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SEAWEED EXTRACTS: AN ECO-FRIENDLY APPROACH TO MITIGATE *Amyloodinium ocellatum* PARASITE OF MARINE FISH

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Doutora Raquel Quintã (S2AQUAcolab)

Doutora Teresa Modesto (University of Algarve)

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Declaração de autoria

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluídas.

Assinatura

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(Bruno Ferreira)

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Labor omnia vincit improbus!

Abstract

The ectoparasite *Amyloodinium ocellatum* poses a significant threat to both brackish and marine warmwater aquaculture fishes, causing severe morbidity and mortality. Current treatments for amyloodiniosis are limited and environmentally unfriendly. Seaweed extracts, known for their antiparasitic, anti-inflammatory, and antibacterial properties, offer a promising direction for addressing this gap. This research was dedicated to investigating the effects of six aqueous extracts from seaweed species, *Asparagopsis armata* gametophyte, *Asparagopsis* sp. tetrasporophyte, *Sphaerococcus coronopifolius*, *Gracilaria* sp., *Halopteris scoparia*, and *Ulva* sp., in delaying the development and/or mortality rates of two life stages of *A. ocellatum*, the tomonts and the dinospores. The study utilized a controlled *in vitro* environment, where each seaweed extract was subjected to various dilutions (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100), along with a control treatment using autoclaved seawater. The inactivation rates of tomonts were monitored over 96 hours, while dinospore mortality rates were observed over 6 hours. The results revealed that none of the extract treatments achieved complete inactivation of tomonts, instead they temporarily inhibited their activity. Notably, the extract from the red seaweed *A. armata* gametophyte displayed the highest effectiveness, with inactivity rates reaching 99% at 24 hours and 48% at 96 hours. Similarly, no seaweed extract consistently induced significant higher mortalities in dinospores when compared to the control group, which exhibited substantial mortality rates. Overall, this study shows the promising outcomes achieved, particularly with the *A. armata* gametophyte extract in tomont inactivation. Besides, optimized outcomes might be expected by utilizing an optimized approach for the methodology of the dinospores trials.

Keywords: Aquaculture, *Amyloodinium ocellatum*, seaweed extracts, natural treatments, parasite management.

Resumo

O ectoparasita *Amyloodinium ocellatum* é o dinoflagelado mais comum e importante que afetas peixes, causando morbidade e mortalidades graves em peixes de aquacultura de água salobra e marinha em todo o mundo. *A. ocellatum* tem um ciclo de vida direto que consiste em três fases distintas. O trofonte, estágio parasitário, que se encontra nas brânquias e no epitélio da pele, que após atingida a sua maturação é libertado do seu hospedeiro e dá origem ao tomonte (cisto de repouso reprodutor) e consequentemente ao dinosporo (flagelado de vida livre) após sucessivas divisões. Atualmente, os tratamentos mais comuns empregados no controlo de *A. ocellatum.* enfrentam desafios significativos. Tendo em conta o exemplo do sulfato de cobre, observa-se que, em doses mais elevadas (10 ppm), esse tratamento não demonstrou eficácia na eliminação dos tomontes, enquanto em relação aos dinósporos, apenas foi necessária uma dose menos concentrada (0.5 ppm) para a sua eliminação. Além disso, é importante realçar que o uso de tais tratamentos pode ter impactos adversos tanto no ambiente aquático quanto na saúde dos próprios peixes, devido à toxicidade associada a essas substâncias. Para contrariar o uso destes tratamentos, recorremos aos extratos de algas marinhas, amplamente reconhecidos pelas suas notáveis propriedades antiparasitárias, anti-inflamatórias e antibacterianas. Essas propriedades são atribuídas a uma variedade de metabólitos secundários presentes nas algas, tais como o polifenol e a fucoxantina, um carotenóide. Estes compostos bioquímicos, presentes nos extratos de algas, desempenham um papel fundamental na atividade antiparasitária, afetando diretamente o parasita em questão. Esta abordagem representa uma direção extremamente promissora para preencher a lacuna existente no tratamento da amiloodiniose. O desenvolvimento de tratamentos naturais baseadas em extratos de algas oferecem a perspetiva de uma alternativa eficaz e menos prejudicial para o ambiente aquático e para a saúde dos peixes em sistemas de aquacultura de água salobra e marinha. Ao compreendermos a relação entre os compostos bioquímicos presentes nas algas e sua capacidade de combater o parasita, podemos avançar na otimização dessas terapias e explorar oportunidades adicionais de aprimoramento, oferecendo assim uma nova possibilidade para o controle bem-sucedido da amiloodiniose.

Este trabalho foi dedicado a investigar os efeitos de seis extratos aquosos de espécies de algas marinhas no desenvolvimento e mortalidade do parasita. Dentro das vermelhas as algas analisadas foram quatro, a *Asparagopsis armata* gametófito, *Asparagopsis* sp. tetraesporófito, *Gracilaria* sp. e *Sphaerococcus coronopifolius*, enquanto que dentro das algas verdes e castanhas foram testadas apenas uma de cada, *Ulva* sp. e *Halopteris scoparia*, respetivamente, no retardamento do desenvolvimento e taxas de mortalidade de duas fases da vida do parasita *A. ocellatum*, tomontes e dinósporos, respetivamente. O estudo utilizou um ambiente controlado *in vitro*, onde cada extrato de alga foi utilizado em várias diluições (1:1, 1:5, 1:10, 1:25, 1:50 e 1:100), juntamente com um tratamento de controle usando água salgada autoclavada. As taxas de inativação dos tomontes foram monotorizados ao longo de 96 horas com quatro tempos de contagem diferentes com intervalos de 24 horas, enquanto que as taxas de mortalidade dos dinósporos foram observadas ao longo de 6 horas, mais especificamente no momento da inoculação dos extratos das algas com os dinósporos (0 horas), na primeira hora, na terceira hora e por fim na sexta hora. Os resultados revelaram que nenhum dos tratamentos com extratos alcançou a inativação completa dos tomontes, no entanto estes inibiram temporariamente a sua atividade. Notavelmente, o extrato da alga vermelha *A. armata* gametófito demontrou a maior eficácia na diluição

1:1. Nessa condição, as taxas de inatividade dos tomontes alcançaram valores de 99% no período inicial de 24 horas e embora tenham diminuído para 48% após 96 horas, essa taxa inicial é altamente promissora. Além disso, observações revelaram que, entre as variedades de algas vermelhas testadas, a alga *Gracilaria* sp., também apresentou resultados notáveis. Este extrato, quando submetido às mesmas condições de diluição, registou um pico de inatividade considerável, atingindo os 63% nas primeiras 24 horas e mantendo-se em 8.5% após 96 horas. Ainda no capítulo da inativação dos tomontes, mais concretamente no seu tempo da primeira divisão, apenas o extrato da alga *A. armata* gametófito, mostrou resultados promissores com a sua primeira divisão apenas a aparecer na diluição 1:1 ao fim das primeiras 72 horas, enquanto que em qualquer outro extrato esta divisão não teve qualquer retardamento, acabando por se começarem a dividir logo nas 24 horas de inoculação. Da mesma forma, nenhum extrato de alga induziu consistentemente mortalidades significativas maiores nos dinósporos em comparação com o grupo de controle, que exibiu taxas de mortalidade muito elevadas. Esta observação pode se revelar de importância fundamental para orientar o desenvolvimento de projetos futuros voltados para o uso de extratos de algas no combate aos dinósporos do parasita *A. ocellatum*. Inicialmente, a expectativa era que a ação dos extratos de algas produzisse resultados mais favoráveis em termos de mortalidade dos dinósporos em comparação com a taxa de inativação dos tomontes, devido à reputação da resistência destes últimos. Surpreendentemente, os resultados deste estudo apontaram para uma dinâmica oposta, onde os extratos de algas não induziram consistentemente taxas significativamente mais elevadas de mortalidade nos dinósporos em comparação com o grupo de controle. Isso sugere que, para alcançar resultados otimizados no que diz respeito aos dinósporos, será crucial melhorar e ajustar a metodologia usada no tratamento desses estágios de vida do parasita. Assim, a pesquisa futura pode-se concentrar na exploração de novas abordagens metodológicas e na identificação de substâncias específicas presentes nas algas que possam ser mais eficazes na redução da mortalidade dos dinósporos, proporcionando assim uma base sólida para o desenvolvimento de estratégias de controlo mais eficazes.

No contexto geral deste estudo, foram alcançados resultados promissores ao utilizar o extrato da alga vermelha *A. armata* gametófito para inativar os tomontes. Este extrato não apenas demonstrou uma notável taxa de inatividade dos tomontes, atingindo uma taxa impressionante de 99% num período de 24 horas, mas também exibiu um impacto significativo no tempo de primeira divisão dos tomontes. No entanto, é importante realçar que, apesar desses avanços promissores, há espaço para melhorias adicionais nomeadamente na metodologia do tratamento dos dinósporos e otimização dos extratos de macroalgas a testar.

Palavras-chave: Aquacultura, *Amyloodinium ocellatum,* extratos de algas, tratamentos naturais, controlo de ectoparasitas.

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Abbreviations

µl	microlitre
µm	micrometre
mL	mililitre
ppm	part per million
rpm	revolutions per minute
ANOVA	analysis of variance
EPPO-IPMA	Estação Piloto de Piscicultura de Olhão – Instituto Português do Mar e Atmosfera
EEMT-IPMA	Estação Experimental de Moluscicultura de Tavira - Instituto Português do Mar e Atmosfera
H_2O_2	hydrogen peroxide
PAA	peroxyacetic acid
UV	ultraviolet
L:D	light:dark ratio
SD	standard deviation
°C	degrees Celsius
IC50	50% inhibitory concentration

1 Introduction

1.1 Aquaculture: production and disease challenges

Over the last decades, global production of cultivated aquatic food increased rapidly and has driven aquaculture to be one of the fastest-growing animal-food-producing sectors (Allison, 2011). Today aquaculture accounts for almost half of the fish consumed worldwide (FAO, 2020) and it is expected to be the greatest supplier of seafood in the future. While this gives a chance to address requirements for food security and relieve pressure on marine resources, it also raises significant sustainability problems due to the stressful conditions, which can occur when fish are reared in captivity (Lieke et al., 2020; Natale et al., 2013). A primary concern for a healthy product and a sustainable production process are infectious diseases, which - both directly and indirectly - account for losses of up to 50 % of the total aquaculture production worldwide according to Assefa & Abunna (2018). Poor water quality, compromised biosecurity measures and high stocking numbers are the main parameters that provide ideal conditions for parasite infestation and reproduction, which can quickly reach pathogenic proportions (Lieke et al., 2020).

1.2 *Amyloodinium ocellatum*: a dinoflagellate parasite

Dinoflagellates are frequently found in aquatic habitats, where they function as the main producers and consumers, by collaborating with a variety of invertebrates to develop symbiotic relationships (Fensome et al., 1999). Of the roughly 2000 aquatic organisms that are known to exist, 140 are parasites, being *Amyloodinium ocellatum* (Brown, 1931), the most prevalent and significant dinoflagellate parasitizing fish and seriously harming aquaculture farms (Drebes, 1984).

