty-b enzyme of *B. neritina*, while Irgarol is the biocide that interacts the least. In addition, the P-II-D1 protein of *U.lactuca* had the least interaction with econea, while the Diuron-P-II-D1 complex had a large number of interactions. Although the number of intermolecular interactions is an important parameter in the analysis of the inhibition properties of a molecule in molecular docking studies, the detection of strong interactions such as hydrogen bonding is of great importance in the discovery of the molecule with the highest performance. According to the results of molecular docking analysis, although the binding affinities are low compared to other complexes, hydrogen bond formation has been detected in the GPCR-Dichlofluanid (Arg 93), GPCR-Gallein (Asn 89 – Val90), COI-Irgarol (Leu 51), P-II-D1-Diuron (Ser 79 – Ser 68) and P-II-D1-Irgarol (Ser 169) complexes.

Table 4.1 The best binding scores of target-ligand complexes.

Species	Complexes	Binding Affinities (kcal/mol)
<i>B. amphitrite</i>	GPCR-econea	-7.0
	GPCR-diuron	-6.0
	GPCR-irgarol	-5.6
<i>H. elegans</i>	COI-econea	-6.0
	COI-diuron	-4.6
	COI-irgarol	-4.4
<i>B. neritina</i>	Cyt-b-econea	-9.2
	Cyt-b-diuron	-6.8
	Cyt-b-irgarol	-6.3
<i>U. lactuca</i>	P-II-D1-econea	-6.7
	P-II-D1-diuron	-5.3
	P-II-D1-irgarol	-5.5

When field test results were compared with *in-silico* scores, a visual correlation was observed. Correlation factors were also evaluated via excel calculation (Table 4.3 – 4.8).

Table 4.2 Best binding pose related Target-Ligand interactions and interaction types

Target	Biocide	Residues	Interaction Types
GPCR	Chlorothalonil	Thr36, Ala35, Val33, Thr32	Hydrophobic
	Dichlofluanid	Asp112, Leu109, Trp108, Thr185, Tyr347, Asn89, Tyr92, Arg93, Tyr322	Hydrophobic, Polar, Hydrogen bond, Pi-Pi
	Diuron	Phe318, Phe319, Tyr322, Leu186	Hydrophobic, Polar
	Econea	Gln188, Leu186, Thr185, Tyr322, Ser196, Arg93, Trp108, Leu109, Asp112, Ile113	Electrostatic, Hydrophobic, Pi-Pi
	Irgarol	Tyr322, Val339, Val342, Thr343	Hydrophobic
	CMPD-101	Cys,116, Phe329, Ile113, Asp112, Leu109, Asn89, Tyr92, Arg93, Thr185, Leu186, Gln188	Hydrophobic, Polar, Pi-Pi
	Gallein	Asp340, Asn89, Val90, Arg93, Leu39, Thr36	Hydrophobic, Polar, Hydrogen bond

Table 4 2 Continues

COI	Econea	Ser37, Val38, Leu41, Leu51	Hydrophobic, Polar
	Diuron	Leu41, Gly54, Leu51	Hydrophobic
	Irgarol	Leu51	Hydrogen bond
Cyt-b	Econea	Gly116, Leu119, Leu120	Hydrophobic, Polar, Pi-Pi
	Diuron	Leu133, Pro134, Leu51, Tyr55, Ala84	Hydrophobic, Electrostatic
	Irgarol	Ala66, Ile69	Hydrophobic
P-II-D1	Econea	Ile290, Ala287	Hydrophobic, Polar
	Diuron	Ser177, Gly178, Asn181, Ser79, Ser68, Ala81	Hydrophobic, Hydrogen bond
	Irgarol	Asp61, Glu333, Ser169, Asp170, Asn181,	Electrostatic

Table 4.3 Biofouling scores, *in-silico* molecular docking scores and calculated correlation coefficient of *B. amphitrite* (Evaluated biofouling score obtained from field tested biocidal-rosin coatings)

Biocide	Biofouling Score	Molecular Docking Score (kcal/mol)	Correlation Coefficient
Non-biocidal	5	0	0.6859
Econea	0	-7.0	
Diuron	2	-6.6	
Irgarol	5	-5.6	

Table 4.4 Biofouling scores, *in-silico* molecular docking scores and calculated correlation coefficient of *B. amphitrite* (Evaluated biofouling score obtained from field tested biocidal-lanolin coatings)

Biocide	Biofouling Score	Molecular Docking Score (kcal/mol)	Correlation Coefficient
Non-biocidal	5	0	0.7946
Econea	0	-7.0	
Diuron	2	-6.6	
Irgarol	4	-5.6	

Table 4.5 Biofouling scores, *in-silico* molecular docking scores and calculated correlation coefficient of *H. elegans* (Evaluated biofouling score obtained from field tested biocidal-rosin coatings)

