RUI MANUEL COUTO DE SOUSA

INACTIVATION TESTS OF THE DINOFLAGELLATE *Amyloodinium ocellatum* PARASITE OF MARINE FISH

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

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Trabalho efetuado sobre a orientação de:

Doutora Florbela Soares (EPPO-IPMA)

Doutor Vincent Laizé (CCMAR)

UNIVERSIDADE DO ALGARVE

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...
Para ser mais
para vencer
esta imensa inércia
é preciso recomeçar
ouvir o bater do coração
deixá-lo partir
levando o alforge
carregado de esperança

Ser de novo
o aprendiz das coisas
simples e vivas.

Viagem, Manuel de Sousa

IPMA
Instituto Português
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Aerial view of the IPMA - Estação Piloto de Piscicultura de Olhão where this work was carried out.

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Abstract

The ectoparasite *Amyloodinium ocellatum* is the most common and important dinoflagellate affecting fish, causing severe morbidity and mortality in both brackish and marine warmwater aquaculture fishes worldwide. *A. ocellatum* has a triphasic life cycle, consisting of a free-living flagellate, the dinospore, a parasitic stage, the trophont, and a resting and reproductive cyst, the tomont. Tomonts of *A. ocellatum* can settle in the bottom of tanks and ponds and resist for long periods of time maintaining the ability to reinfest. Biosecurity in ponds is changeling, due to the size of the aquaculture farms and because they are normally located near natural water reservoirs, which limits the application of aggressive chemical treatments. Chemotherapy has shown efficacy in eliminating the dinospore but not the tomont, which is very resistant to treatments. This work focused on the development of *in vitro* inactivating tests for the cystic stage of the parasite. To propagate the parasite, an infection system with 6 European sea bass *Dicentrarchus labrax* was established with parasites collected during the season of higher prevalence in fish aquaculture production earthen ponds. Preservation tests of the tomont were also performed to reduce the need of keeping the infection system for long periods. Different chemicals and physical methods were tested for their capacity to inactivate the tomonts. Efficacy of the inactivation was evaluated through germination tests, dinospores concentration and motility.. This work showed that tomonts can be inactivated by several chemicals and physical methods, in particular by chemicals with a lesser impact on the environment, like peroxides. this work also presents the first documented results on the efficacy of UV-C irradiation on tomont inactivation. Overall, this study shows that *A. ocellatum* treatment and prevention can be achieved following more sustainable environmental strategies.

Keywords: Aquaculture, fish pathology, earth ponds, parasite prevention, tomonts.

Resumo

O ectoparasita *Amyloodinium ocellatum* é o dinoflagelado mais comum e importante que afeta peixes, causando morbidade e mortalidade graves em peixes de aquacultura de água salobra e marinha em todo o mundo. *A. ocellatum* tem um ciclo de vida trifásico, consistindo num flagelado de vida livre, o dinósporo, um estágio parasitário, o trofonte, e um cisto de repouso reprodutor, o tomonte. Os tomontes de *A. ocellatum* podem instalar-se no fundo de tanques e tanques de terra e resistir por longos períodos mantendo a capacidade de reinfestação. A biossegurança em tanques de terra é desafiante, devido ao tamanho das estruturas de aquacultura e por estarem normalmente localizadas próximo de reservatórios naturais de água, o que limita a aplicação de tratamentos químicos agressivos. Os tratamentos químicos têm-se demonstrado eficazes na eliminação do dinósporo, mas não do tomonte, que é muito resistente aos tratamentos. Este trabalho teve como foco o desenvolvimento de testes de inativação *in vitro* para a fase cística do parasita. Para a propagação do parasita, foi estabelecido um sistema de infeção com 6 robalos *Dicentrarchus labrax* e parasitas recolhidos durante a estação de maior prevalência em viveiros de produção de aquacultura de peixes. Foram realizados testes de preservação do tomonte para reduzir a necessidade de manter o sistema infecioso por longos períodos. Diferentes testes químicos e físicos foram aplicados ao estágio de tomontes. A eficácia da inativação foi avaliada por meio de testes de germinação, concentração e motilidade de dinosporos. Este estudo mostrou que os tomontes podem ser inativados por diversos produtos químicos e desafios físicos. Resultados promissores foram obtidos com produtos químicos de menor impacto ambiental, como os peróxidos. Além disso, são apresentados os primeiros resultados documentados sobre a eficácia da irradiação UV-C em tomontes. No geral, este estudo mostra que o tratamento e a prevenção do *A. ocellatum* podem ser alcançados seguindo estratégias ambientais mais sustentáveis.

Palavras-chave: Aquacultura, patologia de peixes, tanques de terra, prevenção de parasitas, tomontes.

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Abbreviations

µL	microlitre
µm	micrometre
ANOVA	analysis of variance
CaO	calcium oxide
$CuSO_4.5H_2O$	copper sulphate pentahydrate
ELISA	enzyme linked immunosorbent assay
EPPO-IPMA	Estação Piloto de Piscicultura de Olhão – Instituto Português do Mar e Atmosfera
H_2O_2	hydrogen peroxide
LIN	linearity
M	molar
mL	millilitre
NaOCl	sodium hypochlorite
PCR	polymerase chain reaction
ppm	part per million
SEM	scanning electron microscopy
SSW	sterile sea water
UV	ultraviolet
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
w/v	weight per volume

1 Introduction

Parasitic diseases attributable to obligate or opportunistic eukaryotic pathogens have a severe impact on global finfish and shellfish aquaculture, and represent a key constraint to production, sustainability, and economic viability in many regions (Shinn *et al*., 2015). Aquaculture systems are very susceptible to parasitic outbreaks, mainly due to high stocking densities and water quality (Lieke *et al*., 2020). Important shifts in temperature are projected due to climate change which could potentially increase parasitic outbreaks, resulting in severe financial impacts (Cascarano *et al*., 2021).

1.1 The dinoflagellate *Amyloodinium ocellatum*

Dinoflagellates are a diverse group of protists that inhabit a wide variety of habitats, from freshwater to open ocean, having a central role in the planktonic food web (Horiguchi, 2015). During reproduction, as part of their life cycle, dinoflagellates produce resting cysts that form a 'seed bank' in sediments, from which waters can be repopulated (Zonneveld & Pospevola, 2015). There are approximately 150 parasitic dinoflagellate species; among them, *Amyloodinium ocellatum* is the most common and important dinoflagellate parasitizing fish causing great damages to aquaculture farms (Coats, 1999; Noga, 2012). It has the capacity to infest both elasmobranchs (cartilaginous fish) and teleosts (bony fish) (Lawler, 1980), but also shrimps and bivalves (e.g., the pacific oyster *Crassostrea gigas*), and there are reports of hyperparasitism of the fish parasite *Neobenedenia melleni* (Colorni, 1994; Aravidan, 2007; Sousa, 2015). *A. ocellatum* causes serious morbidity and mortality in both brackish and marine warmwater fishes farmed in aquaculture facilities worldwide (Noga, 2012). It rarely causes epidemics in wild populations; the best documented outbreak was in fish of a hypersaline inland lake (Salton Sea) in Eastern California, USA (Kuperman & Mattey, 1999). *A. ocellatum* can appear in different types of rearing tanks and systems, like salt pans, semi-intensive earthen ponds, cement tanks, fibre glass tanks and even in cages (Rigos *et al.,* 1998; Noga, 2012.) The disease, amyloodiniosis, is a major bottleneck for semi-intensive aquaculture production in Southern Europe because it affects most fish farms, causing extremely high mortalities (Soares *et al.*, 2011). An economical loss of near 4000 € was estimated for an outbreak in an earth pond in Portugal with gilthead seabream (*Sparus aurata*) and meagre (*Argyrosomus regius*) (Shinn *et al.,* 2015). Climate change can also become a potential stressor, since higher temperatures favour the propagation of the parasite and could lead to more outbreaks in aquaculture farms (Reid *et al*., 2019).

1.2 Biological cycle of *A. ocellatum*

A. ocellatum has a direct, but triphasic life cycle that can be completed in less than a week in favorable environmental conditions (Noga, 2012). Trophonts are spherical or oval and live attached to the gills or skin of fish (Figure1B and 1C). They are normally 20-120 µm in length just prior to detachment from the host (Paperna, 1984b). Trophonts remain attached to the fish by root-like structures (rhizoids) that firmly anchor the parasite to the epithelium. After reaching maturity, trophonts gradually detach from the host (Masson, 2009). After detachment, rhizoids retract within 2 to 5 min and a solid body wall is formed, transforming the parasite into a cyst, the tomont (Figure 1D to 1G). Tomonts start dividing consecutively within 3-6 h of detachment, giving rise to a maximum of 256 tomites (Masson, 2009). In the last division, each tomite divides to form two dinospores that rupture the membrane and swim away (Figure 1A).

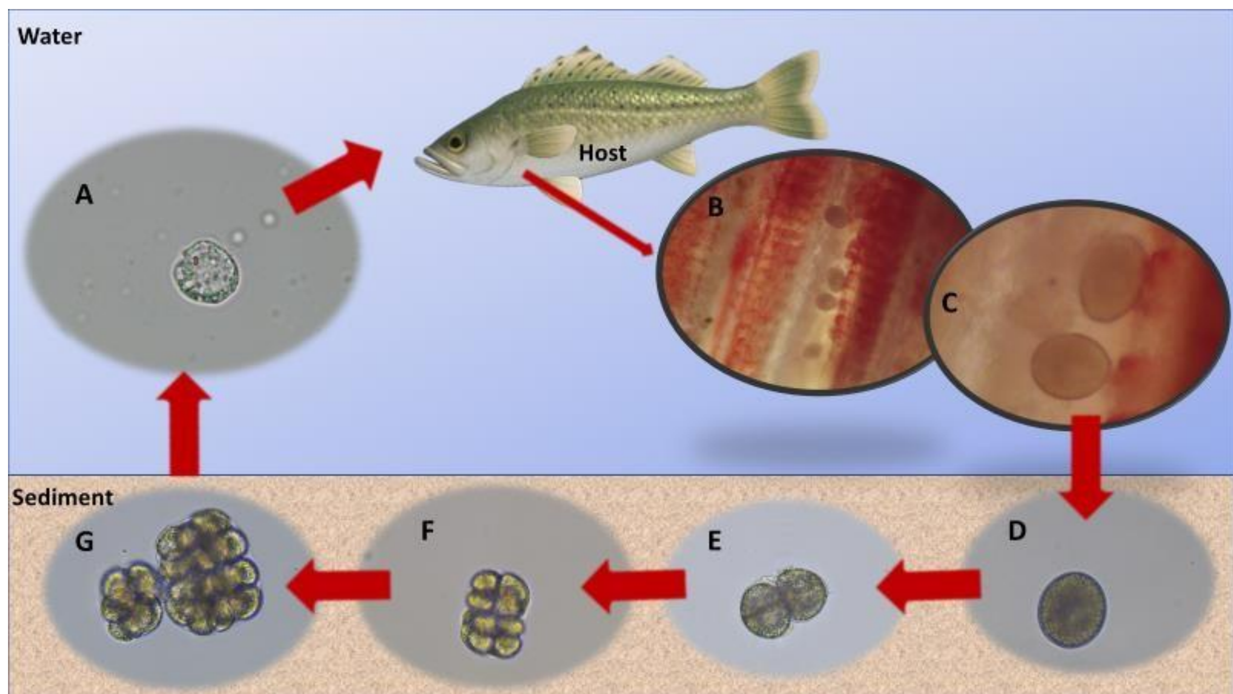

Figure 1. Biological cycle of *Amyloodinium ocellatum.* **A** - free-living dinospore (1000x), **B** and **C** - trophonts in fish gills (100x and 400x), **D** to **G** several development stages of tomonts (400x).

The number of divisions before sporulation are related to the initial size of the trophonts. Large trophonts undergo more divisions and produce more dinospores (Paperna, 1984b). Dinospores constitute the free-swimming, infective stage in the life cycle of *A. ocellatum.*They have a body of approximately 12-15 µm long and 8-14 µm wide that contains two flagella, one transversal and one longitudinal, backwardly directed (Landsberg *et al*., 1994). Once dinospores attach to a fish, they transform into trophonts. The presence of a red stigma or eyespot in all three life stages is characteristic of *A. ocellatum,* which trivial name is derived from this feature (Masson, 2009).

1.3 Environment adaptation of *A. ocellatum*

Temperature and salinity are the primary environmental modulators of *A. ocellatum* pathogenicity, with greater virulence at higher temperatures (Kuperman & Matey, 1999; Paperna, 1984b). In temperate regions, infection by *A. ocellatum* is therefore a problem only during warmer months (Soares, 2011). Geographic isolates vary greatly in temperature and salinity tolerance, with tolerance appearing to reflect the environmental conditions. Optimal temperature ranges for germination are from 23 to 28°C and germination normally stop at temperatures below 15°C (Paperna, 1984b). *In vitro* tests with Red Sea isolates (a high salinity sea) showed that *A. ocellatum* was able to complete divisions at 10-60‰ salinity. Bellow 10‰ division was not complete (Paperna, 1984b). In contrast, isolates from the estuarine regions can cause epidemics at 3‰ salinity (Lawler, 1980). Temperature can affect salinity tolerance, which usually narrows as one deviates further from the optimal temperature range. However, *A. ocellatum* of the Salton Sea was more pathogenic when temperatures were very high (39-41°C), even though the salinity was also very high (46‰). There are also evidences that low dissolved oxygen may be associated with outbreaks of some epidemics (Kuperman & Matey, 1999).

1.4 Pathogenicity and symptomatology of *A. ocellatum*

Clinical signs of amyloodiniosis include changes in fish behaviour, with jerky movements, swimming at the water surface, anorexia, depression, dyspnea and pruritis (Lawler, 1980; Soares, 2011). The gills are usually the primary site of infestation, but heavy infestations may also involve the skin, fins, eyes, and oro-pharyngeal cavity (Byadgi *et al.*, 2019) (Figure 2). Heavily infested skin may have a dusty appearance, consequently the disease is sometimes called 'velvet disease', but this is an uncommon finding and fish often die without obvious gross skin lesions (Levy, 2007). Fish with infected skin may display signs of 'flashing' or rubbing on tank walls, the substrate, or other structures in their environment.

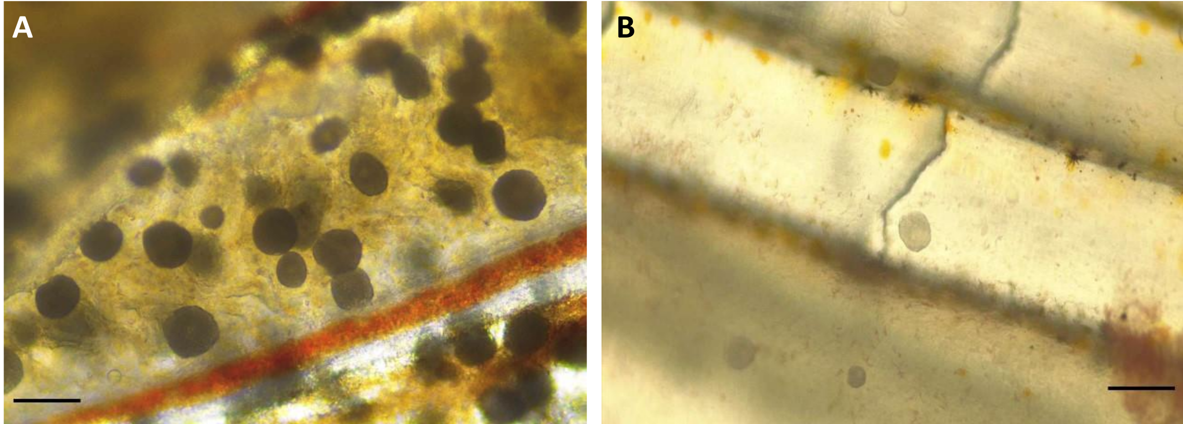

Figure 2. Trophonts of *Amyloodinium ocellatum*. on fish gill lamella (A) or on fish fin. Scale bar = 100 µm

Feeding behavior likely will be poor and some fish may appear emaciated. The key lesion caused by *A. ocellatum* infection is the destruction of epithelialcells. Characteristic histological findings include gill hyperplasia, lamellar fusion, inflammation, haemorrhage, and necrosis (Saraiva *et al.*, 2011; Marques *et al.*, 2019; Byadgi*, et al.*, 2019). Death is usually attributed to anoxia and can occur within 12 h especially in cases of heavy infestation (Lawler, 1980) (Figure 3). Osmoregulatory impairment and secondary microbial infections due to severe epithelial damage may also be important causes of debilitation and death (Noga, 2012).

Figure 3. Fish mortality in earth pond caused by *Amyloodinium ocellatum*. Photo taken at EPPO-IPMA.