The ectoparasitic dinoflagellate *A. ocellatum* is indigenous to temperate and tropical regions all over the world. This parasite has a wide geographic spread and is non-specific in its host selection, it affects more than 100 species of farmed fish and crustaceans (Lawler, 1980; Moreira et al., 2022). Additionally, this parasite has the ability to act as a hyperparasite, infecting other parasites commonly found on fish, such as *Neobenedenia melleni* (MacCallum) (Monogenea: Capsalidae), which affects Gilthead seabream (*Sparus aurata* L.) (Colorni, 1994). *A. ocellatum* represents a serious

threat to semi-intensive aquaculture, particularly in Southern Europe, especially in the warmest months, causing serious morbidity and mortality in both brackish and marine water, since warmer temperatures encourage the parasite's growth and could cause more outbreaks in aquaculture farms (Lawler, 1980; Noga, 2012; Soares et al., 2012).

Furthermore, due to their sensitiveness to temperature, parasites are also one of the groups most vulnerable to global climate change since it has a direct impact on their life cycle, transmission, and host biology (Adlard et al., 2015). This vulnerability is compounded by the potential for higher temperatures to extend the transmission season, leading to an overall increasing in the prevalence of illness and spread of epidemics (Karvonen et al., 2010). The economic consequences can be severe for affected producers, as evidenced by documented incidents such as the outbreak of *A. ocellatum* in a Greek sea cage in 1997, which targeted Gilthead sea bream (*S. aurata),* caused 75% mortality (30000 juveniles) with estimated losses rounding 3000 US$. Similarly, a separate incident in a Portuguese earth pond, affecting both Gilthead seabream (*S. aurata*) and meagre (*Argyrosomus regius*), resulted in an economic loss of approximately 4000 € (Shinn et al., 2015).

1.3 Biological cycle of *A. ocellatum*

A. ocellatum follows a direct life cycle consisting of three distinct stages (Figure 1.1). The completion of the life cycle varies, typically taking 5 to 7 days, influenced significantly by environmental factors such as salinity and temperature (salinity 30-35) and 23-27ºC (Francis-Floyd & Floyd, 2011).

Trophont (parasitic adult stage) is primarily found in the gills and skin epithelia (adhesion sites) (Noga, 2012). It attaches to its host using a rhizoid root-like structure that penetrates deep into the host epithelium, inflicting considerable damage to the tissue at the attachment site (Francis-Floyd & Floyd, 2011). Upon reaching their maturity (60-90 µm), each trophont gradually detaches from their host and within 2 to 5 min the rhizoids retract, and a solid body wall begins to form, marking the parasite’s transition into a cystic stage, the tomont (Masson, 2009; Oestmann & Lewis, 1996). Tomonts (cystic reproductive stage) have the capacity to asexually produce up to 256 new specimens (dinospores) through multiple divisions (Noga, 2012). Notably, larger trophonts undergo a greater number of divisions, resulting in the production of a higher quantity of dinospores. This relationship between trophont size and division dynamics

highlights the significance of size variations in influencing the reproductive potential and subsequent population dynamics of the parasite (Paperna, 1984). Dinospores (free swimming infective stage) possess a body size ranging approximately from 12 to 15 µm in length and 8 to 14 µm in width, characterized by the presence of two flagella: one oriented transversely and another directed longitudinally, moving in a backward direction (Landsberg et al., 1994; Noga, 2012).

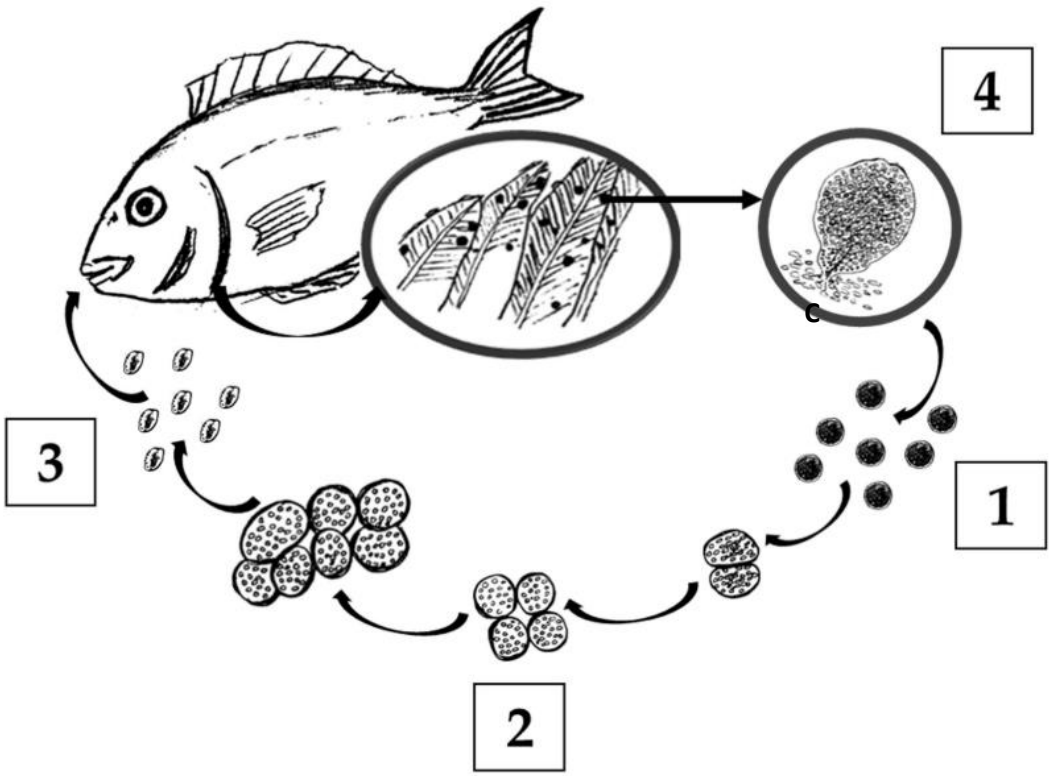

Figure 1.1. Biological cycle of *Amyloodinium ocellatum*, comprising the encysted tomont stage **(1)**, which transitions into tomites **(2)** followed by the dinospore free-living stage **(3)** and the parasitic trophont stage **(4)**. Original from (Moreira et al., 2022).

1.4 Clinical signs and pathogenicity of *Amyloodinium ocellatum* infections

Fish affected by this disease may experience sudden mortality without displaying prominent clinical signs. Nevertheless, in the majority of cases, observable behavioural and physical alterations precede the eventual death. When the gills serve as the primary site of infection, which is frequently observed, respiratory symptoms become the primary clinical indicators. These respiratory signs may encompass heightened respiratory rate, noticeable gill movements, and increased opercular activity, commonly known as piping. Additionally, affected fish may exhibit jerky movements

and a tendency to gather near the water surface or areas with higher levels of dissolved oxygen, alongside a decrease in appetite (Francis-Floyd & Floyd, 2011; Levy et al., 2007; Soares et al., 2011).

In cases where the primary site of infection is the skin, amyloodiniosis can lead to the development of distinctive visual changes in infected fish. These changes may manifest as a white or brown discoloration, often referred to as velvet, or a cloudy appearance. These alterations become particularly noticeable when observed under indirect lighting conditions, such as a flashlight (Levy et al., 2007). In addition, fish experiencing amyloodiniosis affecting their skin may exhibit behavioural indicators such as flashing or actively rubbing against tank walls, the substrate, or other structures within their surroundings. These actions can be observed as attempts to alleviate discomfort caused by the infection (Francis-Floyd & Floyd, 2011). It should be noted that heavy infestations of *A. ocellatum* can extend beyond the skin, affecting other areas such as the fins, eyes, and oro-pharyngeal cavity (Byadgi et al., 2019).

A significant impact of *A. ocellatum* infection is the destruction of epithelial cells, causing noticeable changes in the body. Common histological findings include gill enlargement, fusion of the lamellae, inflammation, haemorrhage and necrosis (Byadgi et al., 2019; Marques et al., 2019). The main cause of death is often lack of oxygen, leading to fatalities occurring within a short period of about 12 hours, especially when the infestation is severe, as described by Lawler (1980).

1.5 Transmission and diagnosis

The transmission of *A. ocellatum* occurs directly between fish through dinospores. These dinospores are mobile in the water and actively seek out new hosts to infect. The stage of infection spreads effortlessly through water as well as through rearing tools such as fishing nets and parameter probes that are utilized in recirculating systems (Noga, 2012). Furthermore, there is experimental evidence confirming that dinospores can also be transmitted through aerosol droplets (Roberts-Thomson et al., 2006). Therefore, this finding elucidates the mechanism by which fish farms, previously unaffected by amyloodiniosis, can become susceptible to *A. ocellatum* following windy atmospheric events such as storms or typhoons (Dequito et al., 2015).

Clinical diagnosis of amyloodiniosis is relatively simple and relies mostly on classical methods. These methods involve direct observation of the target tissues (fins, eyes, skin, and gills) either through a microscope or with the naked eye. When using the

naked eye, parasites can be detected by inspecting the fish against a dark background or by using indirect illumination. Smaller fish can be examined by restraining them in a dish and directly observing them using a dissecting microscope. To confirm the diagnosis, it is necessary to observe trophonts or tomonts attached to the epithelium. As trophonts often detach shortly after the host's death, samples of living or recently deceased fish are examined using light microscopy (Francis-Floyd & Floyd, 2011; Noga, 2012).

1.6 Treatments and prevention of amyloodiniosis

In general, the treatment approach revolves around eliminating the free-swimming dinospores before they can attach to a new fish host or eradicating the trophonts from the fish, thereby disrupting the life cycle (Francis-Floyd & Floyd, 2011; Soares et al., 2011). Due to the multi-stage life cycle of *A. ocellatum*, effectively controlling an outbreak requires multiple treatments or prolonged treatment durations (exceeding 10 to 14 days) to ensure complete eradication (Francis-Floyd & Floyd, 2011). Currently, copper sulfate is widely utilized as the primary chemical for the control of amyloodiniosis, it is toxic to dinospores, but also to most invertebrates (crustaceans and gastropods) and algae and has been a standard treatment for years (Francis-Floyd & Floyd, 2011; Noga, 2012). The recommended concentration range of copper levels are 0.12-0.15 ppm for a duration of 10-14 days to ensure complete elimination of every life stage. Despite doses of 0.5-10 ppm being lethal to sporulating tomonts, and dinospores, it cannot interrupt the division process of encapsulated tomonts (Lieke et al., 2020; Noga, 2012).