Biocide	Biofouling Score	Molecular Docking Score (kcal/mol)	Correlation Coefficient
Non-biocidal	5	0	0.7946
Econea	0	-7.0	
Diuron	2	-6.6	
Irgarol	4	-5.6	

Table 4.6 Biofouling scores, *in-silico* molecular docking scores and calculated correlation coefficient of *H. elegans* (Evaluated biofouling score obtained from field tested biocidal-lanolin coatings)

Biocide	Biofouling Score	Molecular Docking Score (kcal/mol)	Correlation Coefficient
Non-biocidal	5	0	0.7165
Econea	1	-6.0	
Diuron	3	-4.6	
Irgarol	5	-4.4	

Table 4.7 Biofouling scores, *in-silico* molecular docking scores and calculated correlation coefficient of *B. neritina* (Evaluated biofouling score obtained from field tested biocidal-rosin coatings)

Biocide	Biofouling Score	Molecular Docking Score (kcal/mol)	Correlation Coefficient
Non-biocidal	5	0	0.8356
Econea	0	-6.0	
Diuron	2	-4.6	
Irgarol	4	-4.4	

Table 4.8 Biofouling scores, *in-silico* molecular docking scores and calculated correlation coefficient of *B. neritina* (Evaluated biofouling score obtained from field tested biocidal-lanolin coatings)

Biocide	Biofouling Score	Molecular Docking Score (kcal/mol)	Correlation Coefficient
Non-biocidal	5	0	0.7392
Econea	0	-9.2	
Diuron	3	-6.8	
Irgarol	5	-6.3	

In certain studies where the antifouling abilities of econea were examined in the publications, field tests were also carried out. The outcomes of our *in-silico* studies agreed with the literature's field findings.

Kartal and Sarıışık (2020) were studied the antifouling performances of ZnO, Cu₂O, zinc borate, and econea on ultra-high molecular weight polyethylene fishing nets with the layer-by-layer deposition approach. For six months, nano-biocide-coated fishing nets were set near a fish farm in the Aegean Sea. According to the findings of Kartal and Sarıışık (2020), nano-econea films are an efficient antifoulant against fouling microorganisms. Using a layer-by-layer deposition method, Kartal and Sarıışık (2020) were investigated the antifouling capabilities of ZnO, Cu₂O, B₂O₆Zn₃, and econea on aquaculture nets. According to the study, aquaculture nets with nano-biocide coatings were positioned close to an aquaculture farm in the Aegean Sea for six months. The

results were showed that, nano-econea films are an effective antifoulant against fouling microorganisms. In another study, the toxic effects of econea, capsaicin and triphenyl borane investigated on Algae *Chlamydomonas reinhardtii*, *Daphnia magna* and *Danio rerio* by Oliveira et al. (2017). Among the booster biocides that the researchers tested, the strongest inhibitory effect was determined by econea against the growth of green alga *Chlamydomonas reinhardtii*. Sen et al. (2020) investigated the antifouling performance of econea on fish cage nets. The authors used two different commercial antifouling paint as a positive control and they also created a new formulation with econea as a main biocide. While the commercial paints (AP-1 and AP-2) contained 35% and 20% (w/w%) biocidal copper, the new formulation included 3.4% (w/w%) econea. According to the authors, econea evaluated as excellent biocidal compound compare to traditional copper-based paints. Non-release technology was experimented for econea and diuron by Silvia et al. (2019). The polyurethane and polydimethylsiloxane used as binders. Econea and diuron covalently bond to the binders via isocyanate linker. According to results, PDMS based econea shows highest antifouling performance. Ashraf and Edwin (2016) synthesized a polyethylene glycol-methacrylate copolymer-based hydrogel containing nano copper oxide by microwave assisted method in their study. The biofouling resistance of the synthesized hydrogel was covered in nylon fishing nets and tested in field trials. Ashraf and Edwin (2016) tested the physicochemical and morphological properties of the newly synthesized copolymer supported hydrogel by means of UV-vis, FTIR, TEM and SEM analyses. It was determined that the bio-coating resistance of nano-copper oxide-containing polyethylene glycol-methacrylate copolymer-based hydrogel coated nets was very high against the control group after 90 days in the marine area. Bloecher and Floerl (2020) tested the efficacy of new generation antifouling coatings developed for fishing nets through field trials. The starting point of the study is the use of high levels of copper oxide as a biocide in traditional coatings in order for fishing nets to gain antifouling effect, and the fact that copper is significantly harmful to environmental health, and therefore it is desired to use lower rates of biocidal copper in new generation coatings. In this study, Bloecher and Floerl (2020) tested seven different commercial antifouling coatings, one of which was the conventional type. In the study, it was found that new types of low copper content coatings are rapidly invaded by