1.5 Diagnostic of amyloodiniosis

Diagnosis of amyloodiniosis relies on classical methods, i.e. the direct observation of clinical signs and microscopy, but molecular techniques, .e.g. the detection of the parasite by polymerase chain reaction (PCR), are increasingly used to confirm the initial diagnostic. Classical methods consist of a direct observation of the target tissues (fins, eyes, skin, and gills) using a microscope (Figure 4) or even by eye. In the latter case, parasites can be detected by inspecting the fish against a dark background or by indirect illumination (Noga, 2012). Smaller fish can be restrained in a dish and directly examined using a dissecting microscope. For a confirmative diagnostic, trophonts or tomonts must be observed attached to the epithelium. Since trophonts often detach shortly after host death, snips of living or recently dead fish are examined using a light microscopy (Noga, 2012). Baths with freshwater can also be used to dislodge the parasites from the tissues of the fish, and then perform an analysis of the deposit that is formed (Oestmann & Lewis, 1995; Noga, 2012). Another approach is the detection of the parasite presence on skin and gills with the use of dilute Lugol's iodine, which reacts with the starch vacuoles in *A. ocellatum*, colouring in brown (Massimo, 2019). Histopathology can also be used for diagnosis but the fact that trophonts can dislodge during fixation may difficult the assessment of the severity of the infestation (Noga, 2012). Molecular techniques, e.g. PCR and loop-mediated isothermal amplification (LAMP), were recently developed to detect *A. ocellatum* (Levy *et al*., 2007; Picón-Camacho *et al.*, 2013). These techniques are very sensitive and can detect the parasite even at very low concentrations, e.g. 10 dinospores/mL of water (Levy *et al.*, 2007). Thus, they can allow for a sensitive monitoring of pathogen load in susceptible fish populations (Noga, 2012; Soares, 2011). Immunological approaches such as ELISA (enzyme linked immunosorbent assay) can also detect specific antibodies in fish recovering from amyloodiniosis outbreaks or that have been experimentally exposed to the parasite (Cecchini *et al.,* 2001). This assay could be useful for monitoring levels of protection in susceptible populations, as elevated antibody titers have been associated with resistance (Cecchini *et al*., 2001). Finally, proteomic technology may contribute in the future to determine antigenic molecules of the parasite, which remain to be identified. Their identification could help in the development of targeted therapies or more effective prophylaxis (Massimo, 2019).

Figure 4. Microscopic diagnostic of *Amyloodinium ocellatum*. Photo taken at EPPO-IPMA.

1.6 Current knowledge on treatment and prevention of amyloodiniosis

A. ocellatum is a virulent parasite and infections must be treated as quickly as possible to avoid catastrophic consequences. The free-living dinospores are susceptible to chemical treatment, but trophonts and tomonts are more resistant (Soares, 2011). To date, copper sulphate is the most used chemical to control amyloodiniosis epizootic episodes (Noga, 2012, Soares, 2011). Free copper ion is the active component and its concentration should be maintained at 0.12-0.15 ppm for 10-14 days to control the infection (Noga, 2012). A combined influx of freshwater to reduce salinity to 10 ppt and a lower dose of copper sulphate (0.1 ppm) showed positive results in trials with the European sea bass *Dicentrarchus labrax* (Bessat *et al.*, 2018). Tomonts are more resistant to copper and can cope with concentrations 10 times higher than those incapaciting dinospores. However, high concentrations of copper should be avoided as it is toxic to fish, invertebrates, and algae. Also, free copper ion is unstable in sea water and levels should be monitored daily to maintain the correct levels (Noga, 2012).

Formalin (37% formaldehyde) is also used to control ectoparasites in fish culture. Flush treatment with 100-200 ppm of formalin for 6-9 h causes trophonts of *A. ocellatum* to detach from gills, but tomonts resume division after removal of the chemical (Paperna, 1984a). More recently, juvenile bullseye puffer fish (*Sphoeroides annulatus*) had the parasite load on the skin and gills significantly reduced upon treatment with 51 ppm of formalin for 1 h or 4 ppm for 7 h, . Fish were reinfested after 15 days but infection was controlled by repeating the treatment, reinforcing the notion that the chemical does not inactivate the tomont stage (Fajer-Avila *et al.*, 2003).

Hydrogen peroxide, H_2O_2, is an alternative and more ecological therapeutant for amyloodiniosis. In the flathead grey mullet *Mugil cephalus,* fry mortality due to amyloodiniosis declined dramatically within 3 d following a treatment with 25 ppm of hydrogen peroxide for 30 min. In the Pacific threadfin *Polydactylus sexfilis,* single treatments with hydrogen peroxide at 75 or 150 ppm for 30 min eliminated trophonts without affecting fish survival (Montgomery-Brock *et al.*, 2001). In the milkfish *Chanos chanos* and mangrove snapper *Lutjanus argentimaculatus* 1 h immersion in fresh water or 1 h immersion in 200 ppm hydrogen peroxide prior to return to clean sea water was equally effective in removing 100% of the trophonts (Cruz-Lacierda *et al.*, 2004). More recently, the treatment of European sea bass *D. labrax*, with 100 and 200 ppm of H_2O_2 for 30 min significantly decreased the number of trophonts observed on the gills (Seoud *et al.*, 2017). Hydrogen peroxide should be used within the therapeutic limits, as it could be lethal to the fish Finally, although hydrogen peroxide reduced fish death associated with amyloodiniosis, it is unknown if tomonts remain viable after the treatment (Montgomery-Brock *et al.*, 2001).
Peracetic acid, a stabilized mixture of acetic acid, H_2O_2 and water, is traditionally used as a disinfectant for agriculture, food processing, and medical and veterinary facilities, and more recently in wastewater treatment, commercial laundries and in aquaculture (Lieke *et al.*, 2020). It has been reported that treatment with 8 to 15 ppm of peracetic acid for 1 h was effective at killing all stages of the fish parasite *Ichthyophthirius multifiliis*, including cysts (Picón Camacho, 2010). Although supporting evidence are currently missing peracetic acid is seen as a good candidate for controlling amyloodiniosis. In addition, its degradation does not produce toxic residues (acetic acid is quickly metabolized by microorganisms), thus it could represent an environment friendly solution (Lieke *et al.*, 2020).
The application of the oral antimalarial chloroquine diphosphate was effective in controlling amyloodiniosis at a rate of 50 mg/kg body weight, in the red drum *Sciaenops ocellatus* (Lewis *et al.*, 1988). Through water baths, chloroquine diphosphate was also effective against dinospores at 5-10 ppm but did not influence tomont division (Noga, 2012; Ramesh-Kumar *et al.*, 2015). The water soluble ionophorous antibiotic, 3,N- methylglucamine lasalocid, was effective in reducing the tomont division rate. At concentrations higher than 0.001 ppm, motile dinospores did not excyst, and *in vitro* studies showed that trophonts infection was significantly reduced at 0.1 ppm and was eliminated at 1 ppm. Further tests with red drum fry exposed to dinospores showed a reduction of 80% in trophonts on gill filaments at a concentration of 0.1 ppm, and a total prevention at 1 ppm (Oestmann & Lewis, 1996a).

In a recent study,tomatine, a glycoalkaloid derived from the tomato *Solanum lycopersicum*, and 2',4'- dihydroxychalone, isolated from the plant *Zuccagnia punctata,* showed promising results in inactivating the activity of the dinospores. No effect was registered on the cystic form (Tedesco *et al.*, 2020). Nauplii of the brine shrimp *Artemia salina* were also tested as a biological mean to reduce dinospores. In tanks infected with dinospores and stocked with *A. salina* nauplii, red drum had 65% fewer trophonts in their gills than those from tanks without nauplii (10.75 vs 3.75 trophonts/filament). *In vitro* studies also showed that dinospores were eliminated after 20 h in the presence of 1 nauplii for every 2500 dinospores and after 8 h in the presence of 1 nauplii for every 1250 dinospores (Oestmann *et al.*, 1995). The practical application of this method has not been demonstrated yet.

1.7 Control of amyloodiniosis in earth ponds

As previous referred, tomonts of *A. ocellatum* can settle at the bottom of tanks and ponds and resist for long periods of time maintaining the ability to reinfest. Biosecurity in ponds is changeling, due to the size of the aquaculture farms and because they are normally located near natural water reservoirs, which limits the application of aggressive chemical treatments (Yanong, 2013). Cleaning the organic matter and drying the earth pond are consider essential between crops, but in some regions, drying may not be possible due to the weather. Disinfection of bottom pond was achieved with different chemical and physical techniques (Boyd, 2003; OIE, 2009). The application of quicklime (calcium oxide) or active chlorine to bottom earth ponds are common practices. Since chlorine is highly toxic to aquatic animals, it is recommended to neutralize it with sodium thiosulphate before any discharge (OIE, 2009). No information is however available on the effect of quicklime and active chlorine on the cystic form of *A. ocellatum*. Methods using UV light or microwave are promising, for soil disinfection although supporting evidence still remain to be collected can potentially became available for agriculture and experiments should be taken to understand its potential for parasite inactivation in soils (Mun *et al*., 2009; Desogus *et al.*, 2016).

2 Objectives

- Develop and optimize an infection system for propagating and monitoring the dinoflagellate *A. ocellatum;*
- Identify the proper temperature preserve the tomont stage of A. ocellatum without jeopardizing viability or initiate sporulation.
- Register the morphology of each life stage of the parasite *A. ocellatum* through scanning electron microscopy (SEM);
- Determine the best method to inactivate the cystic form of *A. ocellatum*, through a series of *in vitro* chemical and physical tests, and propose a novel set of measures to help prevent the propagation of the parasite in earth ponds.

3 Materials and methods

3.1 Collection of *Amyloodinium ocellatum*

During the summer of 2020, adult specimens of European sea bass *Dicentrarchus labrax* and gilthead seabream *Sparus aurata*, were regularly sampled from earth ponds as part of the biosecurity program established at the EPPO-IPMA. Gills were observed microscopically (40-100x magnification) for the presence of parasites and infected gills were collected for parasite recovery. For this, gills were placed in a 250 mL transparent glass jar with 100 mL of distilled water (Autostill 4000x) at room temperature, then gently stirred for 1 min. Water was filtered on a 200-µm nylon mesh to remove large debris, then on a 30-µm nylon mesh to collect the trophonts. Parasites were collected in a glass jar by washing the 30 µm nylon mesh sterile seawater sterilized (SSW, autoclaved at 120ºC for 20 min). Washing and filtering processes were repeated 3 times and finally tomonts were collected into 15-mL tubes. Tomonts were counted using a Sedgwick-Rafter chamber under a Leica DMIL LED inverted microscope (100x magnification) equipped with phase contrast. Collected parasites were used for initial tests and to seed the infection system.

3.2 Infection system

The infection system consisted of a 600-L fibre glass tank filled with 500 L of sand- filtered seawater containing 35‰ of salt, thermostatted at 24ºC, and maintained under constant aeration. A total of 15000 tomonts collected from earth ponds of EPPO-IPMA were used to initiate the infection system. Bioballs previously used for *A. ocellatum* infection experiments were also included in the tank. Six adult European sea bass *Dicentrarchus. labrax*, (83.67±4.03 g, 21.63±0.38 cm) reproduced and grown at EPPO-IPMA were introduced into the infection system. Monitorization of the infection was done by controlling the concentration of dinospores in the water (Abreu *et al.*, 2005). Briefly, 10 mL of water from the infection system was collected into 15 mL tubes supplemented with 20 µL of Lugol's solution and centrifuged at 160 *g* for 10 min in a Hermle Z383K centrifuge. The top 9 mL of the supernatant were discarded, and the bottom 1 mL was used for counting dinospores in a Segwick-Rafter chamber. Tomonts were collected when the concentration of dinospores in the water reached 10 dinospores/mL and following a protocol adapted from Bower *et al.* (1987). Briefly, infected fish were placed into a container with 2 L of freshwater (0‰ salinity) for 1 min. Then 2 L of filtered seawater (35‰ salinity) was introduced, which level up the salinity to 17.5‰, and fish was maintained into the container for an additional 1 min before fish were returned to the

infection tank. The water in the container was filtered with a 200-µm nylon mesh to remove large debris and a 30-µm nylon mesh to collect the parasites. Parasites were washed 3 times with sterile seawater (35‰) and collected into 15 mL tubes. Tomonts was counted in a Sedgwick-Rafter chamber as described in section 3.1, and concentration of tomonts was adjusted to 2500 tomonts/mL. Antibiotics (100 UI of penicillin and 0.1 mg/mL of streptomycin; Sigma-Aldrich) were also included. Tomonts were then used for preservation tests. The infection system was maintained functional until preservation tests were concluded.

3.3 Preservation tests

To perform the inactivation tests during the winter/spring period without the need to maintain the infection system functional for long periods, attempts were made to conserve the tomonts at a reduced temperature. Two experiments were performed with different objectives. Low temperature (5ºC) to evaluate the capacity of resistance of tomonts to low temperature and medium temperature (13-15ºC) to evaluate if tomonts could be stored at higher temperatures without starting the division process.

3.3.1 Low temperature refrigeration (5ºC)

Tomonts were placed into a Hotpoint SB 1801 AA fridge at 5ºC. Short (24h-48h) and long (21d-30d) periods of preservation were tested. At appropriate times, 20 µL of the refrigerated tomonts were diluted in 180 µL of sterile seawater (35‰), placed in a 96-well plate (flat bottom) and incubated at 23ºC in a refrigerated incubator (WWR INCU-LINE IL 53), following the protocol developed by Paperna (1984b). Sporulation of the tomonts was evaluated every 24 h till emergence of dinospores, with a Leica DMIL LED inverted microscope (100x magnification).

3.3.2 Medium temperature refrigeration (13ºC and 15ºC)

Tomonts were placed into a Electronia HS-125WEN fridge at 13ºC or15ºC (Figure 5A) and sampled as described above. Division of the tomonts was evaluated every 24h with a Leica DMIL LED inverted microscope (40x magnification). Temperature fluctuation on the fridge was registered with a Traceable memory thermometer (VWR) (Figure 5B).

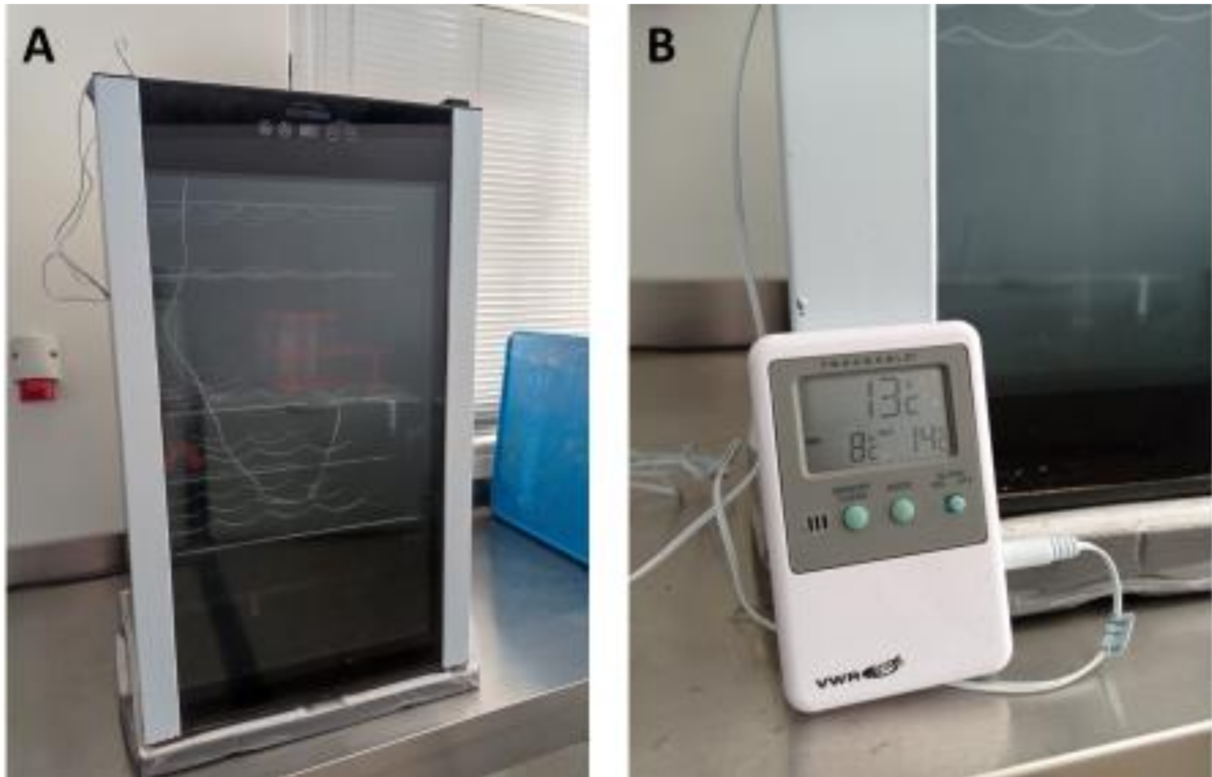

Figure 5. Equipment used for the medium temperature refrigeration (13ºC and 15ºC). (A)- HS-125WEN fridge (Electronia); (B)- Trace memory thermometer (VWR).