Additionally, in fish culture, formalin (37% formaldehyde) serves as another treatment method to control this parasite. By subjecting the fish to a flush treatment with 100-200 ppm of formalin for 6-9 hours, the trophonts of *A. ocellatum* detach from the gills. However, it should be noted that tomonts resume their division once the chemical is removed (Paperna, 1984). In recent studies, the application of formalin as a treatment method has shown promising results. For instance, when juvenile bullseye puffer fish (*Sphoeroides annulatus*) were treated with either 51 ppm of formalin for 1 hour or 4 ppm for 7 hours, a significant reduction in parasite load on the skin and gills was observed. However, it should be noted that fish were reinfested after 15 days, indicating that the treatment does not effectively inactivate the tomont stage.

Nevertheless, the infection was successfully controlled by repeating the treatment, highlighting the potential of formalin as a viable option for managing amyloodiniosis (Fajer-Ávila et al., 2003).

An alternative and more environmentally friendly therapeutic agent is hydrogen peroxide (H2O2). Single administrations using this treatment at concentrations of either 75 or 150 ppm effectively eliminated *A. ocellatum* trophonts in the Pacific threadfin (*Polydactylus sexfilis*) without any adverse effects on the fish. In a subsequent trial, a single treatment with 75 ppm hydrogen peroxide significantly reduced the levels of *A. ocellatum* infestation, and a second treatment after 6 days successfully reduced trophonts to undetectable levels (Montgomery-Brock et al., 2001). In more recent investigations, the treatment of European sea bass (*Dicentrarchus labrax*) with 100 and 200 ppm of H2O2 for 30 minutes exhibited a significant decrease in the number of trophonts observed on the gills (Seoud et al., 2017).

Along similar lines, PAA (peroxyacetic acid or acetylhydrogen peroxide), a stabilized mixture of acetic acid, H2O2, and water, has gained prominence as a disinfectant widely utilized in diverse sectors, including agriculture, food processing, and medical and veterinary facilities (Lieke et al., 2020). Notably, PAA has found applications in wastewater treatment, commercial laundries, and aquaculture in multiple countries, indicating its growing significance in recent times (Straus et al., 2018). It has been reported that treatment with 8 to 15 ppm of peracetic acid for 1 hour effectively eliminates all stages of the fish parasite *Ichthyophthirius multifiliis*, including cysts (Picon Camacho, 2010). This success can be attributed to the ability of peracetic acid, similar to hydrogen peroxide, to generate hydroxyl radicals that oxidize enzymes and proteins, as well as increase cell wall permeability by disrupting sulfhydryl and sulfur compounds. In particular, the heightened potency of peracetic acid, owing to its greater fat solubility, highlights its potential as an effective treatment option in various sectors, including aquaculture and related industries (Kitis, 2004).

According to the demand for environmentally friendly disease control methods, researchers have also been investigating alternative approaches. Among these alternatives, the utilization of natural products derived from various organisms, such as plants (essential oils), animals (chitosan), and seaweeds, has gained significant attention (Vatsos & Rebours, 2015).

1.7 Seaweed extracts as a potential natural treatment for parasite control

Seaweeds offer a vast array of compounds, such as polysaccharides like fucoidan, phytochemicals including phlorotannins, carotenoids, minerals, peptides, and lipids, which hold promise in parasite control (Holdt & Kraan, 2011). These biologically active natural products found in seaweeds possess a diverse range of properties, from antibacterial and anti-inflammatory to anthelmintic effects (Blunt et al., 2013). Moreover, seaweeds are an abundant source of secondary metabolites, boasting a wide variety of structurally diverse compounds like polyphenols, alkaloids, terpenes, and halogenated compounds (Watson & Cruz-Rivera, 2003). It has multiple functions, including defense against herbivores, fouling organisms, and pathogens. They also provide protection against UV radiation and allelopathic agents. Seaweeds commonly employ chemical defense mechanisms that inhibit the formation of biofilms, with numerous secondary metabolites demonstrating bacteriocidal or bacteriostatic properties. These characteristics highlight the diverse and potent nature of seaweed secondary metabolites in safeguarding against various threats and inhibiting the growth of harmful bacteria (Steinberg et al., 1997).

Furthermore, seaweeds exhibit antimicrobial and antiparasitic activities primarily through the phenolic and polysaccharide components found within them. These compounds can act by either inhibiting the growth of microorganisms (stasis) or directly destroying them (cidal) (Abu-Ghannam & Rajauria, 2013; Chiboub et al., 2017). The antimicrobial action of polyphenols, in particular, is thought to be attributed to their capacity to modify the permeability of microbial cells (Bajpai et al., 2008). These inherent characteristics of seaweeds underline their potential as a valuable resource for combating parasites and highlight the broad spectrum of biologically active compounds they contain.

Despite the limited number of studies investigating the antiparasitic properties of seaweed, one noteworthy example is the study conducted by Moo-Puc et al. (2008). This research showcased the direct antiprotozoan effects of organic extracts derived from various seaweed species against *Trichomonas vaginalis* trophozoite. However, the foundation of my thesis lies in the study focused on tackling the monogenean ectoparasite *Neobenedenia* sp., where researchers explored the potential of 8 different seaweed extracts *in vitro*, such as *Asparagopsis taxiformis*, *Gracilaria edulis*, *Hypnea musciformis*, *Caulerpa taxifolia*, *Derbesia tenuissima*, *Ulva* sp. and *Dictyopteris delicatula* (Hutson et al., 2012) . The results of the study revealed that extracts from two

specific seaweeds, *Ulva* sp. and *A. taxiformis*, exhibited notable effects to control some aspects of *Neobenedia* sp. life cycle. These extracts demonstrated the ability to delay embryonic development and inhibit egg hatching. Notably, *A. taxiformis* showcased the highest efficacy, effectively inhibiting the embryonic development of *Neobenedenia* sp. and significantly reducing hatching success to 3% compared to the 99% observed in the seawater control group (Hutson et al., 2012). These findings suggest that *A. taxiformis* holds significant potential for further development as a natural treatment option. Furthermore, these results can serve as a foundation for exploring its efficacy against other parasites, such as *A. ocellatum.*

2 Objectives

The objective of this study is to determine, in a controlled *in vitro* environment, the effect of the extracts of six species of seaweed in delaying the development and inducing mortality for each stage of *A. ocellatum*, tomonts and dinospores, respectively.

3 Material and Methods

3.1 Seaweed collection and extracts preparation

Six seaweed species were chosen to test the bioactivity of extracts on two different stages (tomont and dinospore) of *A. ocellatum*. Being the red seaweeds composed by *Asparagopsis armata* gametophyte, *Asparagopsis* sp. tetrasporophyte, *Sphaerococcus coronopifolius* and *Gracilaria* sp.; the green seaweed, *Ulva* sp.; and the brown seaweed *Halopteris scoparia*. Seaweeds were harvested from Praia dos Arrifes, Albufeira in spring on a low tide (1.0m), apart from *Gracilaria* sp. that was captured from a neglected earthen pond at Aqualvor Lda, Alvor, a sustainable aquaculture company, in the same day and *Asparagopsis* sp. tetrasporophyte that was being cultivated in the laboratory at EEMT-IPMA, Tavira. The seaweeds harvested from the field were transported in buckets filled with seawater until arrival at EEMT-IPMA, where it was firstly quickly washed with freshwater and then with UV-filtered seawater to remove the adhering sediments, small organisms (invertebrates) and microorganisms, such as ciliates, in order to provide a controlled and relatively sterile environment. Following that, each seaweed species, were placed in individual tanks with aeration and photoperiod 12:12 (L:D). Nutrient supplementation and water replacement were conducted on a weekly basis. *A. armata* gametophyte was the first one being used due to its difficulty to maintain under cultivation. As a result of that, it was kept only 16h before extract preparation.

The preparation of the seaweed extracts followed a protocol that was adapted from Hutson et al., (2012) with minor adjustments. Each seaweed species went through an additional rinse in freshwater, followed by a washing with UV-filtered seawater to detach the last remains. The washed seaweed was then treated with betadine diluted in autoclaved seawater at a ratio of 1 mL/10 mL for 30 seconds to prevent contamination of microorganisms. This was followed by three autoclaved seawater baths´ to remove any residual betadine. Afterward, the seaweed was dried, weighed, and blended with autoclaved seawater at a wet weight to volume ratio of 0.1g/mL for 2 minutes. The resulting solution was poured into a Schott bottle and placed on a magnetic stirrer (900rpm) at room temperature of 24ºC for 4 hours to allow mixing. To remove any debris or unwanted particles, each solution was sieved through a filter (25µm) into a beaker and then into 50 mL centrifuge tubes. The tubes were spun at 4211g for 5 minutes at 4ºC. The supernatant was transferred to a clean Schott bottle (Figure 3.1) and

stored at 4°C. The tomont wells were provided with the most recently prepared extract (1 day), whereas the dinospores were incubated in the wells following a 5 day interval from the time the extract was prepared. This incubation occurred immediately after the conclusion of the tomonts experiment. To prevent any degradation or loss of natural products through storage, extracts were freshly made for each seaweed species before use and discarded at the conclusion of each experiment.

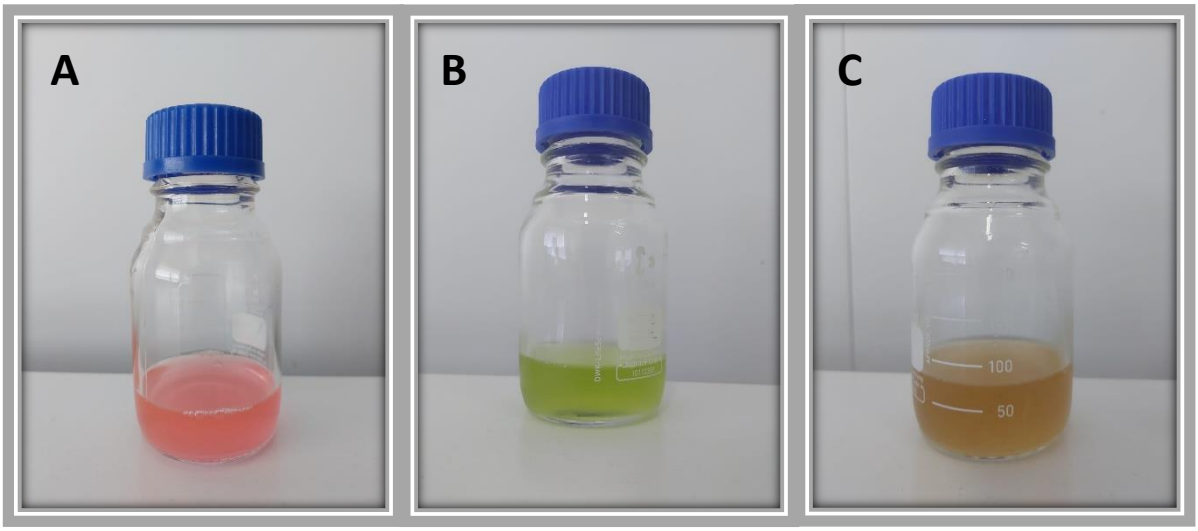

Figure 3.1. Freshly made extracts of three different seaweed species. **A** - *Asparagopsis armata* gametophyte, **B** - *Ulva* sp., **C** - *Halopteris scoparia*. Original from the author.