biofouling organisms compared to traditional coatings, but biocidal copper rates can be reduced thanks to the right biocide combinations. Through two distinct trials, the study by Santos et al (2020) intends to assess several materials used in the management of biofouling in tropical seas. The primary goals of the study, according to Santos et al., were to (a) compare the bioaccumulation in various types of nylon nets coated with and without copper-based antifouling paint, (b) compare the bioaccumulation in copper alloy metal nets, (c) identify the organisms responsible for bioaccumulation in tropical waters, and (d) assess the impact of a zinc anode on the loss of copper alloy metal exposed to the bioaccumulation process (2020). Mono and multi filament nylon fishing nets were used in the study. Copper-based antifouling paint was chosen as “International Copperkote® Triton”. The field trial took a total of 8 weeks. As a result of the study, it was found that monofilament nylon fishing nets biocoating later than multi-filament ones, the pores of the multi-filament fishing nets are completely closed at the end of the 56th day, the nets covered with antifouling paint still have eye openings at a level that can be used, the dry mass of the creatures that cling to the surface after the field trial. When measured, mono-filament fishing nets are less covered than multi-filament ones (mono:300g, multi:400g), organisms that cause bio-coating problems; Mollusca, Ectoprocta, Chordata, Arthropoda species were found to be dense. Yu et al., (2019) aimed to develop nano-copper (CuNP) doped HDPE mono-filament fishing nets and to determine the antifouling properties of the new filament. Copper nanoparticles were modified using organic surfactant. HDPE-CuNP mono-filaments were obtained using the melt-spin method. Thanks to SEM analysis, it was determined that the CuNP dispersion was homogeneous. The CuNP release amount of the newly synthesized filaments was tested in artificial sea water in the laboratory and it was determined that it released a very high amount of copper within 50 minutes (30mg/50min). At the end of their study, it was decided that the new filament is an effective product for the short-term biofouling problem by looking at the copper release rate. MacKenzie et al., (2021) tested the use and performance of biocide-free new generation anti-adhesive antifouling coatings on fishing nets. In the study, the amount of attachment of *Mytilus edulis* species, which cling to fishing nets, to coatings that do not contain biocide was tested. MacKenzie et al., (2021) used white and black nylon fishing nets in their experiments. Finsulate® (black and white color),

Netminder® (white color), and white Dyneema® (white color) coatings were purchased commercially and covered on nets and field trials began. Compared to the control group, only the Netminder® (white color) coating delayed the bioaccumulation process by two weeks. The field trials lasted for 17 weeks in total and at the end of the study, it was found that the antifouling effects of the biocide-free coatings were very insufficient against the bioaccumulation process. Kalantzi et al., (2016) tested the effects of copper alloy fishing nets and copper-containing antifouling coatings on environmental health and their effectiveness against the biofouling problem caused by mussels. In their experiments, copper alloy fishing nets and nylon fishing nets coated with traditional copper-containing antifouling paint were tested in the marine area for one year. They determined the amount of copper released from the nets by taking samples from the sediment, mussels and cultured fish in the area where the nets are suspended at regular intervals, and analyzed the bio-signal chemistry (acetyl choline, catalase, glutathion transferase, metallothioneine) of the living things. In the first 6 months of the study, they observed that copper alloy fishing nets released more copper to the environment than traditional coatings, and the released copper was more effective on living biota. At the end of the study, no copper accumulation was observed in the sediments. In addition, no anomaly was found in bio-signal chemicals in samples taken from living things. The amount of copper released into the nature has not exceeded the limit set by the Food and Agriculture Organization (FAO). Edwards et al., (2014) tested the effectiveness of various commercial antifouling dyes on fishing nets to reduce bioaccumulation in fish farming cages in Colombia. The main purpose of the study is to test the antifouling effects of new generation biocide-free coatings on fishing nets in order to reduce the environmental damage of copper-containing antifouling paints. Edwards et al., (2014) selected 5 different non-biocidal commercial dyes for this study (non-biocidal: Dyneema, Netpolish, Aquacoating, and ThornD). Netrex copper-containing paint was used as a positive control. Field trials lasted for a total of 8 months. At the end of the study, copper-containing antifouling paint showed the highest performance compared to those that did not contain biocides, and the effectiveness of those that did not contain biocides was found to be insufficient in the bioaccumulation process. Lainioti et al., (2021) produced environmentally friendly cross-linked antifouling coatings in their study. As an antimicrobial polymer;