3.4 Scanning electron microscopy

The morphology of the different stages of the parasite *A. ocellatum* was determined by scanning electron microscopy (SEM), following a protocol adapted from Gómez-Lizárraga *et al.* (2019). To obtain different development stages, tomonts were incubated for 96 h, 72 h, 48 h and 24 h in a 96-well plate (flat bottom) at 23ºC.At appropriate times, tomonts were transferred with a micropipette to 1.4 mL of 2% glutaraldehyde (buffered with 0.1 M sodium cacodylate) into a 2 mL centrifuge tube. After 90 min at room temperature, microtube was centrifuged at 160 *g* for 10 min, and supernatant was discarded. Tomonts were washed 3 times with 1.5 mL of distilled water to prevent the formation of NaCl crystals then dehydrated using increasing ethanol series (10%, 30%, 50%, 70%, 90% and 99%). In the final step tomonts were gently suspended and 50 µL of the final suspension were incorporated in each stub with double carbon tape. Preparations were left to dry for 12 h and tomonts observed under a scanning electron microscope (Hitachi TM4000 Plus).

3.5 **Inactivation tests**

3.5.1 Chemical tests

Chemical compounds to be tested were selected based on their usage in aquaculture and interest for parasite control programs. Copper sulphate ($CuSO_4.5H_20$ 25% Cu), was purchased from Cuprosulf Vallés, burnt lime (CaO), was purchased from Quimirraia, formalin (formaldeheyde at 37%) was purchased from Sigma Aldrich, sodium hypochlorite (NaOCl at 13%) was purchased from SPC Pro, hydrogen peroxide (H_2O_2 at 35% was purchased from Sigma Aldrich,Virkon S was purchased from Dupont, Ox-Virin (Grupo OX) was kindly provided by TLH Lda. Incubation was performed in 96-well plates (flat bottom) at 23ºC in a refrigerated incubator (WWR INCU-LINE IL 53). Tomonts were continuously exposed to selected chemical for 72h. To simulate bath treatment, tomonts were also exposed to the chemicals for short periods (1 h, 2 h and 4 h). All tests were done in triplicate and included a negative control. A summary of the tests is presented in Table 1.

3.5.1.1 Copper (Cu)

Stock solutions of copper sulphate pentahydrate ($CuSO_4.5H_2O$) were prepared at 1, 10 and 100 ppm of Cu in SSW. Then 200 µL of the preserved tomonts (2500 tomonts/mL) were added to a 2 mL microtube supplemented with 800 µL of the Cu solution plus sterile sea water (SSW) to achieve a final concentration of 0.1, 1 and 10 ppm of Cu. Incubated at 23ºC for the selected time of exposure (1 h). For the 72 h continuous exposure, the incubation was done directly in the 96 well plate with 40 µL of tomont plus a mixture of copper stock solution and SSW. At appropriate times, tomonts were washed by adding 1000 µL of SSW and centrifuged at 200 *g* for 10 min. Top part of the supernatant (1800 µL) was removed and tomonts were washed again with 1800 µL of SSW. After 3 washes, tomonts were resuspended in a final volume of 800 µL of SSW, distributed in a 96-well plate (200 µL per well, in triplicate) and incubated at 23ºC.

3.5.1.2 Formalin

Stock solutions of formalin (37% formaldehyde) were prepared at 100, 1000 and 10000 ppm in SSW. Inactivation tests followed the protocol described in section 3.4.1.1 and formalin was added to achieve concentrations of 10, 100, 250, 500, 750 and 1000 ppm. Time of exposure to formalin was increased from 1 h to 2 h and 4 h for the minimum concentration with total inactivation on the continuous exposure. Removal of formalin was performed as described in 3.4.1.1. Additionally, 200 µL of tomonts exposed to 250, 500 and 750 ppm of formalin for 1h at 23ºC

were used for motility tests.

3.5.1.3 Sodium hypochlorite

Stock solutions of sodium hypochlorite (13% active chlorine) were prepared at 100, 1000 and 10000 ppm of active chlorine in SSW. Tomonts were exposed to sodium hypochlorite at 10, 100, 250, 500, 750 and 1000 ppm and incubated following the protocol described in 3.4.1.2.

3.5.1.4 Hydrogen peroxide

Stock solutions of hydrogen peroxide (35% H_2O_2) were prepared at 100, 1000 and 10000 ppm in SSW. Tomonts were exposed to hydrogen peroxide at 10, 100, 250, 500, 750 and 1000 ppm and incubated following the protocol described on 3.4.1.2. Additionally, 200 µL of tomonts exposed to 250, 500 and 750 ppm of H_2O_2 for 1h at 23ºC were used for motility tests.

3.5.1.5 Ox-Virin - Peracetic acid

Stock solutions of peracetic acid were prepared at 100, 1000 and 10000 ppm by diluting a commercial solution of peracetic acid 5% (Ox-Virin®, Grupo OX) in SSW. Tomonts were exposed to peracetic acid at 10, 100, 250, 500, 750 and 1000 ppm and incubated following the process described above in section 3.4.1.2. Additionally, 200 µL of tomonts exposed to 250, 500 and 750 ppm of peracetic acid for 1h at 23ºC were used for motility tests.

3.5.1.6 Calcium oxide (CaO)

A solution of 10% (w/v) CaO in distilled water was used to prepare stock solutions at 100, 1000, 10000 ppm in SSW. Since CaO acts as an alkaline agent, the pH of each solution was measured using the Fisher Brand Accumet AB150 pH Benchtop Meter. Tomonts were exposed to CaO at 10, 100, 1000 and 10000 ppm and incubated following the protocol previous described in 3.4.1.2.

3.5.1.7 Virkon S

Virkon S is a commercial oxidizing disinfectant blend containing potassium peroxymono-sulphate, sodium hexametaphosphate, sodium alcylbenzenesulphonate, malic acid, sulphamic acid, sodium chloride, fragrance and an indicator dye. A solution of 10% (w/v) Virkon S in distilled water was used to prepare stock solutions of 100, 1000, and 10000 ppm in SSW. Tomonts were exposed to VirkonS at 10, 50, 100 and 1000 ppm and incubated following the

protocol previous described in 3.4.1.2. Additionally, 200 µL of tomonts exposed to 10 and 100 ppm of Virkon S for 1h at 23ºC were used for motility tests.

Table 1. Chemical tests performed to assess *Amyloodinium ocellatum* tomonts inactivation (each test was performed in triplicate and included a negative control).

Compound	Dose (ppm)	Exposure time (h)	Number of incubations
Copper sulphate (25% copper)	0; 0.1; 1; 10	1; continuous	24
Formalin (37% formaldehyde)	0; 10; 100; 250; 500; 750; 1000	1; 2; 4; continuous	84
Sodium hypochlorite (13% active chlorine)	0; 10; 100; 250; 500; 750; 1000	1; continuous	42
Hydrogen peroxide (35%)	0; 10; 100; 250; 500; 750; 1000	1; continuous	42
Ox-Virin (5% peracetic acid)	0; 10; 100; 250; 500; 750; 1000	1; continuous	42
Calcium oxide (10%)	0; 10; 100;1000;10000	1; continuous	30
Virkon S	0; 10; 50; 100; 1000	1; continuous	30

3.5.2 Physical tests

Tests were performed in triplicate and included a negative control. A summary of the tests is presented in Table 2.

3.5.2.1 Low salinity

SSW was diluted with distilled water to prepare water at a salinity of 5 and 10‰. Salinity was measured with a digital refractometer (HANNA Instruments, HI96822). 20 µL of tomont suspension was added to 180 µL of the low salinity water and incubated in a 96-well plate (flat bottom) at 23ºC. For 0‰ salinity, tomonts were first washed with distilled water to remove most of the salt. Briefly, 250 µL of tomont suspension was added to 9.75 mL of distilled water into a 15 mL centrifuge tube. After a centrifugation at 160 g for 10 min, 9.5 ml of the supernatant was removed, and pellet was resuspended in 0.5 mL of distilled water. Suspension was incubated at 23ºC into a 96-well plate (200 µL per well) for different times, then 150 µL of the hyposaline solution was replaced with an equivalent volume of SSW (35‰).

3.5.2.2 Desiccation

Aliquots (20 µL) of *A. ocellatum* were placed onto a concave microscope slide and left to dry at

room temperature. Slides were washed at 30 min, 1 h, 4 h and 24 h post-desiccation with SSW and the tomonts were recovered into a 15 mL tube then incubated into 96-well plates at 23ºC as described before.

3.5.2.3 Ultraviolet radiation

Exposition of the tomonts to ultraviolet C radiation (UVC) was conducted using a UV apparatus (P.W.Allen & Co. A425, 220-240 volts) equipped with a mercury lamp (Phillips TUV 8W), emitting nearly monochromatic UV radiation at 254 nm (Figure 6). Intensity of the light was set to 1.16 mW/cm^2, according to the data provided by the manufacturer. 20 µL of tomont suspension was added to a concave microscope slide and exposed beneath the apparatus. Exposure times were 10, 20, 30, 40, 50, 60, 90, 120 and 300 s. Each test was performed in triplicate. Recovery and incubation of tomonts was performed as described in the section 3.5.2.2.

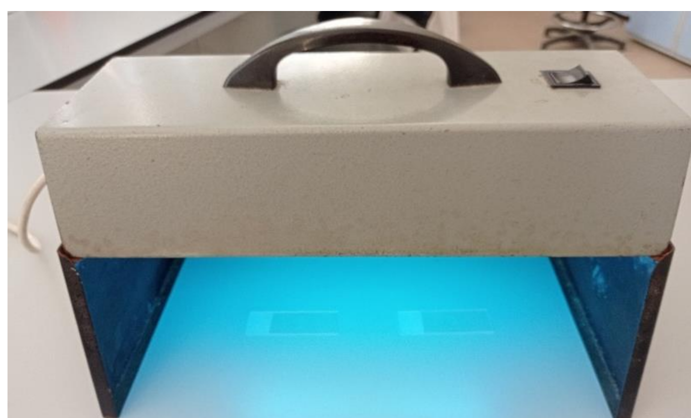

Figure 6. Ultraviolet apparatus (P.W.Allen & Co. A425, 220-240 volts) equipped with a mercury lamp (Phillips TUV 8W).

Table 2. Summary of physical tests for *Amyloodinium ocellatum* tomonts inactivation.

Test	Treatment/dose	Exposure time	Number of incubations
Hyposalinity	0‰, 5‰, 10‰, 35‰	1 h, 2 h, 4 h, continuous	48
Desiccation		30 min, 1 h, 4 h, 24 h	15
UVC	1.16 mW/cm^2	10 s, 20 s, 30 s, 40 s, 50 s, 60 s, 90 s,120 s, 300 s	30

3.6 Evaluation of tomonts inactivation

3.6.1 Sporulation analysis

Sporulation was monitored under a Leica DMIL LED inverted microscope equipped with phase contrast (100x magnification) at 24 h, 48 h and 72 h. Tomonts that did not undergo at least one division within the first 72 h were considered inactive. Division process was evaluated by total tomite production per tomont. Tomonts at intermediate stages of division were attributed the higher value of tomites of that division (Table 3).

Table 3. Classification of *Amyloodinium ocellatum* tomont division.

Division	Tomites/Tomont	Points attributed to tomites
0	1	1
1	2	2
2	3-4	4
3	5-8	8
4	9-16	16

3.6.2 Dinospore production

Dinospore concentration was assessed at 96 h of incubation. Dinospores were counted from the 96 plate well plate using a Leica DMIL LED inverted microscope (100x magnification). Non-motile dinospores in the bottom of the well were counted as dead dinospores. Then 10 µL of Lugol's solution was added to each well of the 96-well plate, and after total dinospores were counted after 10 min. Number of live dinospores was determined from the difference between total and dead dinospores.

3.6.3 Dinospore motility

Swimming behaviour of the dinospores was assessed with the OpenCASA software using ImageJ, following the protocol by Zhao *et al.* (2019). A sample of 20 µL was taken from the additional 200 µL of the chemical incubation and placed onto a concave microscope slide and observed using a Leica DMIL LED inverted microscope. Dinospore movements were recorded at 24.5 fps for 10 s at 100x magnification using a digital camera (Swift, SC1003). OpenCASA is a software developed by Alquézar-Baeta *et al*. (2019) that has a plugin to register motility parameters, based on previous works (Wilson-Leedy & Ingermann, 2007). Settings were adjusted to the dinospore size (Figure 7) and curvilinear velocity (VCL, µm/s), average path

velocity (VAP, μm/s), straight-line velocity (VSL, μm/s) and linearity (Lin, %) were automatically determined. Motility analyses were performed for formalin, H_2O_2, Ox-Virin and Virkon S.

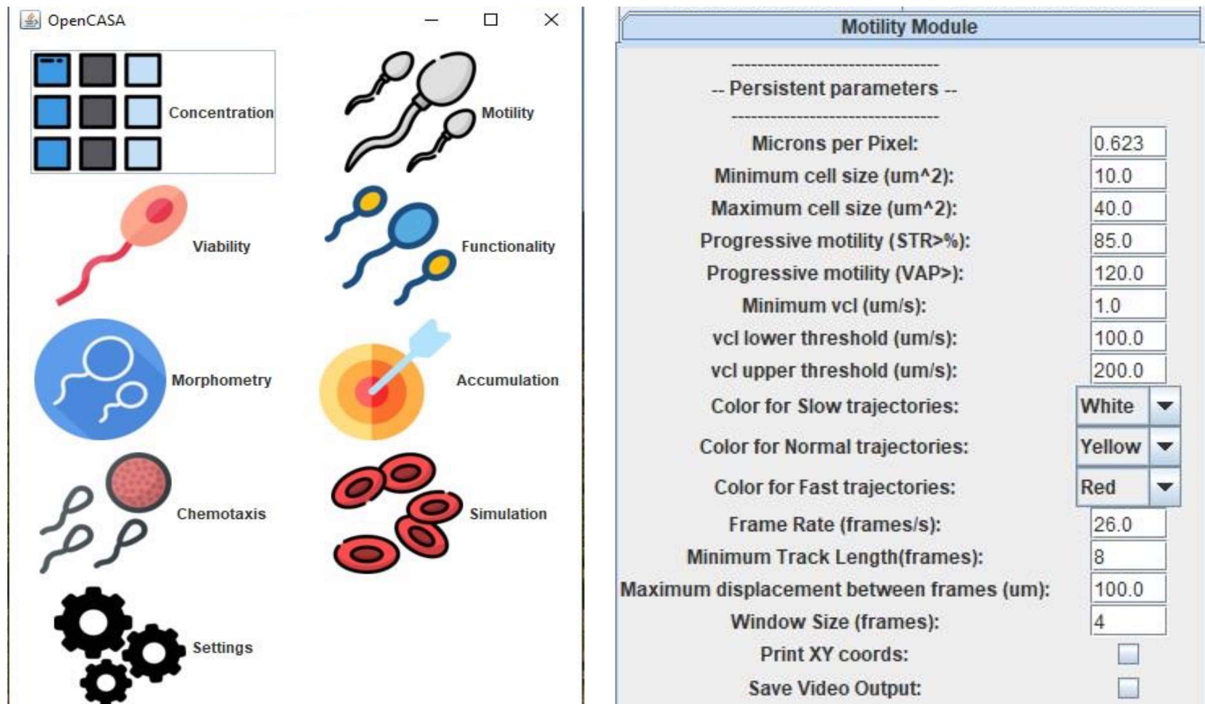

Figure 7. Settings of the motility module from the OpenCASA software for *Amylodinium ocellatum* dinospore motility analysis.

3.6.4 Statistical methods

Prior to analysis, normality assumption and homogeneity of variance were verified using Shapiro-Wilk's and Levene's tests, respectively. Data was expressed as mean ± standard deviation (SD). One-way ANOVA was performed to evaluate the effects of the treatments. The post-hoc Tukey-Kramer multiple comparison test was also performed to compare treatment groups to control. Differences with a p-value less than 0.05 were considered statistically significant. All statistical tests were performed using RStudio version 1.2.5001.

4 Results

4.1 Tomont production in the infection system

The infection system was maintained functional for 36 days. During this period, it was possible to recover a total of 282,000 tomonts (Figure 8). Dinospores were observed 4 days after the activation of the system, and a maximum of 800 dinospores/mL was obtained on day 29. Based on preliminary tests, dinospore levels higher than 100 dinospore/mL could lead to fish death. To keep dinospores level under control, 30% of the tank water was renewed on the same day of tomont collection. Infection was maximal on day 29 with 25666 tomonts/fish recovered. On this day, two fish eventually died. For this reason, 75% of the tank water was renewed to reduce the level of dinospores. On day 36, last tomonts were collected, and fish were euthanized. The system was maintained without thermostat, aeration and fish, for hibernation. The system was reactivated after preservations tests were concluded to produce tomonts for the inactivation tests.