3.2 Parasites infection and collection

The first contamination was done using refrigerated tomonts previously stored on EPPO-IPMA. The first attempt served as a pre-trial, since the viability of tomonts were under 30%. However, it was possible to use these tomonts and reinfect 2 new fishes and with that, start the first trial with fresh tomonts and so on.

The infection system was created to assure the freshest tomonts and the highest viability possible of it. In order to do that, the infection system was prepared every 3 days prior to infection for fish acclimatation to the tank environment. These plastic tanks (60 L) were filled with 40 L of sand-filtered seawater (salinity 35) and maintained at room temperature (25ºC) under constant aeration (Figure 3.2).

Figure 3.2. Infection tank with a styrofoam cover to prevent contaminations of other experiments

Every week, 2 juvenile specimens of Gilthead seabream *Sparus aurata* were transported from juvenile section of EPPO-IPMA using a net and placed into the infection tanks, where the fish were kept under control for the rest of the contamination. Fish were deprived of food for the 3-day acclimation period and the subsequent 2 infection days. During the entire acclimation period, tomonts for the upcoming treatment were kept under incubation. This duration precisely aligned with the time allocated for tomont incubation. In the beginning of the first treatment, 30000-50000 refrigerated tomonts were incubated in 50mL falcon containing autoclaved seawater (autoclaved at 120ºC for 20min) at 25ºC for 72h. At the end of this period, tomonts had already developed to the dinospore stage and were ready to infect the fish. However, in order to not kill the fish with an overdose of dinospores, it was decided that the amount of the dinospores would not surpass 100000 per fish. To avoid this situation, after every weekly 3 days incubation period, dinospores were counted before the infection. For this, 20 µL were pippeted from the incubated falcon into a concave microscope slide and analyzed under a Leica DMIL LED inverted microscope (10X magnification) the number of dead dinospores. To estimate the number of living dinospores, the procedure was the same as described in the topic 3.3.2. Following that, fish were infected with the dinospores and after 48h, euthanized, in order to start the seaweed extracts experiments.

In order to collect parasites, gills were checked at the Leica DMIL LED inverted microscope for the presence of trophonts. Once the parasite was detected, each gill was stirred for 30 seconds with autoclaved seawater into a 500 mL beaker. Water was filtered via a 200 µm nylon mesh to remove big particles before passing through a 30 µm nylon mesh to capture trophonts. Parasites were captured into a 500 mL beaker by washing the 30 µm nylon mesh with autoclaved seawater and finally tomonts were collected into a 50 mL falcon and counted on the inverted microscope (10X magnification) using a concave microscope slide. Parasites that were collected were used for initial tests and the leftovers were preserved on a Electronia HS-125WEN fridge between 5º-15ºC for future trials.

3.3 Effects of seaweed extracts *in vitro* on *Amyloodinium ocellatum* inactivity/mortality

The effect of seaweed extracts on the mortality of two life stages of *A. ocellatum* tomont and dinospore, were tested *in vitro* on a 96 well-plate (flat bottom) in 6 different concentrations (dilutions 1:1, 1:5, 1:10, 1:25, 1:50 and 1:100). Each dilution had 6 replicates, in addition to an autoclaved seawater control, (6 replicates) and 10 µL tomonts/dinospores, previously stored on autoclaved seawater in 50 mL falcons, were introduced into the wells along with autoclaved seawater, to achieve a total volume of 210 µL per well, including the corresponding extract dilution. In order to homogenize the falcons and have nearly an equivalent quantity of tomonts/dinospores within each 10 µL, particular attention was given to a crucial step involving the agitation of falcon tubes. Tomonts, due to their resilient cysts, vigorous shaking was possible and this process had to be repeated for every single well, due to their quick settle in the bottom of the falcon. On the other hand, for delicate dinospores, gentle agitation was only done every 6 replicates, with the intention of avoiding harming these free-living flagellates.

Dilutions were chosen based, not only through a study (Hutson et al., 2012), that showed promising results on managing monogenean infections, but also on the outcome of the pre-trials performed with 3 different seaweeds, *Ulva* sp., *Halopteris scoparia* and *Plocamium cartilagineum*, at 2 concentrations (1:1 and 1:10) that also showed positive results in the inactivation of tomonts at the dilution 1:1.

3.3.1 Tomonts inactivity

Several seaweed extracts were tested on their effects on the inactivity of tomonts. During the experiment, tomonts were incubated on a VWR INCU-Line IL56PR Premium incubator, on 96-well plates at 25ºC. The effect of extracts on tomonts inactivity was examined following 96h exposure. Their development was tracked every 24h on a Leica DMIL LED inverted microscope (10X magnification) by checking inactivated tomonts and tomonts in division. The state of inactivity was assigned to the tomonts when they did not undergo any division.

$$\text{Inactivity (\%)} = \frac{\text{Inactive Tomonts (t)}}{\text{Initial Tomonts (t0)}} * 100$$

Where: t = time in hours (24, 48, 72 and 96); t0 = tomonts' initial quantity

3.3.2 Dinospores mortality

To assess the impact of different seaweed extracts on dinospores mortality, 96-well plates with a flat bottom were utilized for analysis over a period of 6 hours exposure. The time intervals selected for examination were at 0h, 1h, 3h, and 6h, each time mark, non-motile dinospores were counted. The addition of 10 µL of Lugol's solution after the final sampling, the 6 hour mark was required to fixate motile dinospores. Five minutes after lugol addition, every dinospore on the bottom of the well were counted and the difference between total and dead dinospores was used to calculate mortality, and posterior mortality rate according to the equation:

$$\text{Mortality rate (\%)} = \frac{\text{Mortality (t)} * 100}{\text{Mortality L}}$$

Where: t = time in hours (0,1,3 and 6); Mortality L = Mortality accessed after Lugol was added.

3.4 Statistical analysis

Before conducting the analysis, data was assessed for normality assumption and homogeneity of variance through the Shapiro-Wilk's and Levene's tests, respectively. If both assumptions were met, a one-way ANOVA was used to evaluate within each time point the effects of the treatments. Subsequently, if significant differences were found, the post-hoc Tukey-Kramer multiple comparison test was conducted to compare the effect of different extract dilutions between groups. For data that did not fit the assumptions of the parametric test, a logarithmic transformation was attempted. If assumptions of normality and homogeneity of variance were not accomplished, a non-parametric Kruskal-Wallis test was conducted; if significant differences were observed, a Wilcoxon test was used for each group. The data was presented as the mean ± standard deviation (SD). Statistical significance was determined based on a p-value of less than 0.05. All statistical analyses were performed using RStudio version 4.2.2.

4 Results

4.1 Tomonts inactivity

4.1.1 *Asparagopsis armata* gametophyte extract

In the undiluted extract treatment (1:1) the first tomont division was only observed at 72h, while in all other treatments, divisions were observed since the 24h sampling. In fact, tomonts' division on treatment with this extract dilution was significantly delayed throughout the 96h exposure period, as it is perceptible when comparing this treatment with the control and with all other extract dilutions (p-value < 0.05). Undiluted (1:1) extract shows a range of inactivity between 48-99% for the 96h period and a significantly higher tomont inactivation than all the other extract dilutions (p-value < 0.05), while the control and the other 5 extract dilutions (1:5, 1:10, 1:25, 1:50, 1:100) ranged between 2-23% at this time period (Figure 4.1). Apart from this result, at 24h there was also a significant higher tomonts' inactivation in extract dilutions 1:5 and 1:10 comparing to control and to 1:50 dilution (p-value < 0.05).

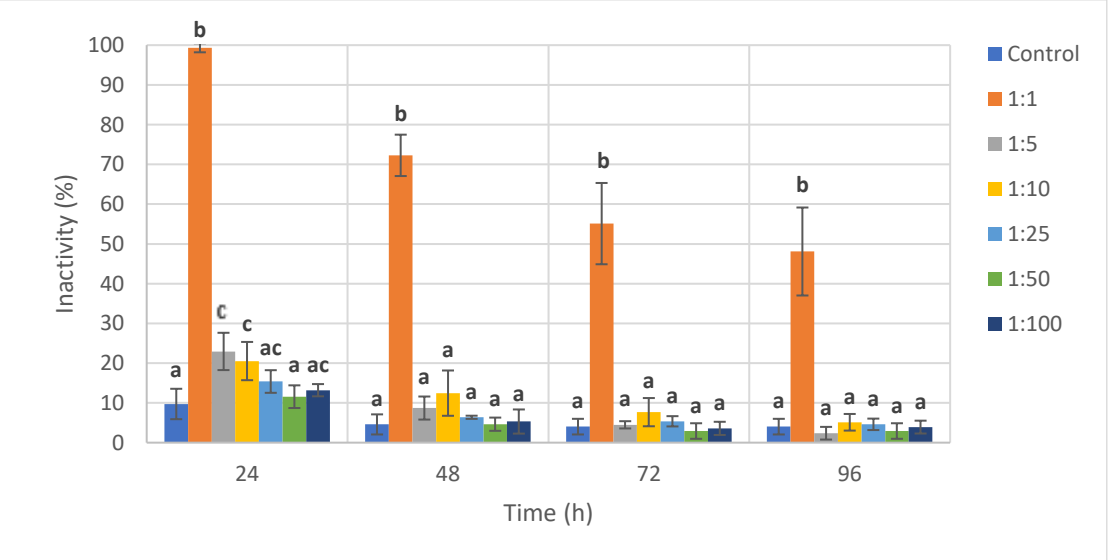

Figure 4.1. Inactivity rate (%) of *Amyloodinium ocellatum* tomonts at different extract dilutions (1:1, 1:5, 1:10, 1:25, 1:50 and 1:100) and a control (autoclaved seawater), when exposed to *Asparagopsis armata* gametophyte extract through a period of 96h. Within each time point, columns sharing a letter do not differ significantly (p > 0.05).

4.1.2 *Asparagopsis* sp. tetrasporophyte extract

The first tomont division occurred in the first 24h in every extract treatment, however there is a notable and significant higher delay over the course of 48 hours

within the undiluted sample, when compared to each extract dilution (p-value < 0.05). Extract dilution 1:1, showed inactivity levels between 50% (24h), and 28% (96h), while other extract dilutions had lower values, ranging between 0-14% in the 96h period (Figure 4.2). Besides this result, there was also a significant higher tomonts' inactivation in extract dilutions 1:10 and 1:25 compared to dilution 1:50 in the first 24h (p-value < 0.05).