P(SSAmC16-co-VBCHAMx) and tertiary polymer; P(SSAmC16w-co-VBCHAMx-co-GMay) was synthesized and covered on fishing nets. In the study, accelerated biofouling test was applied in laboratory environment to test the antifouling properties. The accelerated biofouling experiment was carried out by growing green algae in aquariums and immersing the nets in algae-containing tanks for one month when the appropriate environment is provided. Lainioti et al., (2021), according to the results of their study, P(SSAmC16-co-VBCHAM70) copolymer was found to have a higher antifouling effect compared to the other copolymer. In a one-year development cycle of European sea bass, Yiğit et al., (2017) employed a novel and ecologically friendly copper alloy net material to compare with conventional nylon nets. According to a combination of variables, including fish growth performance, feed consumption, and a bio-economic analysis of initial investment expenses, copper alloy net cages outperform traditional nylon net cages coated with antifouling paint in terms of productivity indices and economic advantages. The findings indicated that copper alloy aquaculture net is a potential replacement element that may be employed in aquaculture sector with a higher financial return. Sen et al., (2020) developed a new and environmentally friendly antifouling paint to protect from the marine field bioaccumulation process, which is a major problem on fishing nets. Econeas has been used as main biocide. Commercial antifouling paints with two different biocidal copper content were provided as positive control. The produced environmental paint and commercial paint applied net samples were released into the marine ecosystem for 6 months. Underwater photographs were taken at periodic intervals and it was observed that invasive organisms settled on the nets. After the field test, the antifouling efficacy of the paints and the structural changes of the nets were assessed using underwater pictures, biomass development, mass differences, and tensile strength tests were also investigated in this study. The findings demonstrate that, copper-free coating formulation (Econeas including formula) has high antifouling properties as commercial antifouling coating. Manufacturers now have another option when deciding which raw materials to use thanks to a new eco-friendly formulation with promising outcomes.

CHAPTER 5

CONCLUSION

Within the scope of this master thesis, it is aimed to determine the performance of antifouling paints by *in-silico* molecular docking analysis. For this purpose, two different field experiments were carried out at İzmir Marina (Location coordinates: 38°24'23"K 27°04'06"D) (First field experiment date intervals: 01 – 21 August 2021 / Second field experiment date intervals: 01.- 21 September 2021) (Prior to the field trial, permission was obtained from the Ministry of Environment, Urbanization and Climate Change of the Republic of Türkiye). In the first field trial, biocide-free lanolin, rosin, styrene acrylic copolymer, paraffin wax were used as the main binder on aquaculture nets.

According to the results obtained from the first field trial, it was discovered that biocide-free rosin and lanolin coatings have antifouling properties and it was decided to use rosin and lanolin in biocidal formulations as the main binders in further tests. In the study carried out within the scope of the thesis, econe, diuron and irgarol were used in formulations at a rate of 3% (w/w) as the main biocidal agent for the second field tests. Aquaculture nets covered with prepared biocidal rosin and lanolin coatings were subjected to the second marine field test. Since biocidal agents are used in the second field test, this is the most important test performed within the scope of the thesis and the results are obtained. At the end of the second field test, the surfaces of the aquaculture nets covered with antifouling coatings were examined and biofouling organisms were detected. According to the results of the second field test, it was determined that *B. amphitrite*, *B. neritina*, *H. elegans* and *U. lactuca* species invaded the aquaculture nets intensively. After the determination of the biofouling species, the metabolisms of these organisms were investigated from the literature and target proteins with vital functions were determined. GPCR for *B. amphitrite*, Cyt-b for *B. neritina*, COI for *H. elegans* and P-II-D1 for *U. lactuca* were selected as target proteins for use in *in-silico* tests. After the target identification step, internet databases were scanned and it was aimed to find the illuminated structures of the proteins. First, the PDB database was searched, but no data were found for the 3D proteins whose

structures were elucidated by XRD, NMR or EM methods for the proteins identified as the target. For this reason, by continuing the research on the Uniprot database, the primary sequences of the target proteins were obtained in FASTA format and artificially modeled by finding suitable template proteins from the SWISSMODEL protein library. After modeling, 3D proteins whose conformational energies were minimized with YASARA server were subjected to molecular docking analysis with econea, diuron and irgarol using the AutodockVina algorithm. As a result of *in-silico* analysis, the antifouling performances of the biocides were determined as econea first, diuron second and irgarol third.

The data obtained by scoring the amount of biofouling on the nets after the field trial was used for the correlation test. The two main parameters of the correlation test are the biofouling scores and the binding affinities obtained after molecular docking. According to the results of the correlation test, a positive correlation was found between the *in-silico* binding affinities and the field test results.

In conclusion, the results of this study demonstrated that *in-silico* methodologies can be used to evaluate the antifouling performance of novel and candidate biocides without the need for field tests. Molecular docking scores represents the antifouling efficacy of newly produced molecules. Discoveries of novel and effective antifouling biocides can be carried out by using the methodology mentioned in this study. The outcomes of this study may open a new gate for development of eco-friendly antifouling paints.

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