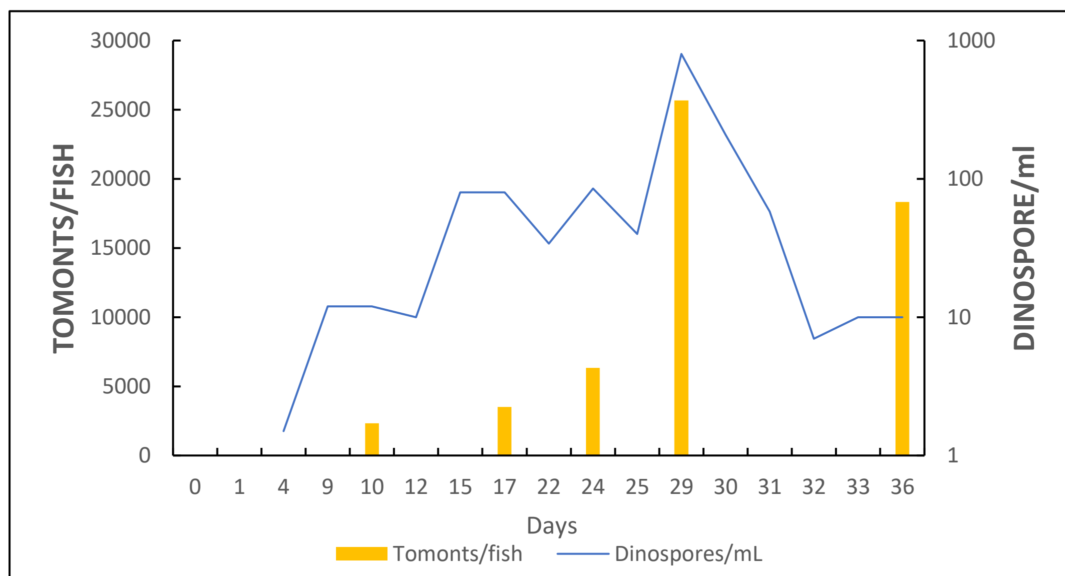

Figure 8. Production of *Amyloodinium ocellatum* dinospores and tomonts in the infection system.

4.2 Preservation tests

4.2.1 <u>Low temperature refrigeration (5ºC)</u>

Tomonts incubated at 5ºC (Figure 9) presented a reduction of their viability throughout time, ranging from 94.38% (T24h) to 59.33% (T30d). There was also a 24 h delay in the sporulation process, with more developed stages of tomonts appearing at 96 h in T21d and T30d, and at 72 h in T24h and T48h.

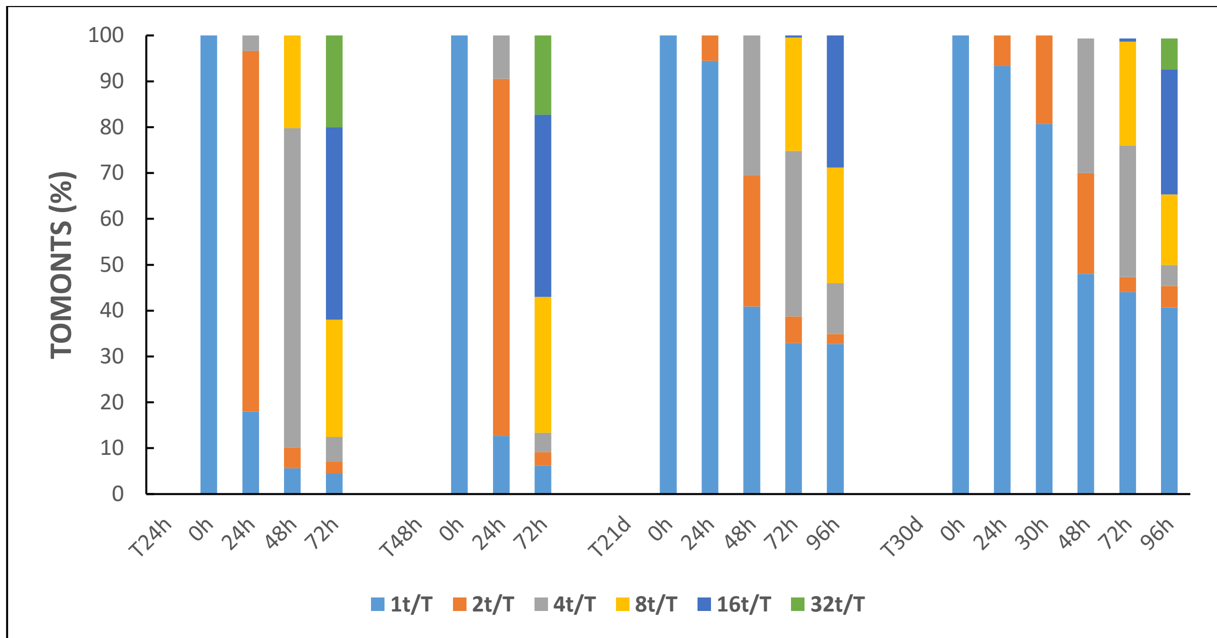

Figure 9. Viability of *Amyloodinium ocellatum* tomonts conserved at 5ºC and incubated at 23ºC for 24 h, 48 h, 21 d and 30 d. 1t/T, tomont at 1 tomite stage; 2t/T, tomont with 2 tomites; 4t/T, tomont with 4 tomites; 8t/T, tomont with 8 tomites; 16t/T, tomont with 16 tomites; 32t/T, tomont with 32 tomites.

4.2.2 Medium temperature refrigeration (13ºC and 15 ºC)

Developing tomonts were observed as soon as 96 h at 15ºC, while at 13ºC development could be delayed to after 96 h (Figure 10). At day 18 a large proportion of sampled tomonts were already in division. Temperature monitoring of the refrigeration showed a high variation in this period (7-18ºC).

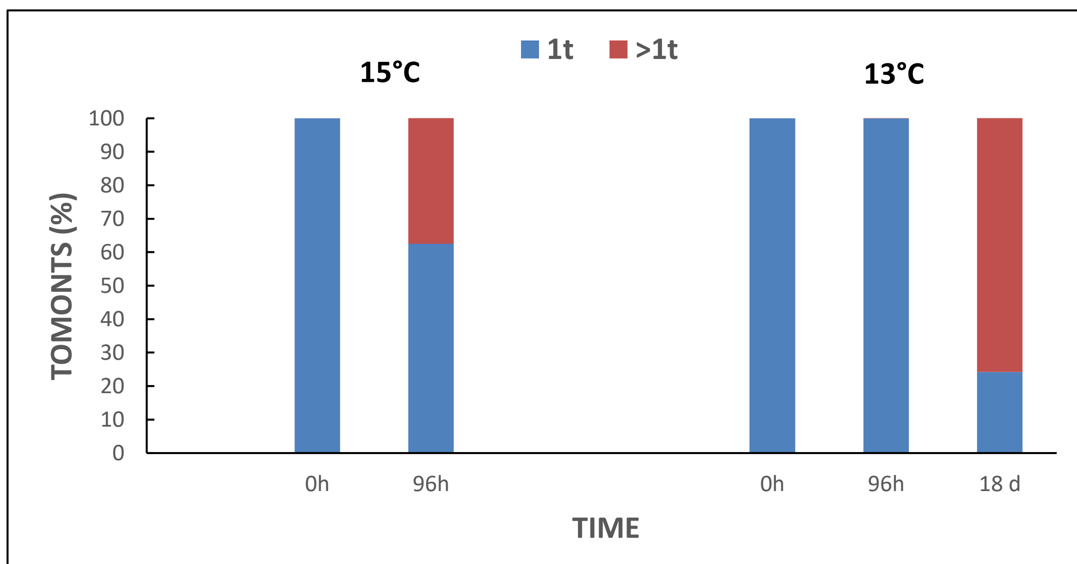

Figure 10. Development of *Amyloodinium ocellatum* tomonts preserved at 13ºC and 15ºC. **1t** indicates tomonts at a single cell stage, and **>1t** indicates tomonts with more than 1 division.

4.3 Scanning electron microscopy

Preliminary tests aimed at validating the protocol developed for scanning electron microscopy (SEM) and adapted from Gómez-Lizárraga *et al.* (2019). It was possible to generate high quality images of the various development stages of *A. ocellatum* (Figure 11A-G). Unfortunately, the image of most delicate form, the flagellate dinospore, revealed a very distorted structure (Figure 1H)

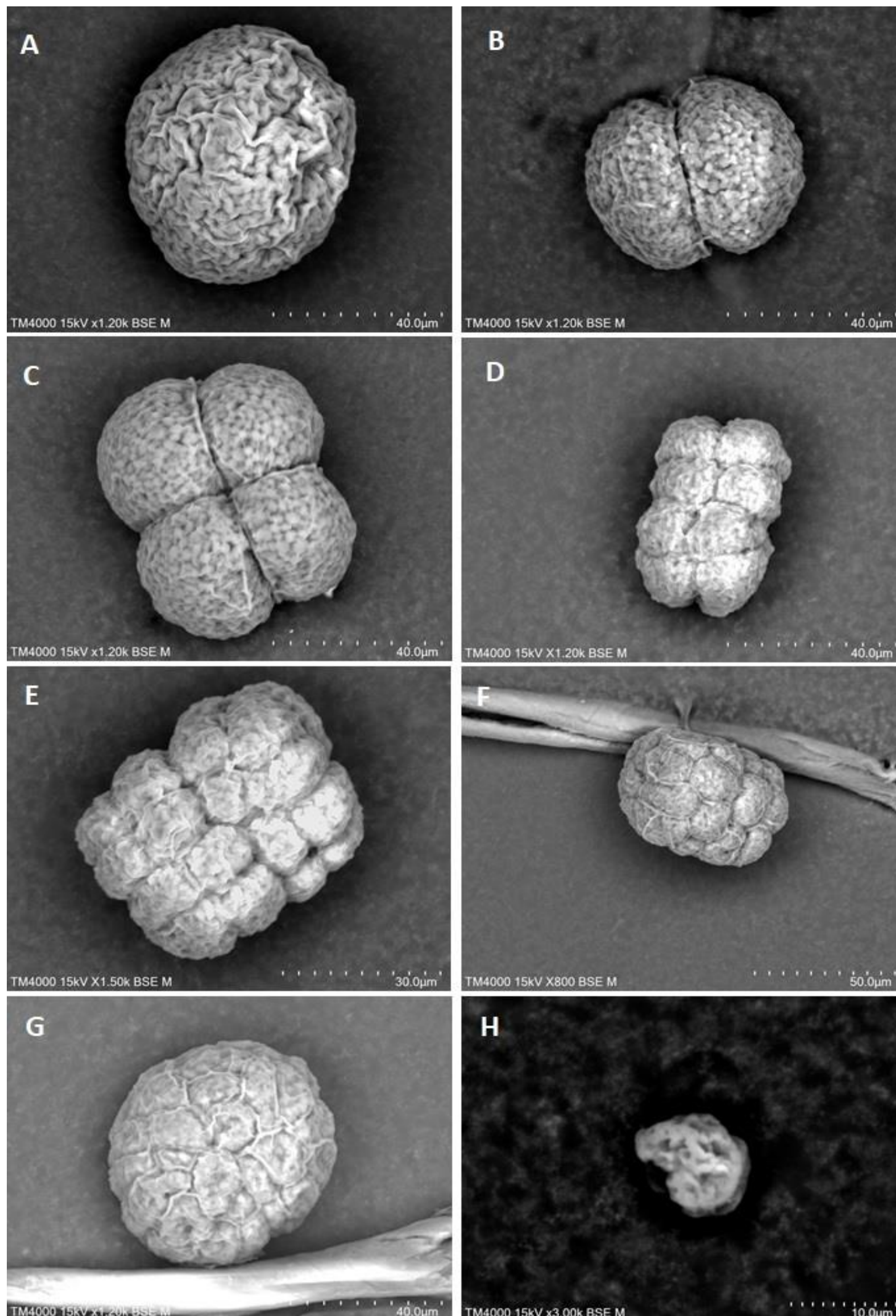

Figure 11. Micrographs of *Amyloodinium ocellatum* development stages by scanning electron microscopy. **A** – tomont; **B** - tomont on first division (2 tomites); **C** - tomont on second division (4 tomites); **D -** tomont on third division (8 tomites); **E** - tomont on fourth division (16 tomites); **F** - tomont on fifth division (32 tomites); **G -** tomont on sixth division (64 tomites); **H**–

dinospore. Scale bar: A, B, C, D, G = 40 µm, E = 30 µm, F = 50 µm and H = 10 µm.

4.4 Inactivation tests

4.4.1 Copper (Cu)

4.4.1.1 Sporulation

Continuous exposure for 72 h of the tomonts to Cu had a significant effect on their division after 48 h of incubation for all the concentrations tested (p-value < 0.001). The highest concentration (10 ppm) of Cu had the highest effect but did not totally impair tomont division (Figure 12A). Exposure of the tomonts to Cu for only 1 h also impaired tomont division after 48 h, although no difference was observed between the concentrations tested (Figure 12B). There was also a significant increase in the proportion of inactive tomonts upon the continuous exposure of the tomonts to 10 ppm of Cu (p-value = 0.008). Exposure to only 1 h resulted in similar results, with 10 ppm significantly increasing the proportion of inactive tomonts. Either treatment was not sufficient to completely inactivate tomonts (Figure 12C, D).

4.4.1.2 Dinospore production

Exposure of tomonts to Cu had a significant impact on total dinospore production even at the lowest concentration tested (0.1 ppm, p-value < 0.001). There was no registered alive dinospore at concentrations of 1 and 10 ppm. For 0.1 ppm dead dinospores were significantly higher than control (p-value = 0.00112) (Figure 12E). One hour exposure to the chemical had a significantly impact on dinospore production. At 1 ppm there was still live dinospores, but not at 10 ppm (Figure 12F).

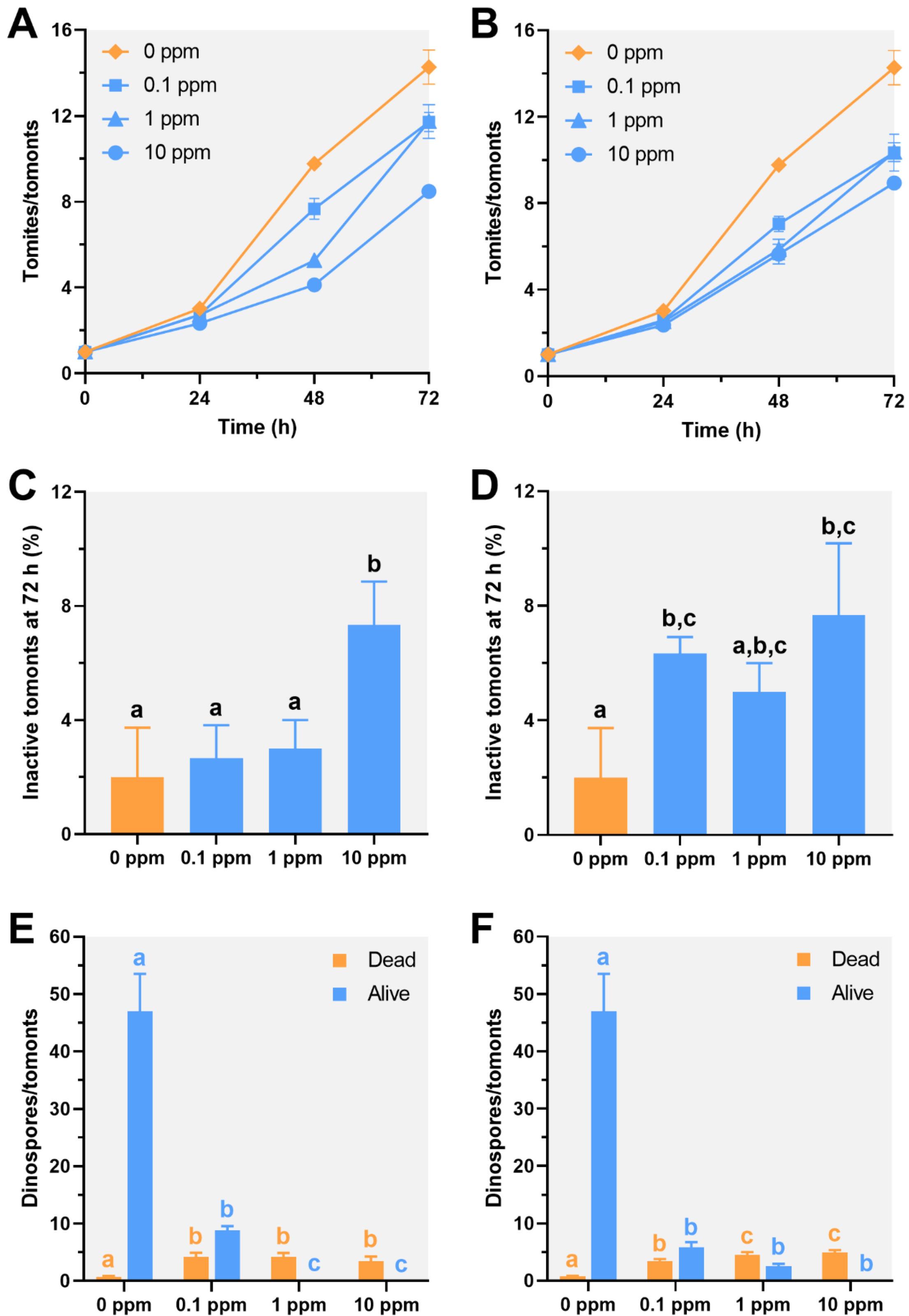

Figure 12. Inactivation of the parasite *Amyloodinium ocellatum* with copper (Cu). **A,B** - Time course of tomont division after a continuous (**A**) and 1 h (**B**) exposure to different Cu concentrations. **C,D** - Proportion of inactive tomonts after continuous (**C**) and 1 h (**D**) exposure. **E,F** - Dinospore production per tomont after continuous (**E**) and 1 h (**F**) exposure to different Cu concentrations. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

4.4.2 Formalin

4.4.2.1 Sporulation

Tomont division was significantly impaired upon a continuous exposure for 72 h to formalin even at a lower concentration (10 ppm, p-value < 0.001). Exposure to 100 ppm had a strong impact on tomont division, but only exposure to 250 ppm was sufficient to completely arrest the division process (Figure 13A). One hour of exposure to formalin at concentrations higher than 100 ppm resulted in impaired tomont division but only 1000 ppm was sufficient to completely arrest the division process (Figure 13B). Increasing the exposure time to 250 ppm formalin significantly lowered tomont division, with the lower tomite/tomont being achieved with 4 h exposure (p-value < 0.001) (Figure 13C). Concentrations higher than 100 ppm increased the number of inactive tomonts upon a continuous exposure. High levels of inactivation (95%) were achieved with 250 ppm (Figure 13D). Exposure for 1 h to the different formalin concentrations also induced tomont inactivation, but a strong inactivation was only obtained with 1000 ppm (99%) (Figure 13E). At least 2 h were needed to significantly increase tomont inactivation with a concentration of 250 ppm of formalin (Figure 13F).