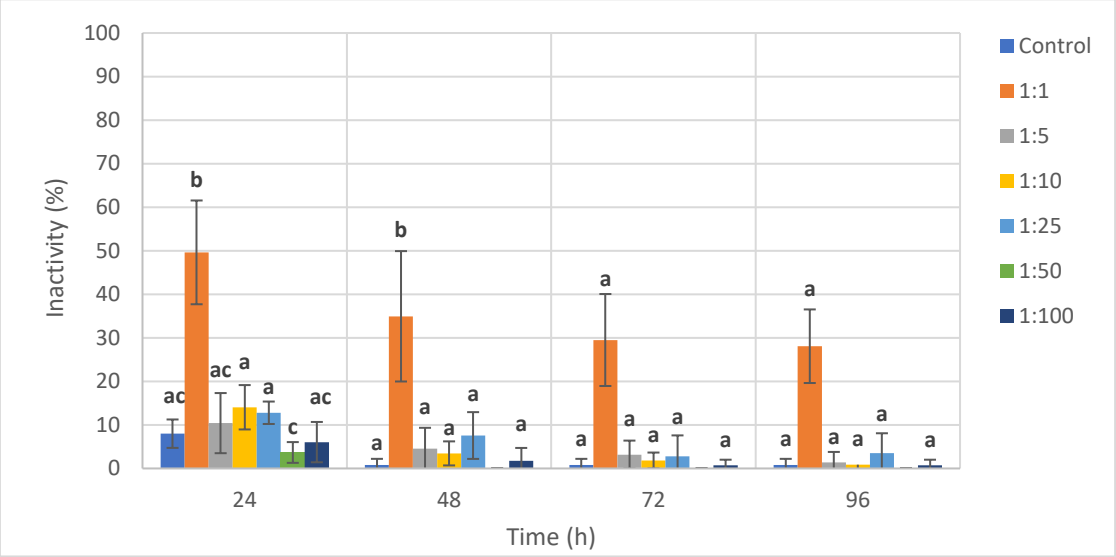

Figure 4.2. Percentage of inactivated tomonts of *Amyloodinium ocellatum* when exposed to *Asparagopsis* sp. tetrasporophyte extract in 6 different dilutions (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100) and a control throughout 96h. Within each time point, columns sharing a letter do not differ significantly (p > 0.05).

4.1.3 *Gracilaria* sp. extract

The first tomont division occurred in the first 24h in every extract treatment, however there is a significantly higher tomonts' inactivation between the extract treatment 1:1 and the control and every other extract dilution at the 24h and 72h mark (p-value < 0.05) (Figure 4.3). Undiluted extract showed the highest inactivity rate through the 96h, with a range of inactivity between 8.5-50%, with the exception of the 48h mark, where extract dilution 1:100 had higher inactivity rate (24%). Control and the remaining extract dilutions showed a range of inactivity between 2.3-35%.

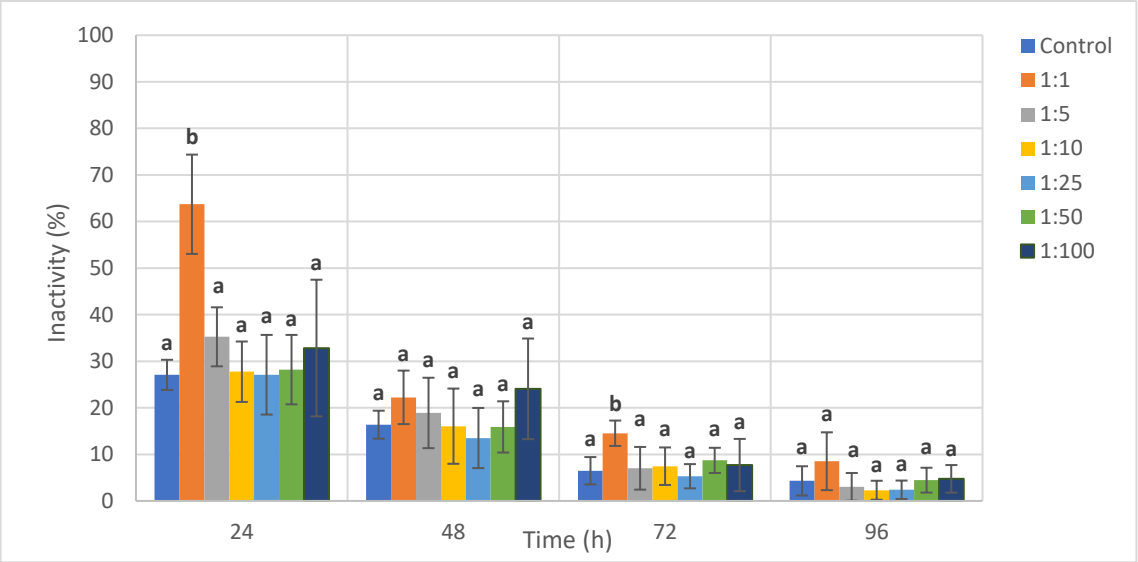

Figure 4.3. Variation in tomont of *Amyloodinium ocellatum* inactivity (%) across various dilutions (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100) and control (autoclaved seawater) upon 96h exposure to *Gracilaria* sp. extract. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.1.4 *Sphaerococcus coronopifolius* extract

The first tomont division occurred in every extract treatment in the first 24h. In fact, tomonts' division on treatment with the *Sphaerococcus coronopifolius extract* was significantly delayed throughout the 96h exposure period, as it is perceptible when comparing this treatment with the control and all other extract dilutions (p-value < 0.05) (Figure 4.4). Undiluted treatment had the highest inactivation rate at 53% in the first 24h and had the lowest inactivity rate at 26% at the 96h mark. Dilution extract 1:1 consistently showed a significantly higher tomonts inactivation throughout the 96h period when compared to every other extract dilution, including the control. In the first 24h, extract dilution 1:5 also showed significantly higher tomonts inactivation between the extract 1:25, 1:50,1:100 and the control (p-value < 0.05) in the first 24h. While in the 48 hours, extract dilution 1:10 showed significantly lower tomont inactivation (p-value < 0.05).

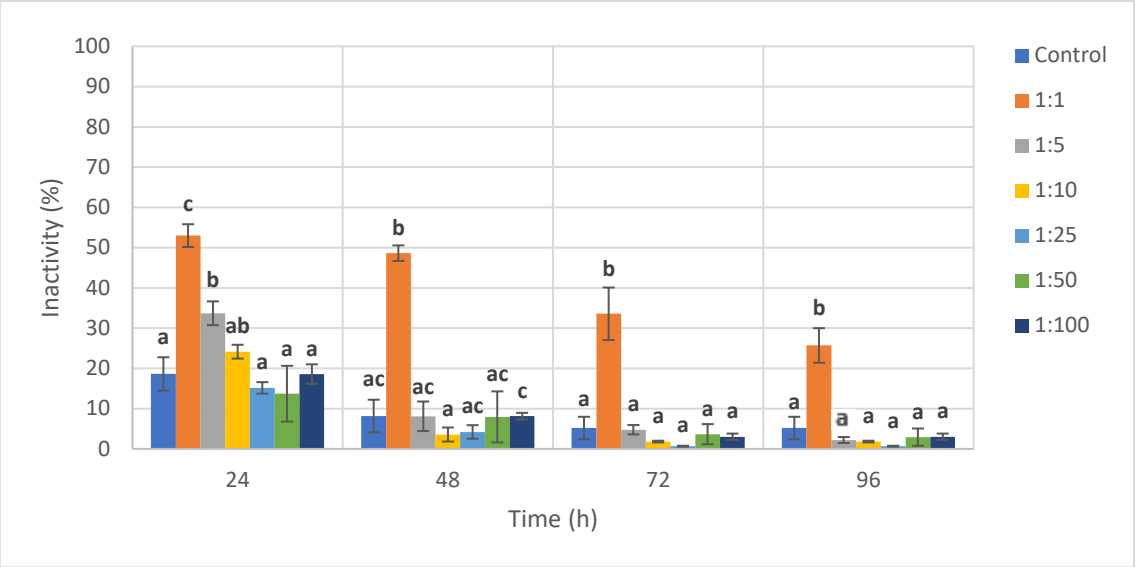

Figure 4.4. Tomonts of *Amyloodinium ocellatum* when exposed to a *Sphaerococcus coronopifolius* extract through a period of 96h at 6 different dilutions (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100) and in the presence of a control. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.1.5 *Halopteris scoparia* extracts

In the first 24h every extract dilution had already developed their first division. Extract dilution 1:1 showed higher tomont inactivation throughout the whole 6h period (Figure 4.5), however without any significant statistical difference between each group.

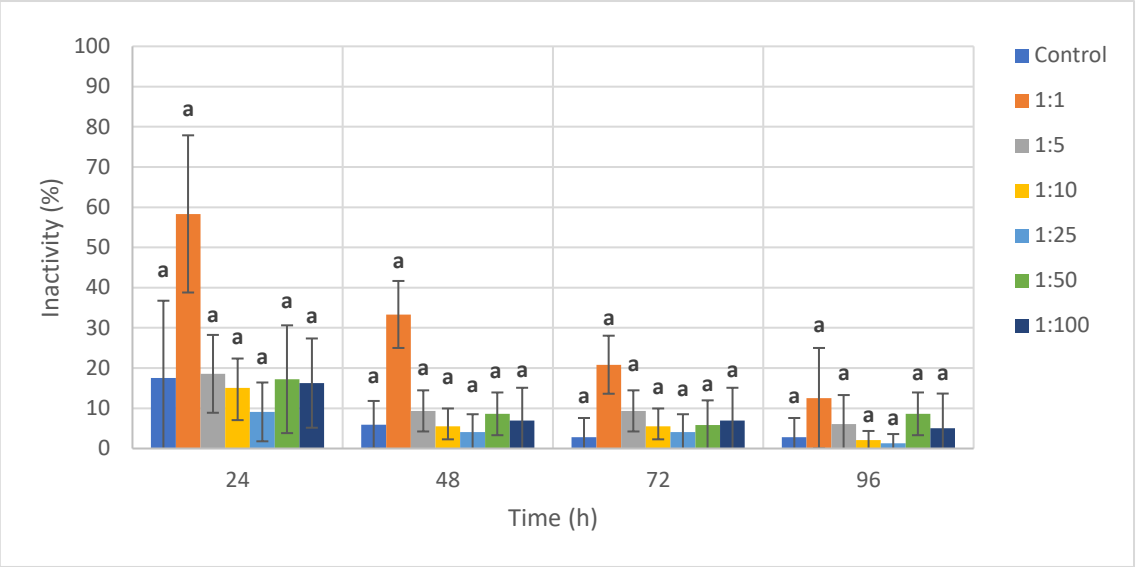

Figure 4.5. Divergence in tomont inactivity (%) of *Amyloodinium ocellatum* at different dilution levels (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100) and in the presence of a control (autoclaved seawater) following a 96h exposure to *Halopteris scoparia* extract. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.1.6 *Ulva* sp. extract

At the 24h mark every extract dilution had already its first tomont division. A continuous exposure to *Ulva* sp. extract showed, in the undiluted treatment, the highest inactivity at the 24h (46%) and the lowest at the 96 hours (20%) (Figure 4.6). A significantly higher effectivity on delaying the division of tomonts in the extract dilution 1:1 was shown, when compared to control and all other extract dilutions (p-value < 0.05) in the first 72 hours. At the 96h mark, besides extract dilutions 1:5 and 1:25 still having a lower tomont inactivation than extract dilution 1:1, these differences were not significant (p-value > 0.05).

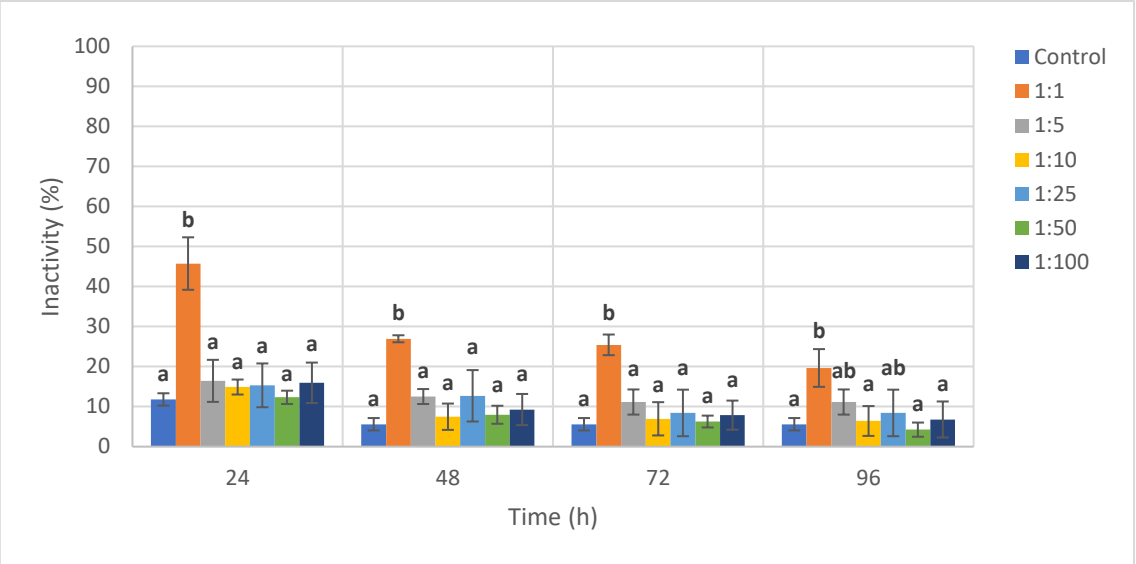

Figure 4.6. Difference in tomonts inactivity (%) of *Amyloodinium ocellatum* at different dilution levels (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100) and in the presence of a control (autoclaved seawater) through a 96h period to *Ulva* sp. extract. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.2 Dinospores mortality

4.2.1 *Asparagopsis armata* gametophyte

Mortality of dinospores when exposed to 6 straight hours of *Asparagopsis armata* gametophyte extract did not show significant statistical differences at any extract treatment (p-value > 0.05). Notably control treatment showed high mortality rates consistently through the whole experiment (Figure 4.7). Extract dilution 1:10 was the only extract that showed higher or similarly mortalities when compared to the control. At the end of the 6 hours extract dilution 1:100 was the one that had the highest mortality (94%).

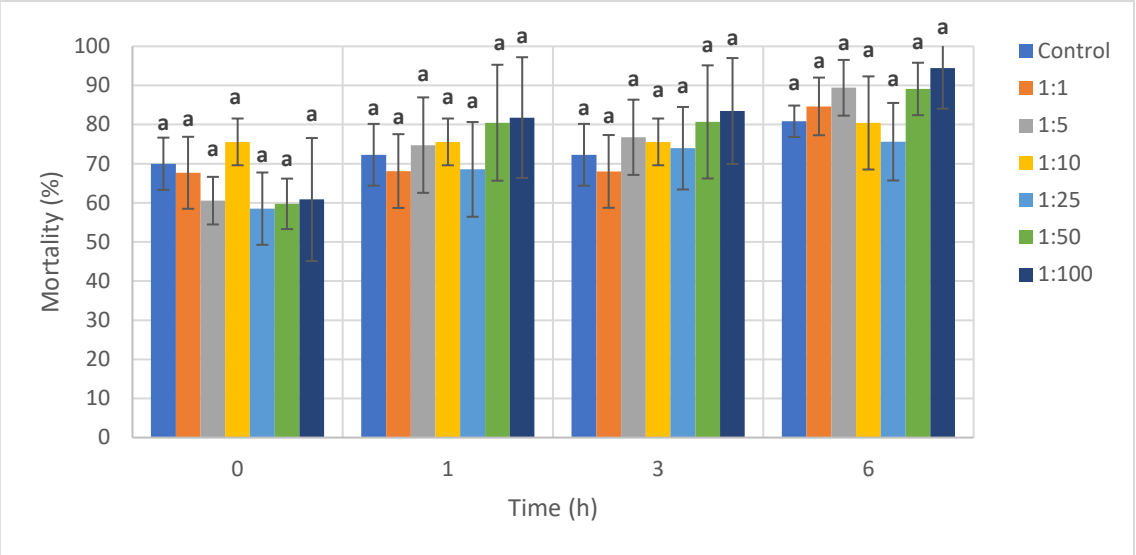

Figure 4.7. Mortality rate (%) of *Amyloodinium ocellatum* dinospores when exposed to *Asparagopsis armata* gametophyte extract in 6 different dilutions (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100) and a control through a period of 6h. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.2.2 *Asparagopsis* sp. tetrasporophyte

Asparagopsis sp. tetrasporophyte extract showed significantly higher mortalities in the extract dilution 1:100 in comparison to the dilution 1:10 and 1:5 (p-value < 0.05) in the first 24 hours (Figure 4.8). But when compared these two specific dilutions 1:100 and 1:10, the extract dilution 1:100 showed significantly higher mortality rate than the extract dilution 1:10 in the 0-hours mark. Regarding the 1st hour of the experiment, extract dilution 1:1 showed significantly higher rates of mortality in comparison to the dilution 1:10 and 1:5 (p-value < 0.05). The extract dilution 1:10 had also a significantly higher mortality than the extract dilution 1:5. In the final hours of the experiment (3 and 6 hours), no significant differences were found between each specific extract treatment (p-value > 0.05). Highest mortality (93%) of the dinospores was reached in the 6th hour in the extract dilution 1:1.

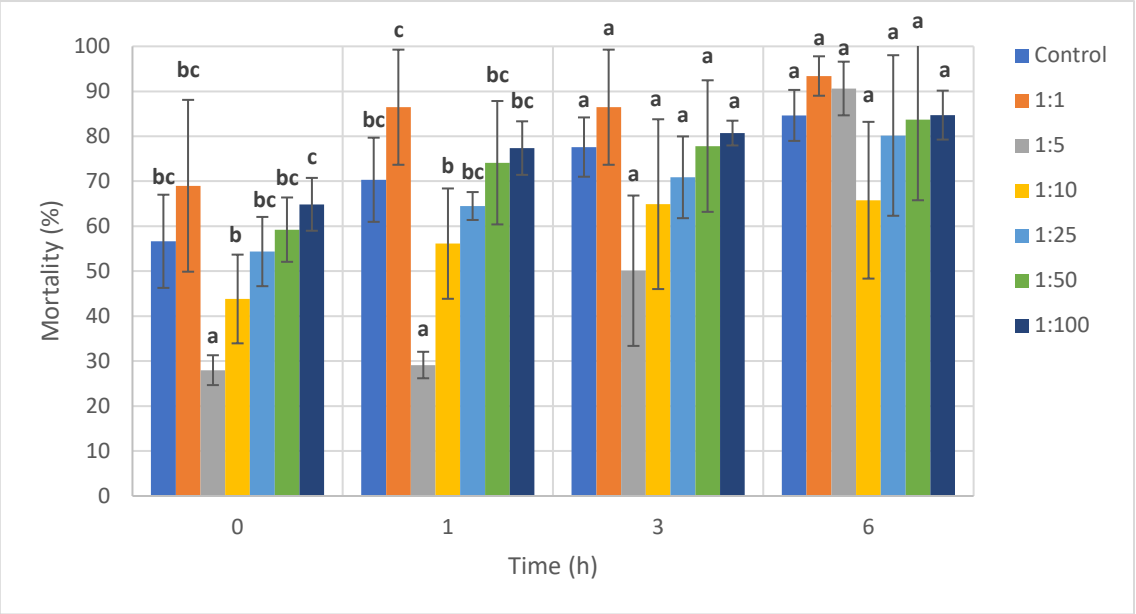

Figure 4.8. Mortality rate (%) of *Amyloodinium ocellatum* when exposed to *Asparagopsis* sp. tetrasporophyte extract for a period of 6 straight hours in various dilutions and a control. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.2.3 *Gracilaria* sp. extract

Continuous exposure to *Gracilaria* sp. extract for 6h showed mortality of dinospores around 100% in every dilution, as well as for the control treatment (Figure 4.9). In the 0h mark, extract dilution 1:50 showed a significantly higher mortality of the dinospores compared to the extract treatment dilutions 1:5 and 1:10 (p-value <0.05. Regarding the 1 and 6-hours mark, significantly differences between extract treatments were not seen. However at the 3rd hour, extract dilution 1:25 showed significantly higher mortality rate when compared to extract dilution 1:5 and 1:10, as well as the extract treatment 1:100 that showed higher mortality comparing to extract dilution 1:10 (p-value < 0.05).