4.4.2.2 Dinospore production

Dinospore production was significantly decreased at all concentrations, even at the lowest concentration tested (10 ppm, p-value < 0.001). Continuous exposure for 72 h to 250 ppm prevented dinospore production (Figure 13G). One hour exposure had a lower impact on dinospore production. Only concentrations higher than 750 ppm totally impaired dinospore production (Figure 13H). Malformations of the dinospores were observed after exposure of the tomonts to formalin for 1 h (Figure 14). These dinospores failed to complete the final division and were released in pairs.

4.4.2.3 Dinospore motility

Dinospores produced after a 1 h exposure of the tomonts to 250, 500 and 750 ppm of formalin had significant lower velocity parameters than control dinospores. Straight line velocity (VSL), circulinear velocity (VCL) and average path velocity (VAP) for 250, 500 and 750 ppm were significantly lower than control values (p-value < 0.00001). Linearity (LIN) was also significantly higher for control dinospores (p-value < 0.0001). No difference was observed among the different concentrations of formalin (Figure 13I).

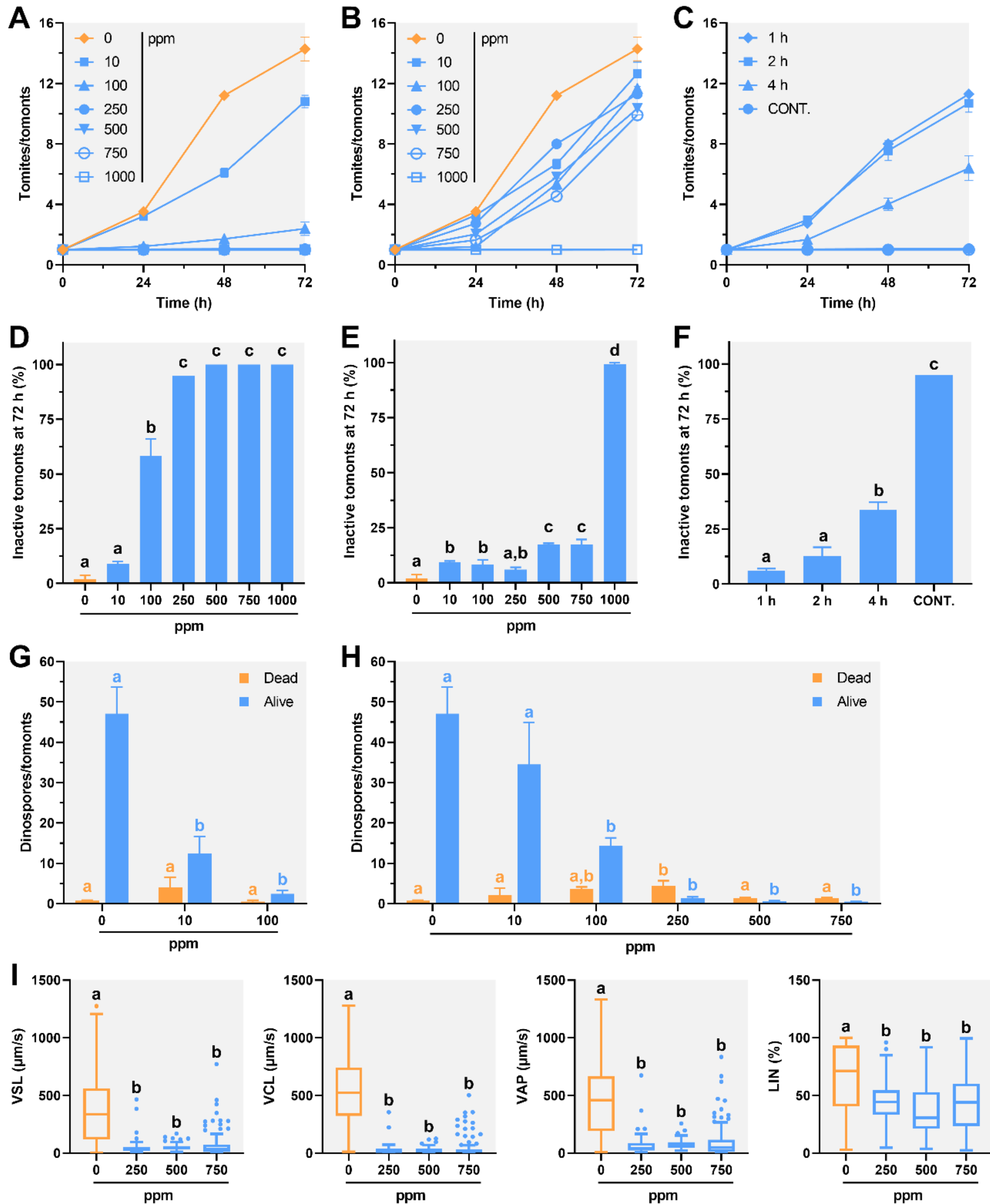

Figure 13. Inactivation of the parasite *Amyloodinium ocellatum* with formalin. **A**,**B** - Time course of tomont after continuous (**A**) and 1 h (**B**) exposure to different formalin concentrations; **C** - Time course of tomont division after different time of exposure to 250 ppm formalin; **D**,**E** - Proportion of inactive tomonts after continuous (**D**) and 1 h (**E**) exposure to different formalin concentrations; **F** - Proportion of inactive tomonts after different time of exposure to 250 ppm formalin; **G**,**H** - Dinospore production per tomont after continuous (**G**) and 1 h (**H**) exposure to formalin; **I** - Motility parameters of dinospores produced from tomonts exposed for 1 h to different concentrations of formalin. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

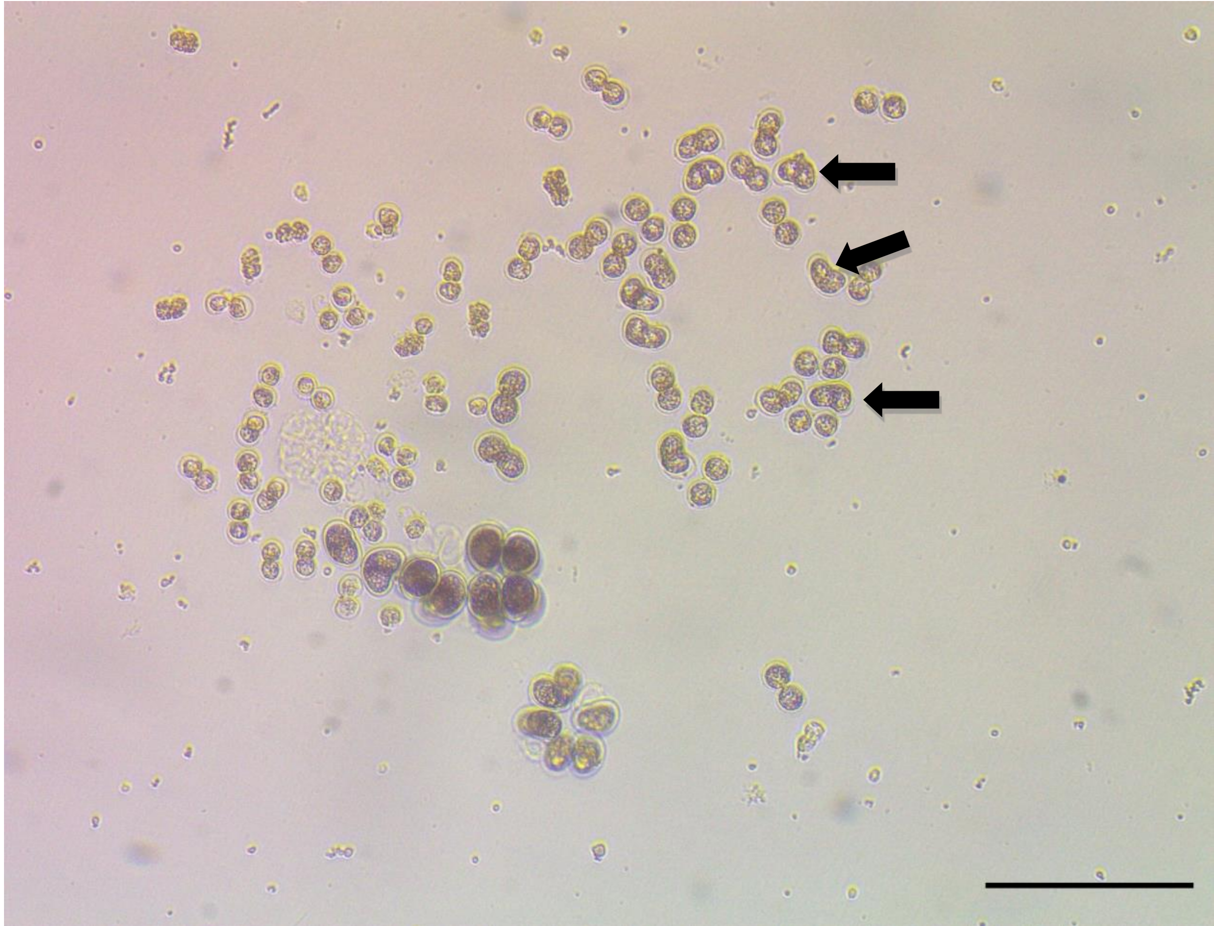

Figure 14. *Amyloodinium ocellatum* dinospores produced from tomonts exposed for 1 h to 250 ppm formalin. Black arrows indicate dinospores with malformations. Scale bar = 100 µm.

4.4.3 Sodium hypochlorite

4.4.3.1 Sporulation

A continuous exposure for 72 h to sodium hypochlorite showed to be effective in reducing tomont division at concentrations as low as 10 ppm, and to completely arrest the division process at 250 ppm (Figure 15A). Exposure for 1 h to the same concentrations revealed a similar trend, with 10 ppm being effective at limiting tomont division and 250 ppm arresting completely the division process (Figure 15B). Tomont inactivation was significantly higher after a continuous exposure to concentrations higher than 100 ppm (p-value < 0.00001) (Figure 15C). Lower concentration (10 ppm) had no impact on tomont inactivation (p-value = 0.990). Similar results were observed for an exposure to the same concentrations for 1 h (Figure 15D).

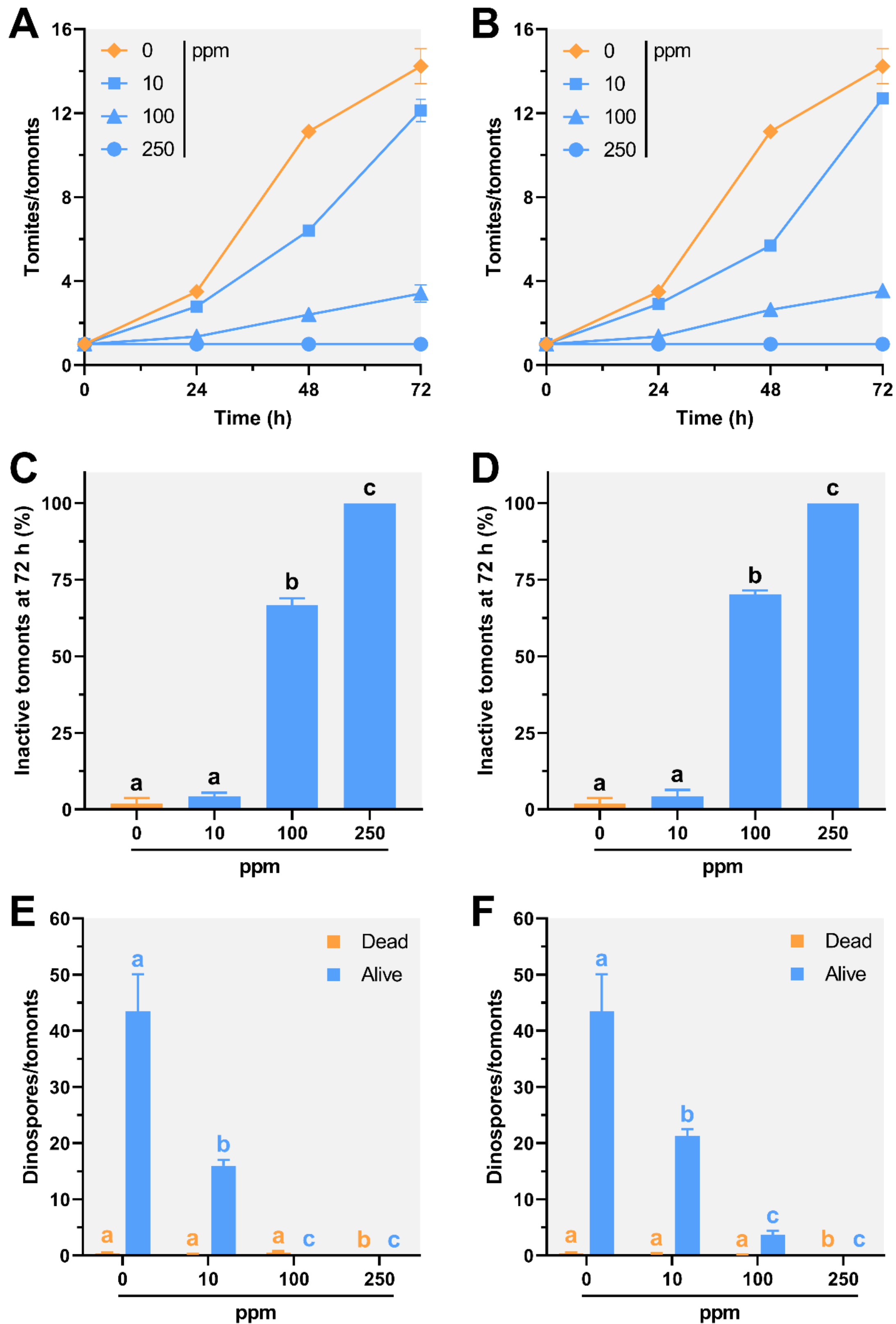

Figure 15. Inactivation of the parasite *Amyloodinium ocellatum* with sodium hypochlorite. **A**,**B** - Time course of tomont division after a continuous (**A**) and 1 h (**B**) exposure to different sodium hypochlorite concentrations; **C**,**D** - Proportion of inactive tomonts after continuous (**C**) and 1 h (**D**) exposure; **E**,**F** - Dinospore production per tomont after continuous (**E**) and 1 h (**F**) exposure to different sodium hypochlorite concentrations. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

4.4.3.2 Dinospore production

Continuous exposure for 72 h to sodium hypochlorite had a significantly effect on total dinospore production even at the lowest concentration tested (10 ppm, p-value < 0.0001). Complete interruption of dinospore production was achieved with continuous exposure to 250 ppm (Figure 15E). In comparison. 1 h exposure showed higher dinospore production but was still significantly lower than control. Exposure to concentration of 250 ppm for 1 h interrupted dinospore production (Figure 15F).

4.4.4 Hydrogen peroxide

4.4.4.1 Sporulation

Tomont division was inhibited upon a continuous exposure for 72 h to concentrations equal or higher to 100 ppm (p-value < 0.0001) and completely arrested at 1000 ppm (Figure 16A). Division was also interrupted at 48 h at 500 and 750 ppm. On the contrary, 10 ppm had no significant impact on tomont division (p-value $= 0.0980$; Figure 16A). Exposure for only 1 h to the same concentrations had a much lower impact on tomont division (Figure 16B) and only 1000 ppm significantly lowered tomont division. A total inactivation of the tomonts was achieved with the highest concentration tested (1000 ppm), although a significant inactivation was obtained with the lowest concentration (10 ppm, p-value $= 0.005$) (Figure 16C). Similar results were obtained with only 1 h of exposure to the same concentrations (Figure 16D).

4.4.4.2 Dinospore production

Dinospore production was significantly lower upon a continuous exposure for 72h to hydrogen peroxide even at the lowest concentration (10 ppm, p-value < 0.001). Concentrations of 250 ppm or higher were able to interrupt dinospore production (Figure 16F). Despite significantly lower, a 1 h exposure to the chemical still impacted dinospore production. The highest concentration could not interrupt dinospore production (Figure 16G).