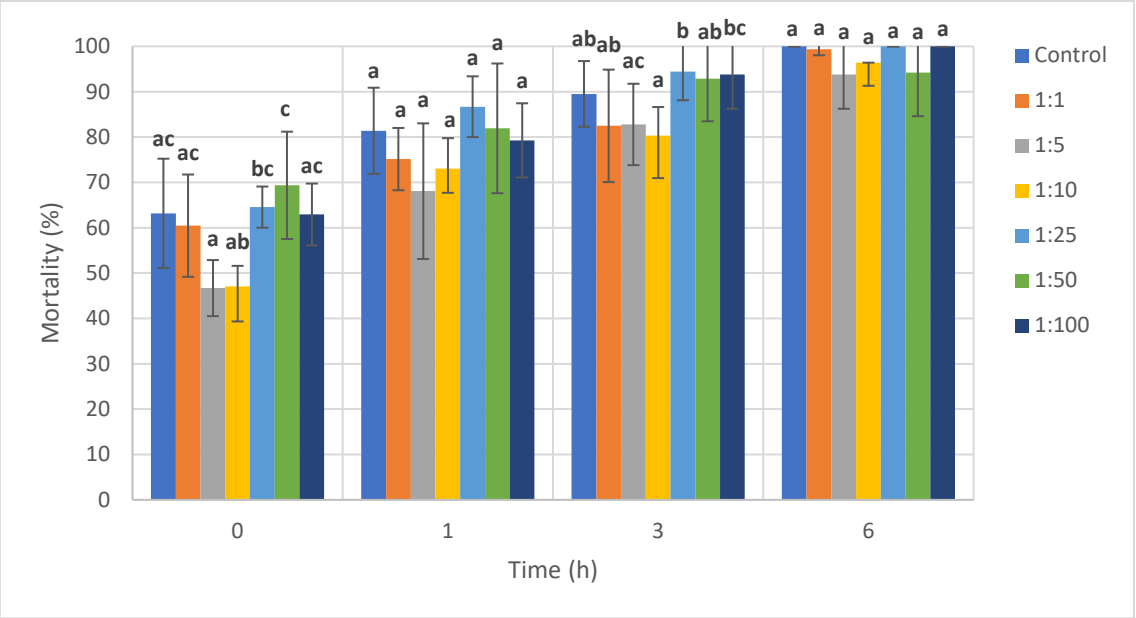

Figure 4.9. Mortality rate (%) in *Amyloodinium ocellatum* dinospores following a 6h exposure to *Gracilaria* sp. extract across 6 different dilution levels and a control. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.2.4 *Sphaerococcus coronopifolius* extract

Sphaerococcus coronopifolius extracts showed a significantly higher mortality of the dinospores in the 0h mark (Figure 4.10), when compared to every other extract treatment (p-value < 0.05), as well as the dilutions 1:5 and 1:100 that registered significantly higher mortality rates between the extract dilutions 1:10 and 1:25 (p-value < 0.05). Regarding the 1h mark, extract dilutions 1:1 and 1:10 showed significantly higher mortalities in comparison to the control group (p-value < 0.05). A similar trend was observed between the 3 and the 6 hours period, with the inclusion of extract dilutions 1:5 and 1:25, which also exhibited significantly higher mortality rates compared to the control group (p-value < 0.05). The highest mortality (93%) was reached in the extract dilution 1:1 at the 6th hour.

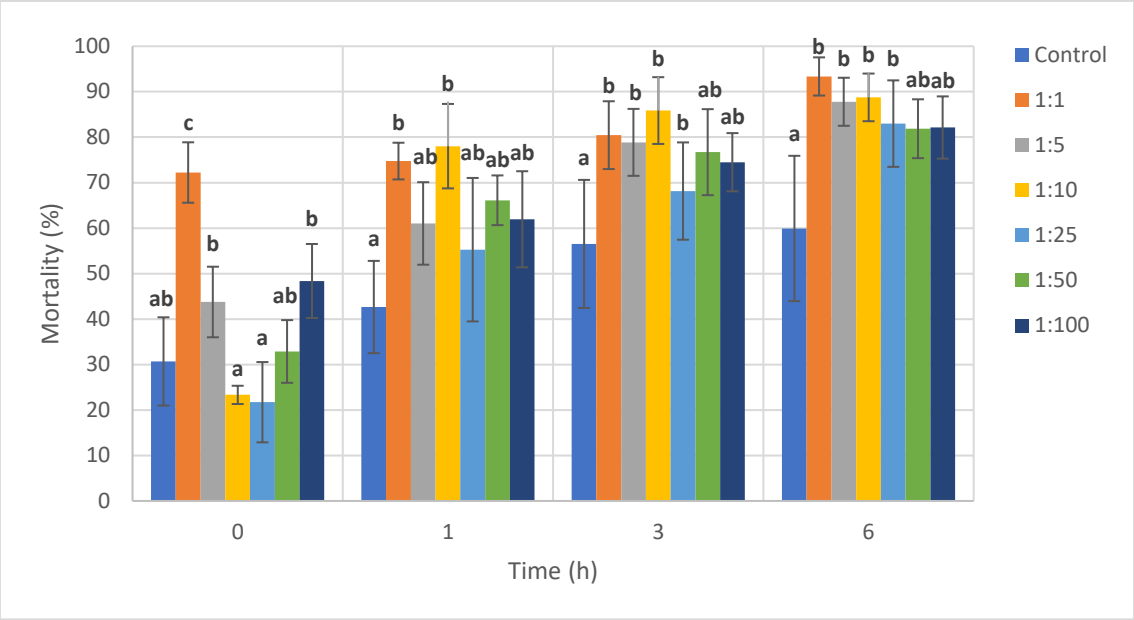

Figure 4.10. Mortality rate (%) of dinospores of *Amyloodinium ocellatum* over a 6h exposure to *Sphaerococcus coronopifolius* extract various dilution levels (1:1, 1:5, 1:10, 1:25, 1:50, 1:100) and a control group. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.2.5 *Halopteris scoparia* extract

Extract dilution 1:25 and 1:50 in the 0h showed significantly higher mortalities when compared to the control treatment (p-value< 0.05). Dinospores exposed at the hour 1 and 3 to *Halopteris scoparia* extract did not show any significant differences between any extract treatment (Figure 4.11). However, in the 6-hour period, significantly higher differences were noted between extract dilution 1:25 when compared to the control treatment and the dilutions 1:1, 1:5 and 1:10 (p-value < 0.05). 100% mortality was reached in the extract dilution 1:25 at this period time (6h).

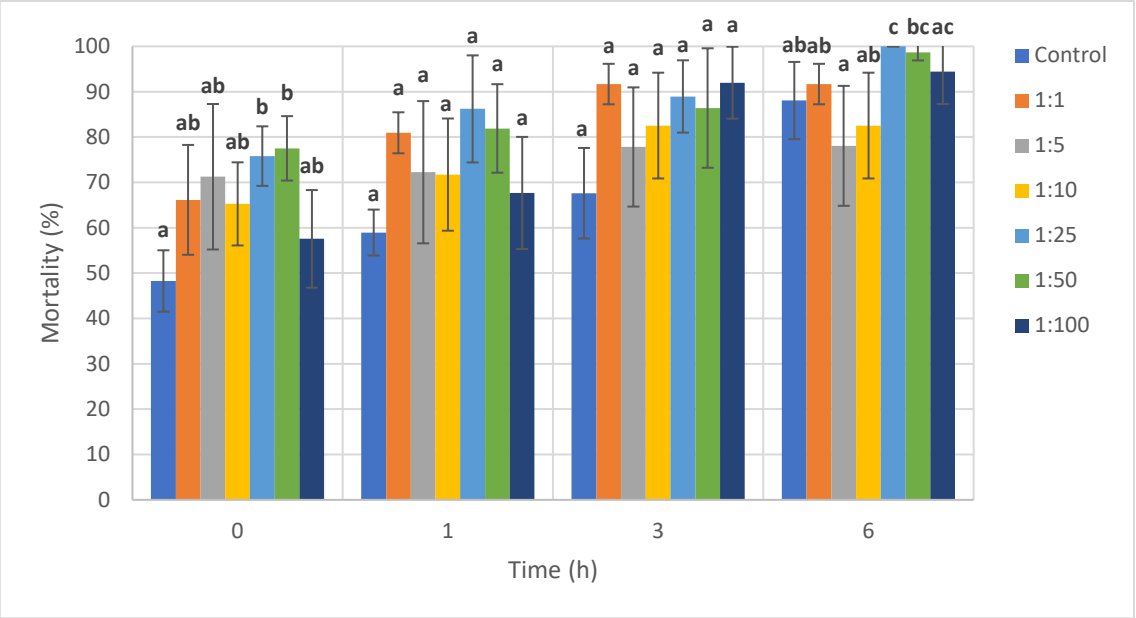

Figure 4.11. Mortality rate (%) of *Amyloodinium ocellatum* dinospores throughout 6h of exposure to *Halopteris scoparia* extract in different dilutions and a control (autoclaved seawater). Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

4.2.6 *Ulva* sp. extract

Dinospores when exposed to *Ulva* sp. extract throughout 6h did not show any significant differences between each extract treatment (p-value > 0.05). The highest mortality (90%) was reached in the extract dilution 1:1 at the last hour (6h) (Figure 4.12).

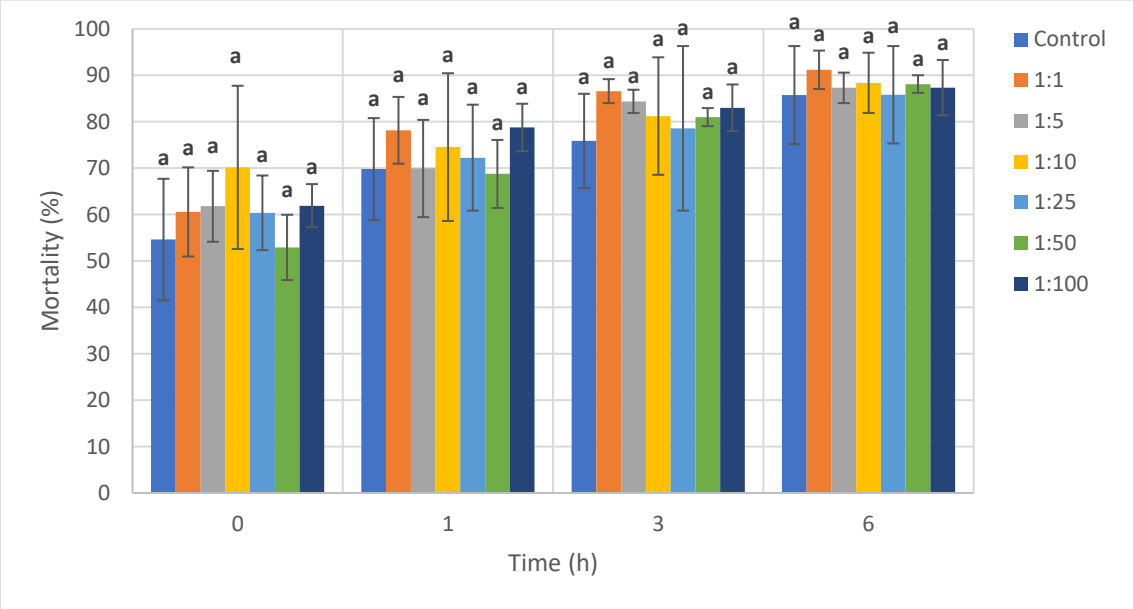

Figure 4.12. Dinospores mortality (%) of *Amyloodinium ocellatum* for a period of 6h when exposed to *Ulva* sp. extract at 6 different dilutions (1:1, 1:5, 1:10, 1:25, 1:50, 1:100) and a control group. Within each time point, columns sharing a letter do not differ significantly ($p > 0.05$).

5 Discussion

Several studies have explored the potential of seaweed extracts in controlling various aquatic parasites, such as *Neobenedeni*a sp. and *Trichomonas vaginalis* (Hutson et al., 2012; Moo-Puc et al., 2008), however there remains a significant gap in research concerning the efficacy of seaweed based treatments specifically targeted at disrupting the life cycle of *A. ocellatum*, This work represents a contribution to filling this gap by introducing environmentally friendly treatment strategies within an *in vitro* condition. It was investigated the impact of six different seaweed extracts, derived from the following species: *A. armata* gametophyte, *Asparagopsis* sp. tetrasporophyte, *Sphaerococcus coronopifolius*, *Gracilaria* sp., *Ulva* sp., and *Halopteris scoparia* on the delay of two life stages of *A. ocellatum*, the tomont and dinospore stage.

Although none of the extract treatments resulted in a complete interruption of the tomont division process within the 96 hours period, it demonstrated the effectiveness of the extracts in the first 24 hours, but over time, their bioactivity is diminished because of possible degradation of the extract and subsequent loss of bioactive compounds. In particular, among the red seaweeds, the extract derived from *A. armata* gametophyte, especially in the extract dilution 1:1, exhibited the ability to delay tomont division with inactivity rates of 99% in the first 24h. Notably, this extract exhibited the highest percentage of tomont inactivity. However, in contrast to the *A. armata* gametophyte extract, the *Asparagopsis* sp. tetrasporophyte extract from the same genus exhibited lower inactivity rates, particularly at the lowest extract dilution (1:1). In fact, within the initial 24-hour period, only a 50% inactivation rate was observed, and there was no observable delay in initial division of tomonts over time, with tomonts sporulating in this time frame. This observation provides valuable insights into managing some aspects of the life cycle of *A. ocellatum* through the incorporation of eco-friendly treatments.

Each taxonomic group of seaweeds exhibits its unique seasonal pattern, resulting in variations in the natural products, including the production of bioquemichal compounds, such as secondary metabolites, polyphenols and halogenated compounds like haloforms, methanes, ketones, acetates, and acrylates (Genovese et al., 2012; Watson & Cruz-Rivera, 2003). These compounds have been documented in the *Asparagopsis* genus and are known for their potent antifungal, antibacterial, and antiprotozoal properties (Vatsos & Rebours, 2015). Moreover, their concentrations can

vary across different parts of the thallus, influenced by reproductive stages and geographical locations (Genovese et al., 2012).

In the present work, it is important to consider the impact of seasonality and the reproductive stage of both *A. armata* gametophyte and *Asparagopsis* sp. tetrasporophyte. While the gametophyte specimens were freshly collected from the field, *A. armata* tetrasporophyte was cultivated in the laboratory for an extended period due to the inherent challenges of sourcing these seaweeds along the Algarve coast. It is reasonable to assume that the field collected seaweed, *A. armata* gametophyte was in an advanced reproductive stage, as some parts of the seaweed had already begun to exhibit whitish discoloration at the time of the extraction. Despite meticulous efforts to isolate and extract only the healthy portions of the algae, it was not possible to ensure a 100% exclusion of any potential less viable parts, which may have led to variations in the quantity of bioactive compounds compared to those collected and cultivated in the lab. These variations could have influenced the initial inactivity rates and subsequent development patterns of tomonts. However, *A. armata* gametophyte, despite reaching an advanced reproductive stage, exhibited the highest inactivity rate of 99% in the first 24h. This highlights the potential of this specific seaweed to delay the development of tomonts.

Another factor that may have contributed to this disparity is the method of blending employed. In contrast to the *A. armata* gametophyte extract, the *Asparagopsis* sp. tetrasporophyte extract appeared notably darker. This difference in coloration can be attributed to the blending process itself. *Asparagopsis* sp. tetrasporophyte possess a unique structure resembling cotton, which, upon contact with the autoclaved seawater used as a solvent, absorb moisture, making the blending process more challenging, resulting in a lesser disruption of cell walls and cellular structures. Consequently, reducing the release of bioactive compounds into the liquid medium.

The observed variations in the tomont division between the *A. armata* gametophyte, that was tested immediately after collection and the one that spent two months in an indoor tank under artificial light (*Asparagopsis* sp. tetrasporophyte) may also be attributed to differences in their UVB exposure. This distinction in UVB radiation exposure holds significance as previous studies have established its influence on the physiological and functional attributes of seaweed communities, including the production of phenolic compounds (Bischof et al., 2006).

The other two red seaweeds, *Gracilaria* sp. and *S. coronopifolius*, both known for their production of bioactive secondary metabolites, also exhibited significant differences in inactivity rates between the 1:1 extract dilution and the other dilutions, as well as the control group treated with autoclaved seawater. Specifically, they showed inactivity rates of 63% and 53%, respectively, in the first 24 hours, whereas the control group had rates of 27% and 19%, respectively. This suggests that within the initial 24 hours, the bioactive compounds may exert a stronger effect, potentially indicating a decline in their effectiveness as time progresses, impacting their ability to interfere with the tomont stage.

Brown seaweeds are also well known for their bioactive compounds, particularly secondary metabolites such as polyphenols and fucoxanthin (Remya et al., 2022). Polyphenols, due to their phenolic characteristics, are recognized for their potent antiparasitic activity. This was corroborated by a study investigating the antiprotozoan activity against *A. castellanii*, which involved several brown seaweeds, including *H. scoparia*. Notably, *H. scoparia* exhibited an IC50 of 105.7 ± 0.3 when using Ethyl acetate as a solvent (Chiboub et al., 2017). These observations support the positive results obtained in the present work in terms of inactivation of tomonts in the extract dilution 1:1. Nevertheless, although differences in tomont inactivity rates within the 96 hours period were evident, statistical analysis did not reveal significant disparities due to the substantial variations between replicates and consequent fluctuations of the standard deviation levels. The presence of fucoxanthin, a natural pigment classified within the carotenoid family, is indicative of brown and green seaweed coloration. Its presence, or lack of, can be observed through changes in color (Pappou et al., 2022; Remya et al., 2022). This observation potentially elucidates the faded color mentioned earlier in the *H. scoparia* and *Ulva* sp. extract at the 96th hour of the tomont experiment. Furthermore, it may contribute to the relatively low inactivity rates observed for tomonts at this time point across all extract dilutions, as the concentration of this secondary metabolite in the liquid medium might be lower.

Regarding the extraction method, it is widely observed that organic solvents are generally more effective in extracting bioactive compounds, particularly secondary metabolites like polyphenol and fucoxanthin, when compared to extracts from polar solvents (water), that tend to exhibit higher antibacterial activity (Pappou et al., 2022; Rosell & Srivastava, 1987). For instance, the types of beneficial substances found in seaweed can vary depending on the solvent used for extraction. Additionally, when a

combination of organic solvent and water is used (aqueous organic solvent), the yield of extracted biocompounds is significantly higher. This was demonstrated in a study by Rajauria et al. (2012), where they found that using a 60% aqueous methanolic solution for the extraction of bioactive compounds from *Himanthalia elongata* resulted in a much greater yield (6.8%) compared to using 100% methanolic extract (1.2%). This highlights the potential for future experiments utilizing similar organic solvents. The temporary inhibitory effect observed in response to water soluble seaweed extract treatments on tomont divisions potentiates the ability of tomonts to either metabolically neutralize or excrete these treatments. This phenomenon is present on a study made by Paperna (1984), where certain chemicals, when administered at higher concentrations, displayed a temporary inhibitory effect on tomont division, which was subsequently reversed upon treatment discontinuation (formalin at 200 ppm and Furanace at 2.5-5 ppm). In contrast, Nitrofurazone at 50 ppm and malachite green at concentrations exceeding 0.1 ppm exhibited an irreversible effect. These observations suggests that if tomonts possess mechanisms to counteract chemical influences, they might similarly respond to water-soluble seaweed treatments.

In general, effective treatments aim to either eliminate free-swimming infective dinospores before they can attach to a new fish host or remove and eliminate the trophonts from the fish, thereby disrupting the parasites' life cycle (Francis-Floyd & Floyd, 2011). By the analysis of the results obtained from the application of the seaweed extracts, it is apparent that these extracts may have an impact on the mortality of dinospores, although the results are not conclusive, one potential contributing factor could be the use of extracts in the dinospore experiment that were not freshly prepared (5 days old), potentially resulting in the loss of bioactive compounds over time. The observed high mortality rates within the seawater control, at times even exceeding the mortality rates in some dilutions, raises doubts regarding the methodology employed for dinospore experiment. Instead of inoculating all 42 wells with their respective extract dilutions consecutively, what results in a long counting period, a better alternative could be doing a sequential inoculation strategy for randomized treatment replicate. This means initiating the inoculation and counting process with fewer replicates and once this set of wells is completed, we can then proceed to inoculate and count the next set of replicates and continue in this manner for all wells. This approach can help mitigate substantial discrepancies in the counting process across various dilutions. Numerous studies, including the work of Paperna (1984), which suggests that copper sulfate, even

at high concentrations of up to 10 ppm, does not stop tomonts division. On the other hand, concentration as low as 0.5 ppm exhibit lethal effects on dinospores. Typically, it has been suggested that drug treatments are primarily effective agains a single life stage the dinospores while tomonts, residing in an encysted reproductive state, inherently possess greater resistance due to their cyst formation. However to achieve a clearer conclusion regarding the duration of this inactivation process, it is recommended to conduct prolonged treatments involving the daily replacement of the liquid medium in each respective well, considering the possibility of the loss of bioactive compounds over the course of the experiment.

6 Conclusions

This study has evaluated different seaweed extracts as potential alternatives to current amyloodiniosis treatments, with a primary focus on their impact on tomont inactivity and dinospore mortality, prioritizing minimal environmental and fish health problems. Notably, the *Asparagopsis armata* gametophyte extract, when employed at a 1:1 dilution, exhibited the highest efficacy (99%) in inactivating the tomont division at the first 24 hours, suggesting its potential as a recommended treatment option. It is also important not to neglect all the other seaweeds species, which exhibited inactivation rates of approximately 50% within the initial 24 hours when exposed to 1:1 dilution. There is also a decrease in efficiency observed for each seaweed extract after the 24h mark, a factor that should be considered in future assessments. Relatively to the dinospores, *Gracilaria* sp. extract achieved a mortality rate of 100% within 6 hours of exposure in both 1:25 and 1:100 dilutions. However, it is important to note that these results should be considered with caution, as the control group also exhibited 100% mortality, raising questions about the real effect of this seaweed on dinospore mortality. Moreover, it is important to acknowledge that none of the seaweed extracts demonstrated complete tomont inactivation for a period longer than 24 hours or a significant complete elimination of infective dinospores over the entire duration. Even so, the results allow us to conjecture that adding extracts from these seaweeds every 24 hours to the fish farming tanks could be a methodology to be considered to mitigate the harmful effects of this parasite.

In summary, seaweed extracts utilising seawater as a solvent are simple and show potential for parasite management in aquaculture**.** However, it is important to understand whether employing any of these extracts commercially is appropriate. The costs of extraction and the potential delivery of the extracts to fish through feeds are the primary issues in this. Additionally, the effectiveness of the produced extracts can also be affected by the extraction process, so testing the dilution (1:1) primarily with the *A. armata* gametophyte extract with a daily exchange of an organic solvent, such as ethanol or methanol, for a more effective extract of the bioactive compounds is a logical extension of this work.

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