4.4.4.3 Dinospore motility

Dinospores produced from tomonts exposed for 1 h to hydrogen peroxide had a significant lower VSL and VCL (Figure 16I and Figure 17). The effect was stronger at 750 ppm (p-value < 0.001). At 250 ppm, VAP was not affected in comparison with control value. Significant differences with control value were observed for LIN at 250 and 750 ppm, but no difference was observed at 500 ppm of hydrogen peroxide.

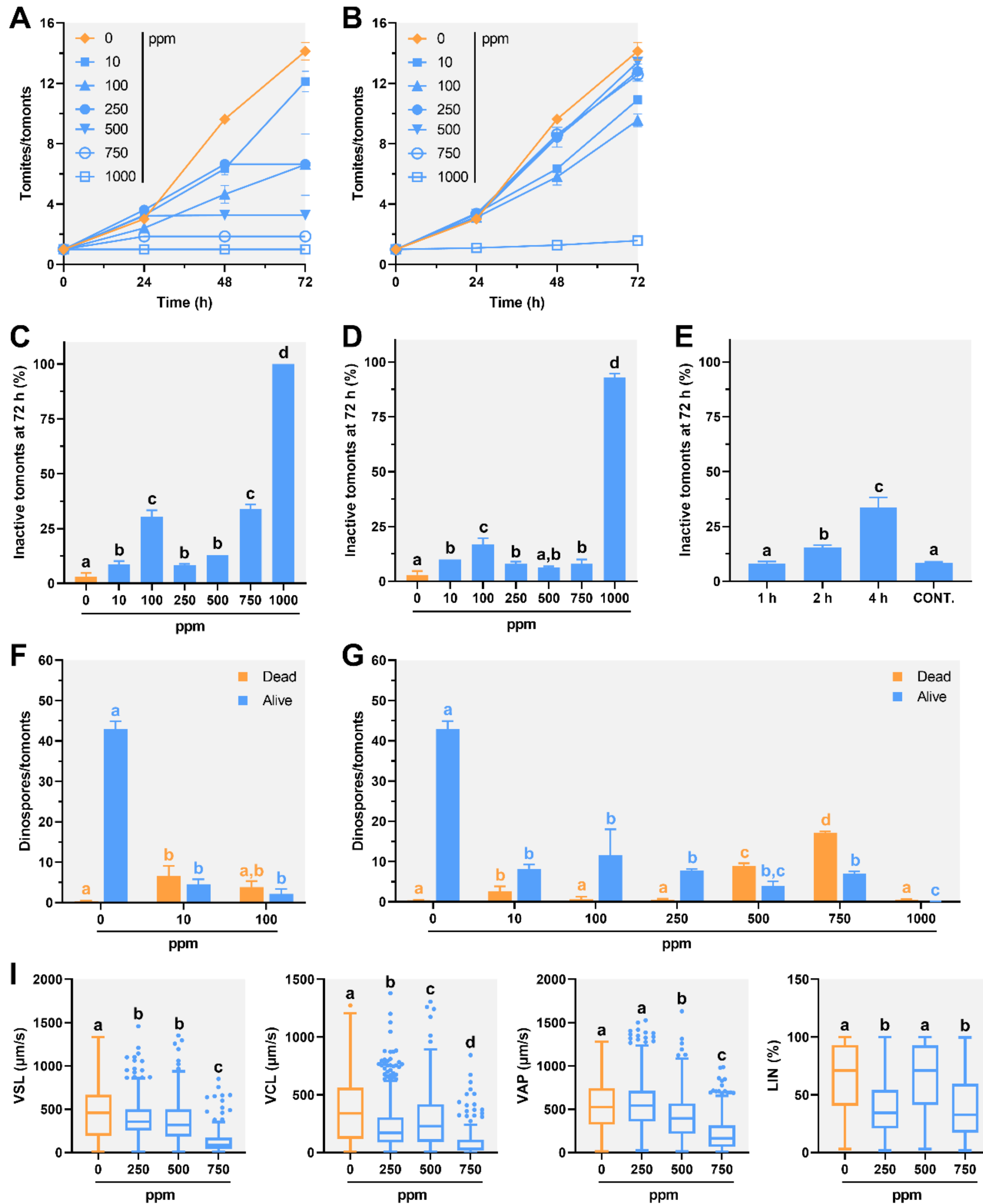

Figure 16. Inactivation of the parasite *Amyloodinium ocellatum* with hydrogen peroxide. **A**,**B** - Time course of tomont division after continuous (**A**) and 1 h (**B**) exposure to different hydrogen peroxide concentrations; **C**,**D** - Proportion of inactive tomonts after continuous (**C**) and 1 h (**D**) exposure to different hydrogen peroxide concentrations; **E** - Proportion of inactive tomonts after different time of exposure to 250 ppm of hydrogen peroxide; **F**,**G** - Dinospore production per tomont after continuous (**F**) and 1 h (**G**) exposure to hydrogen peroxide; **H** - Motility parameters of dinospores produced from tomonts exposed for 1 h to different concentrations of hydrogen peroxide. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

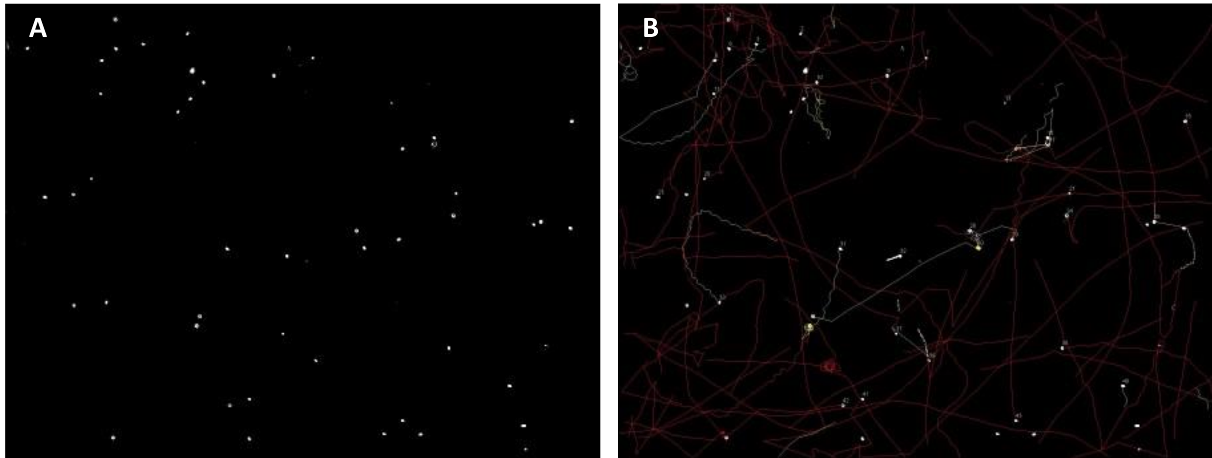

Figure 17. Motility analysis of *Amyloodinium ocellatum* dinospores. **A** - Picture from video of dinospores produced from tomonts exposed to 500 ppm of H_2O_2 for 1 h; **B** - Trajectories recorded with the software OpenCASA for dinospores produced from tomonts exposed to 500 ppm of H_2O_2 for 1 h.

4.4.5 OX-Virin®

4.4.5.1 Sporulation

Continuous exposure for 72 h to OX-Virin® had a significant impact on tomont division even at low concentrations (10 ppm; p-value < 0.0001), with the highest concentration (1000 ppm) completely arresting the division process. Concentrations of 500 and 750 ppm also halted tomont division at 48 h (Figure 18A). Although to a lower extent, 1 h exposure negatively impacted tomont division, while 1000 ppm totally arrested the division process (p-value < 0.001; Figure 18B). Tomonts were fully inactivated at 1000 ppm, although lower concentrations could also impair tomont development to a much lower extent (p-value < 0.001; Figure 18C). One hour exposure of the tomonts to 1000 ppm also strongly impacted tomont division (p-value < 0.001; Figure 18D).

4.4.5.2 Dinospore production

Continuous exposure for 72 h to the chemical had a significant impact on dinospore production, even at the lowest concentration tested (10 ppm, p-value < 0.001). Complete arrest of dinospore production was achieved with concentrations higher than 250 ppm (Figure 18E). One hour exposure had a lesser impact and complete inactivation was achieved at 1000 ppm (Figure 18F).

4.4.5.3 Dinospore motility

VSL was significantly lower for dinospores produced from tomonts exposed to 250, 500 and 750 ppm of OX-Virin® (p-value < 0.001; Figure 18G), but VCL was significantly higher at 750 ppm (p-value = 0.0004). VAP was only affected at 250 ppm while LIN was reduced at all

concentrations (p-value < 0.001).

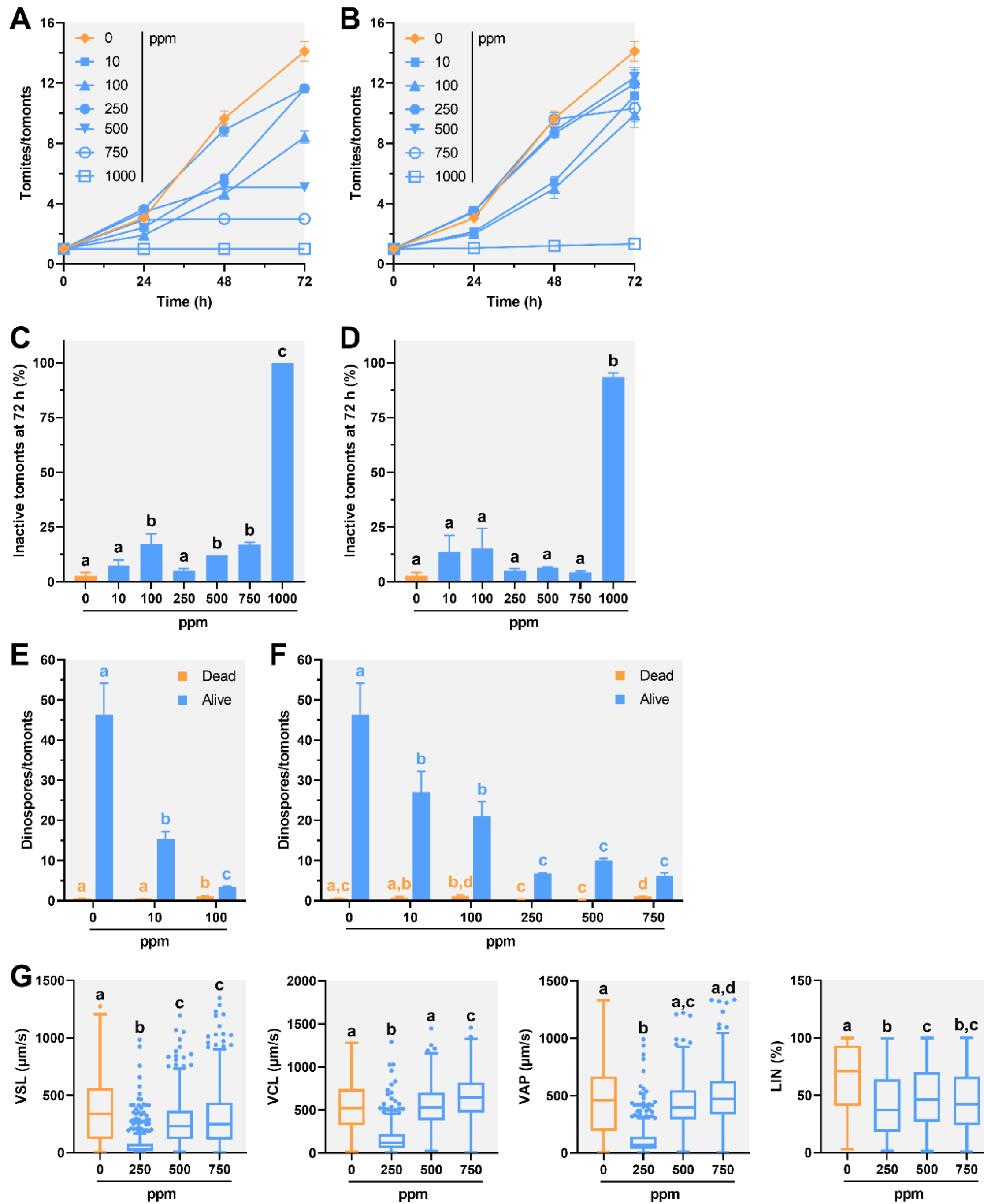

Figure 18. Inactivation of the parasite *Amyloodinium ocellatum* with OX-Virin. **A**,**B** - Time course of tomont division after continuous (**A**) and 1 h (**B**) exposure to different OX-Virin concentrations; **C**,**D** - Proportion of inactive tomonts after continuous (**C**) and 1 h (**D**) exposure to different OX-Virin concentrations; **E**,**F** - Dinospore production per tomont after continuous (**E**) and 1 h (**F**) exposure to OX-Virin; **G** - Motility parameters of dinospores produced from tomonts exposed for 1 h to different concentrations of OX-Virin. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

4.4.6 Virkon S®

4.4.6.1 Sporulation

Continuous exposure of the tomonts to Virkon S® for 72 h significantly impacted their division for concentrations higher than 100 ppm (p-value < 0.001), but complete inactivation was only achieved at 1000 ppm (Figure 19A). A similar pattern was observed for a 1 h exposure to the same concentrations and 1000 ppm arrested tomont division at 48 h and resulted in a significantly lower tomite/tomont value (p-value < 0.001; Figure 19B). Tomont inactivation was significantly higher for 100 ppm (p-value < 0.001) and total inactivation was achieved at 1000 ppm (Figure 19C and Figure 20A). Although to a lesser extent, a 1 h exposure to 1000 ppm of Virkon S also significantly increased the proportion of inactivated tomonts (p-value $<$ 0.001; Figure 19D and Figure 20B).

4.4.6.2 Dinospore production

Total dinospore production was significantly lower upon the continuous 72 h exposure of the tomonts to Virkon S, even at the lowest concentration tested (10 ppm, p-value < 0.001; Figure 19E and Figure 21A). The lowest concentration to arrest dinospore production was 100 ppm. One hour exposure also had a significant impact on dinospore production, even at the lowest concentration tested, but to a lesser extent (Figure 19F and Figure 21B).

4.4.6.3 Dinospore motility

Dinospores produced from tomonts exposed to Virkon S had a significantly lower VSL, VCL and VAP at 10 and 100 ppm (p-value < 0.00001). However, LIN was only affected at 100 ppm (Figure 19G).

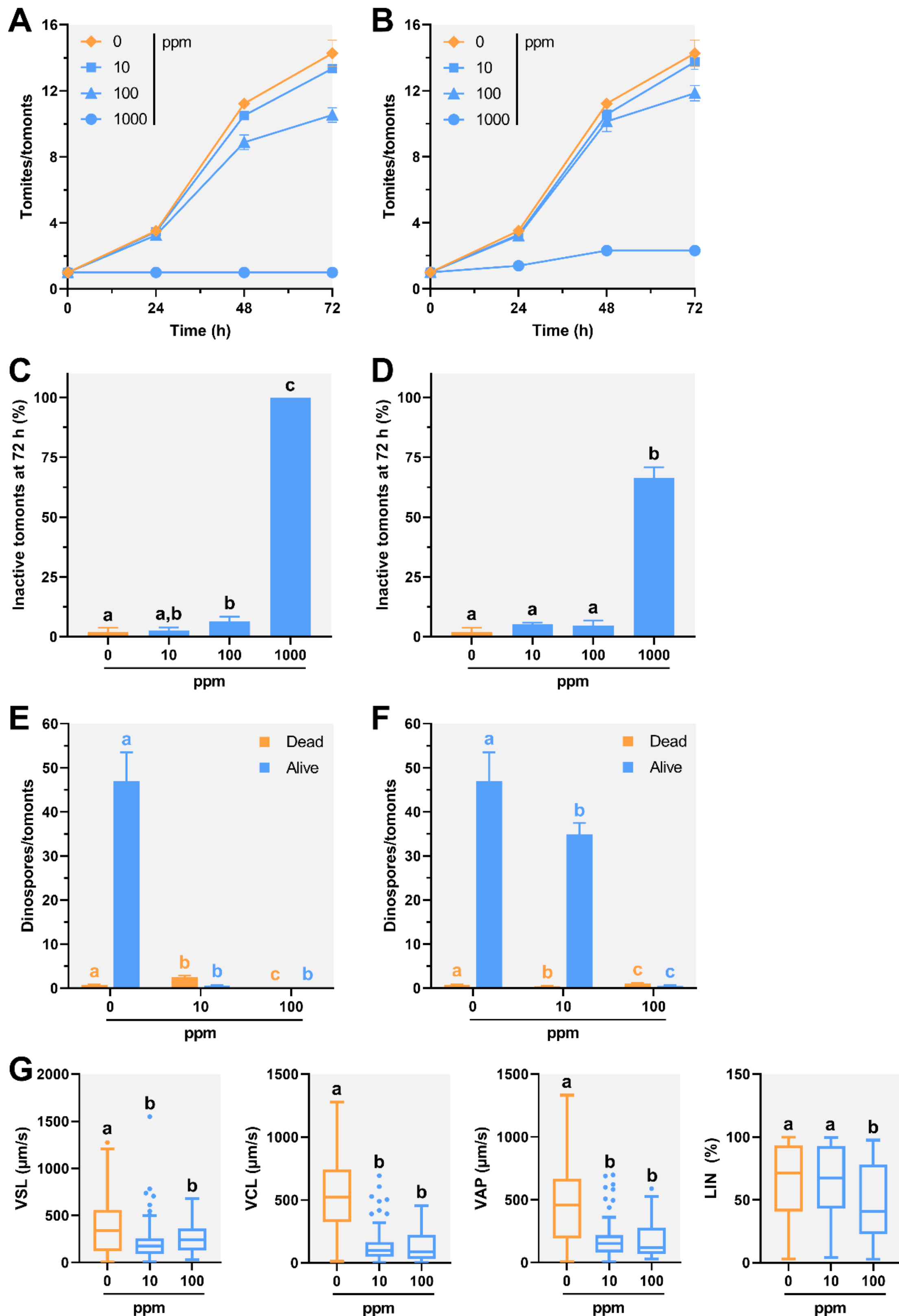

Figure 19. Inactivation of the parasite *Amyloodinium ocellatum* with Virkon S. **A**,**B** - Time course of tomont division after continuous (**A**) and 1 h (**B**) exposure to different Virkon S concentrations; **C**,**D** - Proportion of inactive tomonts after continuous (**C**) and 1 h (**D**) exposure to different Virkon S concentrations; **E**,**F** - Dinospore production per tomont after continuous (**E**) and 1 h (**F**) exposure to Virkon S; **G** - Motility parameters of dinospores produced from tomonts exposed for 1 h to different concentrations of Virkon S. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

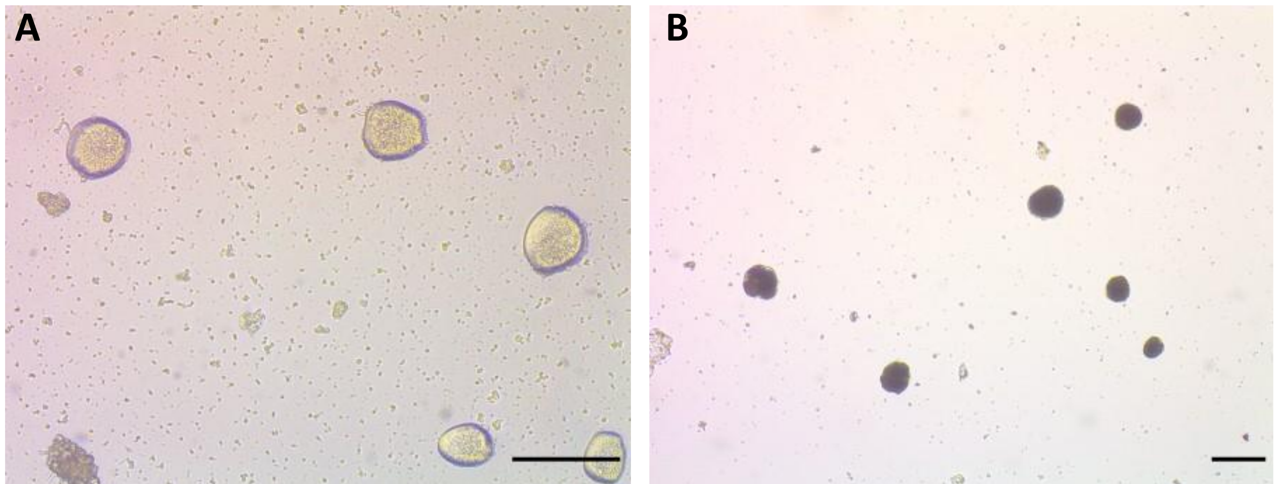

Figure 20. Brightfield micrographs of *Amyloodinium ocellatum* tomonts after exposure to Virkon S. **A** - Continuous exposure to 1000 ppm. **B -** 1 h exposure to 1000 ppm. Scale bar = 100 µm.

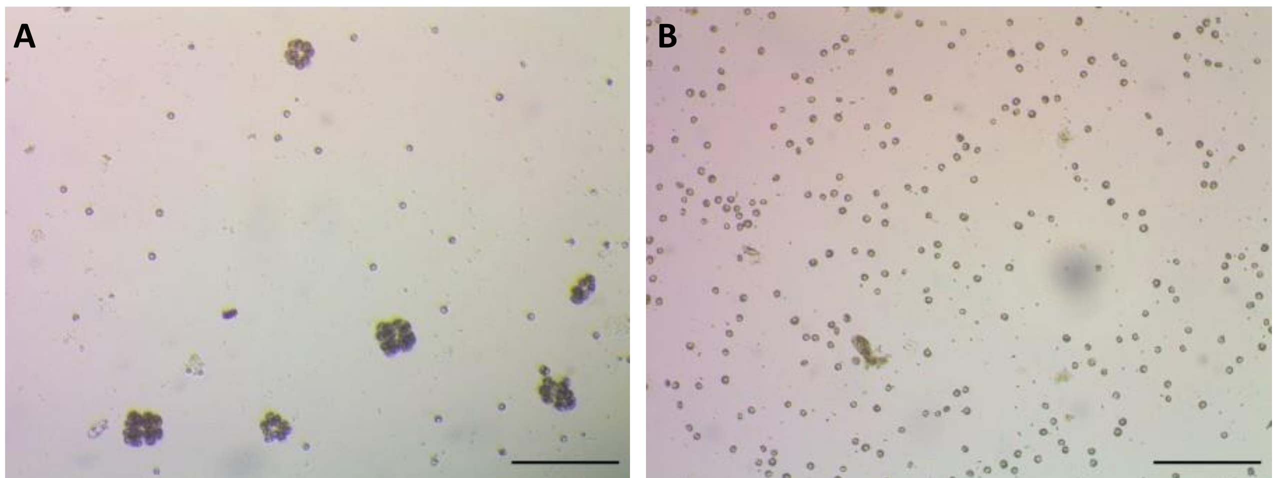

Figure 21. Production of *Amyloodinium ocellatum* dinospores after exposure of the tomonts to Virkon S. **A** - Continuous exposure to 10 ppm. **B** - 1 h exposure to 10 ppm. Scale bar = 100 µm.

4.4.7 Calcium oxide

4.4.7.1 Sporulation

Tomont division was significantly impaired at all the concentrations of calcium oxide (CaO), but total inactivation was only achieved at the highest concentration (10000 ppm; Figure 22A). Only the highest concentration impaired the division of tomonts exposed for one hour to CaO (Figure 22B). Tomont inactivation was significantly increased at concentrations higher than 1000 ppm (p-value = 0.014; Figure 22C). A similar trend was observed upon 1 h exposure to CaO at concentrations above 1000 ppm (p-value = 0.032; Figure 22D). It is worth to note that increased concentrations of CaO resulted in increased pH values (Table 4).

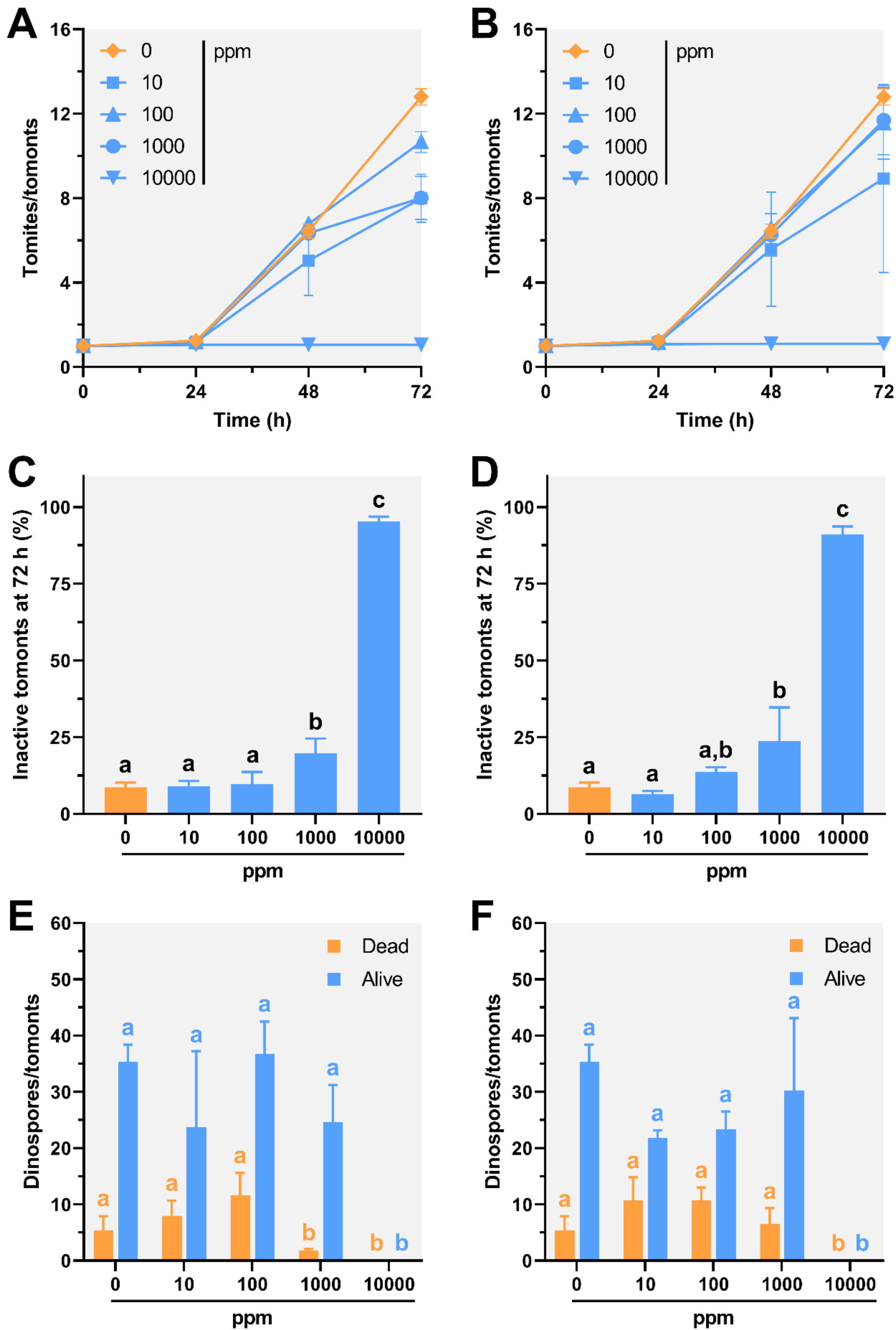

Figure 22. Inactivation of the parasite *Amyloodinium ocellatum* with calcium oxide (CaO). **A,B** - Time course of tomont division after a continuous (**A**) and 1 h (**B**) exposure to different CaO concentrations; **C,D** - Proportion of inactive after continuous (**C**) and 1 h (**D**) exposure to different CaO concentrations; **E,F** - Dinospore production per tomont after continuous (**E**) and 1 h (**F**) exposure to different CaO concentrations. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

Table 4. pH of calcium oxide solutions used to assess inactivation of the parasite *Amyloodinium ocellatum*.

CaO concentration (ppm)	pH
10	9.5
100	11.1
1000	12.2
10000	12.5

4.4.7.2 Dinospore production

The continuous exposure of the tomonts to calcium oxide only triggered a significant effect on dinospore production at the highest concentration tested (10000 ppm; Figure 22E). Similar results were observed when exposure to CaO was reduced to 1 h (Figure 22F).

4.4.8 Salinity

4.4.8.1 Sporulation

Tomont division was impaired upon the reduction of the salinity. At 5‰ and 10‰, the tomite/tomont value was significantly lower than in control (35‰; p-value < 0.001), while division was completely arrested at 0‰ (Figure 23A). Exposure for 1 h to conditions of reduced salinity was enough to significantly impair tomite/tomont division (p-value < 0.001), but not to arrest it, even at 0‰ (Figure 23B). Increasing the time of exposure to 0‰ increasingly impaired tomont division, but complete arrest of division was only achieved with a continuous 72 h exposure (Figure 23C). The proportion of inactive tomonts was significantly higher at 5‰ and 10‰, but complete inactivation was only achieved at 0‰ salinity (Figure 23D). Although to a lower extent, an exposure for 1 h also significantly increased the proportion of inactive tomonts (Figure 23E). Increasing exposure time to 0‰ resulted in significantly higher inactive tomonts (Figure 23F) but continuous exposure (i.e. during 72 h) remained the most efficient method to achieve complete inactivation.

4.4.8.2 Dinospore production

Dinospore production from tomonts exposed to reduced salinity was significantly lower at 5‰, (p-value < 0.001) and 10‰ (p-value < 0.001). No dinospores were produced from tomonts exposed to a 0‰ salinity (Figure 23G). Exposure for 1 h to 5 and 0‰ salinity had a significant negative impact on dinospore production while no effect was observed at 10‰ (Figure 23H). Increased exposure time to 0‰ salinity decreased total dinospore production (Figure 23I).

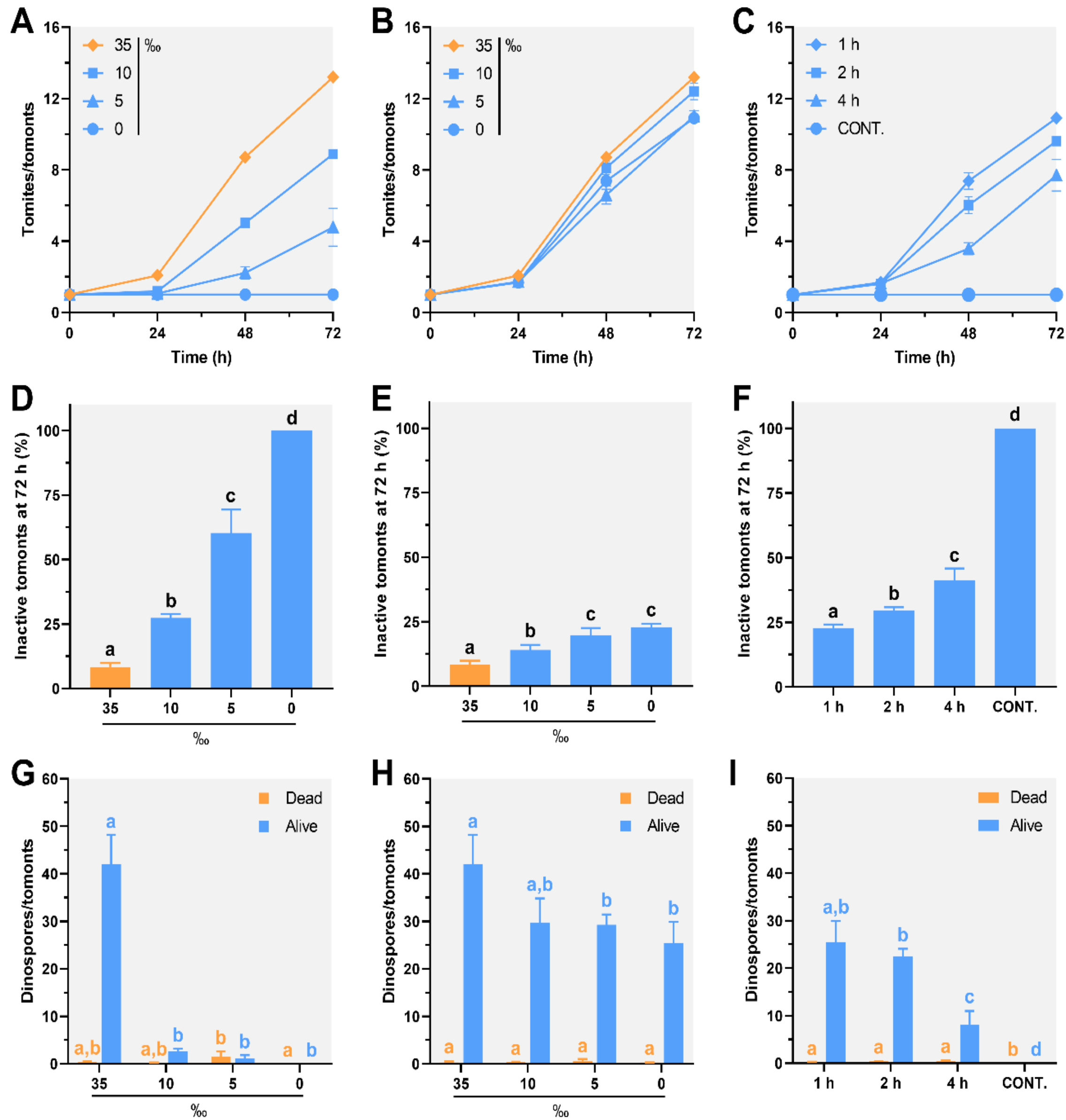

Figure 23. Inactivation of the parasite *Amyloodinium ocellatum* by reduced salinity. **A**,**B** - Time course of tomont division after continuous (**A**) and 1 h (**B**) exposure to reduced salinity; **C** - Time course of tomont division after different time of exposure to 0‰ salinity; **D**,**E** - Proportion of inactive tomonts after continuous (**D**) and 1 h (**E**) exposure to reduced salinity; **F** - Proportion of inactive tomonts after different time of exposure to 0‰ salinity; **G**,**H** - Dinospore production from tomonts exposed continuously (**G**) and for 1 h (**H**) to reduced salinity; **I** - Dinospore production from tomonts exposed for different time to 0‰ salinity. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's *post hoc* test, n=3).

4.4.9 Desiccation

A desiccation of the tomonts for 30 min was enough to achieve a 100% inactivation. Salt crystals were visible around the tomonts (Figure 24A) and an altered morphology was observed for tomonts recovered after desiccation (Figure 24B).

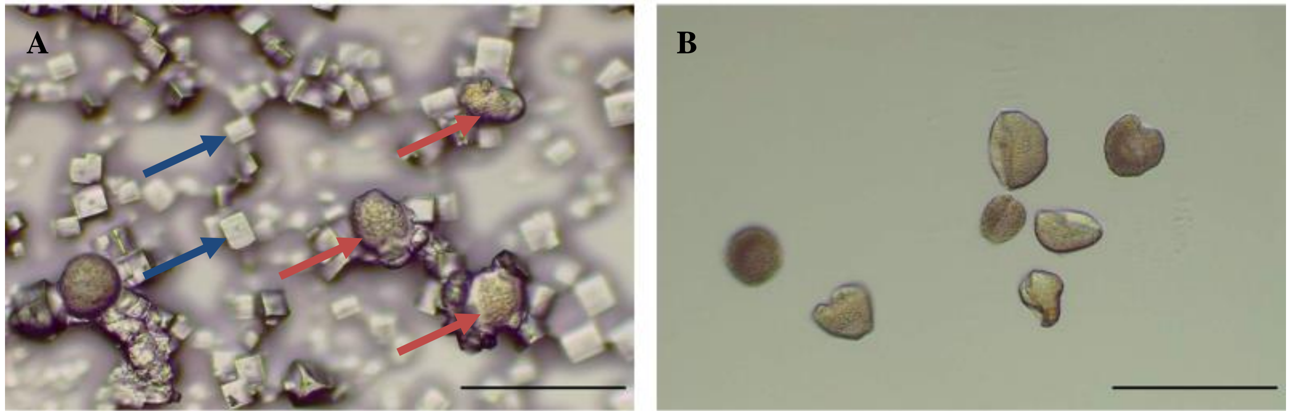

Figure 24. Desiccation of *Amyloodinium ocellatum*. **A**- tomonts after 30 min desiccation Red arrows indicate tomonts and blue arrows indicate salt crystals. **B**-Tomonts of *Amyloodinium ocellatum* recovered after 30 min of desiccation. Scale bar = 100 µm.

4.4.10 UV-C

4.4.10.1 Sporulation

Exposure to UV-C light had a significant impact on tomont division, even at the lowest level tested (10 s equivalent to 11.6 mJ/cm^2, p-value < 0.001). Complete arrest of division was achieved with an exposure of 120 s (equivalent to 139.2 mJ/cm^2; Figure 26A). Increasing exposure time resulted in increased levels of inactive tomonts. Percentage of inactive tomonts was significantly higher even at the lowest level tested. Total inactivation was achieved with 120 s of exposure (Figure 26B).

4.4.10.2 Dinospore production

Increased exposure of tomonts to UV-C light decreased the production of dinospores. An exposure of the tomonts for > 20 s significantly decreased dinospore production, and increased dinospore mortality. An exposure of the tomonts for 40s (equivalent to 46.4 mJ/cm^2) was enough to totally arrest the production of dinospores after 96 h of incubation (Figure 26C).

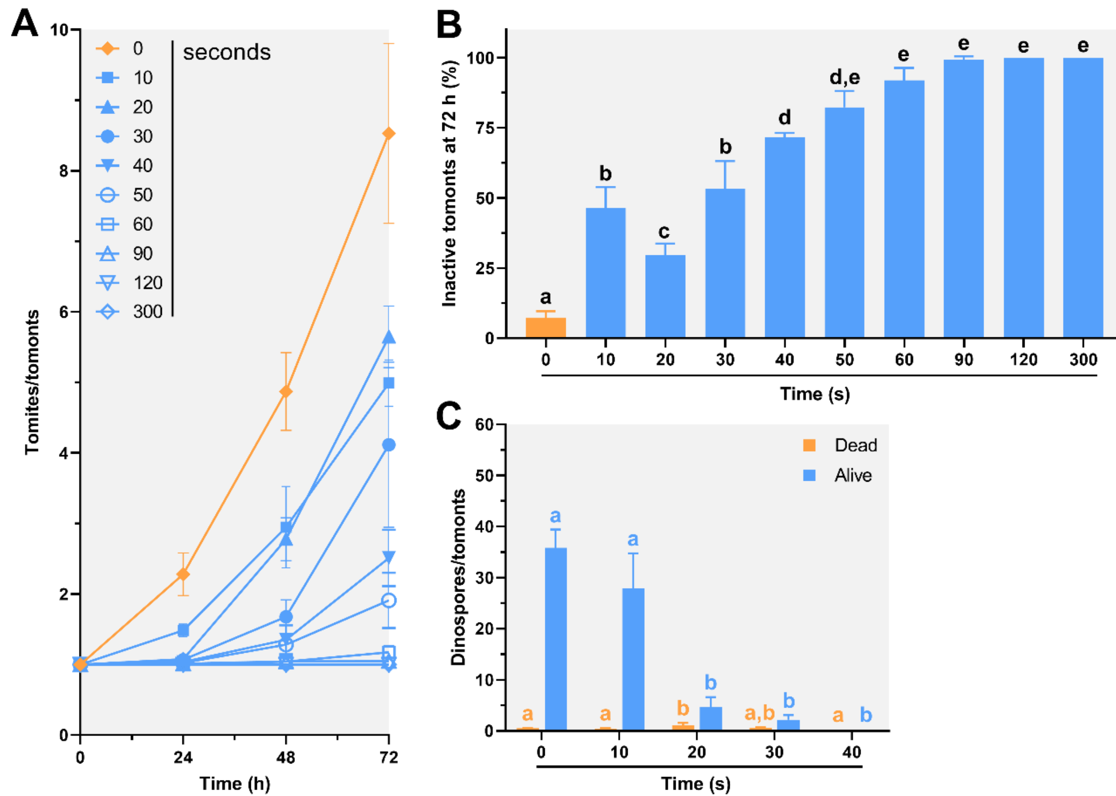

Figure 25. Inactivation of the parasite *Amyloodinium ocellatum* with ultraviolet light. **A** - Time course of tomont division after different time of exposure to UV-C; **B** - Proportion of inactive tomonts after different time of exposure to UV-C; **C** - Dinospore production from tomonts exposed for different time to UV-C. Different letters indicate significant ($p < 0.05$) differences (one-way ANOVA with Tukey's post hoc test, n=3).

5 Discussion

Dinoflagellate resting cysts, as those of *A. ocellatum*, are robust and resistant to environmental stresses (Wang *et al.*, 2018). Several studies have been conducted to investigate methods to control *A. ocellatum* infection, but to date very few studies aimed at evaluating the inactivation of the tomont (Paperna, 1984a). This work represents a continuation of previous studies with the introduction of more environmentally friendly chemicals, like hydrogen peroxide (H_2O_2) and peracetic acid (PAA).

To perform the inactivation tests, a large amount of tomonts was successfully produced using an *in vivo* infection system established according to works previously published (Abreu *et al.*, 2005; Moreira *et al.*, 2017; Moreira *et al.*, 2018). Unfortunately, this system requires the use of adult fish (6 in this work) to host the parasite and their sacrifice at the end of the propagation. To meet the 3R principles, alternatives based on *in vitro* propagation of *A. ocellatum* have been

tried but they remain laborious and require specific equipment (Oestmann & Lewis, 1996; Noga, 1987). The preservation of the tomonts for future usage was also tested during this work. Preservation at a low temperature (5ºC) was possible for short periods (less than 48 h) without impacting the viability of the tomonts. For prolonged periods (until 30 d), viability was reduced from 95% to 56% at 72h of incubation but viable dinospores could still be produced from preserved tomonts. No dividing tomonts were registered at this temperature. Previous works also showed that tomonts of *A. ocellatum* arrest division at low temperatures (5-8ºC) and are unable to further divide after incubation at 20ºC (Paperna, 1984b). This latter work also suggested that some strains of *A. ocellatum* may adapt better to different environment conditions e.g., temperature and salinity. For the present study parasites were collected from fish produced near the Ria Formosa, Portugal, where minimum seawater temperatures reach lower values than the Gulf of Eilat, Israel (12ºC vs 20ºC), which could explain the higher resistance of the parasite to lower temperatures than the previous study (Newton & Mudge, 2003; Gertman & Brenner, 2004; Paperna, 1984b). Preservation at higher temperatures (15ºC and 13ºC) failed to arrest division for long periods (96 h and 18 d, respectively), making tomonts inviable to the inactivation tests. The equipment chosen for this preservation presented a high temperature variability (± 5ºC), that was related with room temperature conditions. Despite the temperature variability, tomonts from this preservation method were able to produce viable dinospores after 3 months (personal observation). Successful preservation of *A. ocellatum* for long periods (3 months) was previously reported at 16ºC but not at 14ºC (Tedesco *et al.*, 2020; Massimo, 2019). At 16ºC, tomonts also showed an asynchronous development (Massimo, 2019). Previous works at 12ºC were successful in preserving trophonts and tomonts of *Cryptocaryon irritans*, a fish parasite ciliate with a life cycle similar to the one of *A. ocellatum,* for long periods (5 and 4 months respectively) (Dan *et al.*, 2009). Cryopreservation could be a solution for the storage of *A. ocellatum* for longer periods, but tentative experiments were unsuccessful (Massimo, 2019; Yang, 2006).

The structure of various development stages of *A. ocellatum* were successfully imaged using scanning electron microscopy. However, the image of the delicate flagellate dinospore revealed a very distorted structure comparing with light microscopy and previous works with scanning electron microscopy (Landsberg *et al.*, 1994), possibly because the treatment with hexamethyldisilazane (HMDS), not available at the time of the experiment, was omitted. HMDS reduces surface tension and shrinkage of cells by giving strength to the sample by cross-linked proteins (Gómez-Lizárraga *et al.*, 2019).

Copper sulphate is registered as a biocide type 2 (algicide) in the European Union, and is currently the most used therapeutant for *A. ocellatum* infestations (Regulation 1033/2013, Noga, 2012, Soares *et al.*, 2012). Our results are in accordance with previous studies, in which $CuSO_4$ was lethal to dinospores of *A. ocellatum* but did not interrupt the division process of the encapsulated tomont (Paperna, 1984a). In previous studies, exposure to 1 ppm $CuSO_4$, equivalent to 0.25 ppm Cu, for 12 to 24 h reduced the reproductive success of tomonts, but dinospores, which could cause reinfestation, were still produced (Paperna, 1984a). In this work, a continuous exposure to 0.1 ppm Cu significantly reduced dinospore production, but total interruption of production of live dinospore was only obtained with 1 ppm Cu. Flush treatments of 1 h were not as effective, since there was still production of live dinospores at 1 ppm Cu. These results validate the premisses used for the established protocols of *A. ocellatum* treatment with Cu (Noga, 2012; Soares *et al.*, 2012; Francis-Floyd & Floyd, 2011). Several *in vivo* studies reported Cu as an efficient therapeutic for *A. ocellatum* (Owatari *et al.*, 2020; Marques *et al.*, 2019; Bessat *et al.*, 2018; Virgula *et al.*, 2017; Abreu *et al.*, 2005). Continuous treatment with a desired therapeutic concentration of 0.15 to 0.2 ppm free Cu ion for 2 to 3 weeks helped to eliminate infection with *A. ocellatum* in marine systems (Francis-Floyd & Floyd, 2011). Treatment with Cu to ensure complete inhibition of tomonts was not obtained in this work even at 10 ppm Cu. Exposure of fish to high concentrations of $CuSO_4$ may lead to death (Tavares-Dias, 2021a). In a study using the European sea bass, exposure to 10 ppm for 24 h and 1 ppm for 96 h triggered 100% mortality, while 1 ppm for 24 h only achieved 16.7% mortality (Díaz-de-Alba *et al*., 2017). In another study using juvenile Senegalese sole (*Solea senegalensis*), exposure to 0.01 and 0.1 ppm Cu resulted in sublethal effects, while exposure to 1 ppm Cu triggered a 26.6% mortality at 24 h and 100% mortality at 96 h (Oliva *et al.*, 2009). Sublethal exposure of fish to Cu causes changes in feeding and swimming behaviour, growth performance, histomorphology of gills, liver, kidney, and spleen, hematology, blood biochemistry, the antioxidant defence system, and oxygen consumption (Tavares-Dias, 2021a). Copper ions also accumulate in the gills, liver, kidney and spleen, and provokes in the gills changes in mucus and chloride cells, hyperplasia and/or hypertrophy of primary and/or secondary lamellae, edema of the gill epithelium, and lamellar fusion (Tavares-Dias, 2021a; Vaz *et al.*, 2019). Invertebrates and algae are also highly susceptible to Cu even at low concentrations. In the marine environment, the concentration of Cu must be monitored frequently because Cu can be unstable (Francis-Floyd & Floyd, 2011; Noga, 2012). Chelated or complexed forms of Cu are sometimes recommended as they reportedly reduce the toxicity to fish. However, the bioavailable concentration is also reduced, which limits the effectiveness

against pathogens (Lieke *et al.*, 2020).

Formalin has been widely used to disinfect water in aquaculture for the prevention of aquatic diseases, and especially against parasitic agents (Tavares-Dias, 2021b; Leal *et al.*, 2018). In this regard, formalin is another documented treatment for amyloodiniosis (Noga, 2012). Results from this study showed that formalin was effective in inactivating the tomont, although high doses were necessary for complete tomont inactivation. We propose that continuous applications of formalin at 100 ppm or 250 ppm in 1-h flush bath are effective treatments to inactivate tomonts of *A. ocellatum*. Previous studies showed that flush treatment with 100-200 ppm of formalin for 6-9 h causes trophonts of *A. ocellatum* to detach from gills, but tomonts resumed division after removal of the chemical (Paperna, 1984b). In the same study, complete inhibition of tomont division was observed only after 24 h exposure to 200 ppm at 27–30°C. After 12 h exposure to 200 ppm formalin, cell division was only reduced by about 40% leaving enough motile dinospores for recurrence. Other studies using juveniles of the bullseye puffer fish (*Sphoeroides annulatus*) showed that lower doses, i.e. 51 ppm for 1 h or 4 ppm for 7 h, significantly reduced the parasite load on the skin and gills, but fish were reinfested after 15 days (Fajer-Avila *et al*., 2003). The acute toxicity (LC50—96 h) of formalin varies widely among fish species (from 0.1 to 640.0 mg/L), and many fish species are sensitive to concentrations close to those required for the treatment of parasitosis (Tavares-Dias, 2021b). Formalin leads to histopathological damages in gills and hematopoietic organs resulting in disturbances to various physiological functions, particularly respiration and hematological and biochemical process (Tavares-Dias, 2021b). Formalin is also a cytotoxic and genotoxic substance, which has been demonstrated to cause DNA strand breakage and cell lysis in humans (Zhang *et al*., 2010). Formaldehyde, the main compound in formalin, is classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1), but is currently approved in the EU as biocide type 2 and type 3, veterinary hygiene use (Regulation 1763/2020; Lieke *et al*., 2020). Because of its established toxicity, extreme care should therefore be taken when using formalin to treat *A. ocellatum* infections.

This work provides for the first time data on the effect of peroxides (hydrogen peroxide (H_2O_2) and peracetic acid (PAA)) on tomonts. A continuous exposure of the tomonts to H_20_2 had a significant impact on their division and dinospore production even at low levels (10 ppm). This study complements the following *in vivo* studies. Montgomery-Brock *et al.* (2001) reported an effect of H_2O_2 on the trophonts of *A. ocellatum* in cultured Pacific threadfin (*Polydactylus*

sexfilis) and demonstrated that a treatment at 75 and 150 ppm H_2O_2 for 30 min could reduce the number of parasites. Cruz-Lacienda *et al.* (2004) reported the successful treatment of infested red snapper (*Lutjanus argentimaculatus*) with 200 ppm of H_2O_2 after 1h. In another study with European sea bass, a treatment with 100 and 200 ppm of 20% H_2O_2 for 30 min significantly decreased *A. ocellatum* load on gills (Seoud *et al*., 2017). However, the effect of H_2O_2 on tomonts was overlooked in this latter study. In the studies by Montgomery-Brock *et al.* (2001) and Cruz-Lacienda *et al.* (2004), fish were removed from the infected tanks after treatment, a situation that may not be possible in an earth pond aquaculture site. In fact, despite significantly reducing the reproductive capacity of the parasite, our results showed that H_2O_2 treatment for 1 h could not arrest tomont division and dinospore production, even at a high dose (1000 ppm).

Peracetic acid, which is a stabilized mixture of acetic acid, H_2O_2, and water has been receiving more attention as a disinfectant in aquaculture (Lazado *et al.*, 2020). PAA has proven to be an effective treatment against various aquatic pathogens including fungi, bacteria, virus, and protozoa (Good *et al*., 2020; Marchand *et al*., 2012). In our experiment concentrations of 10 ppm of OX-Virin® (5% peracetic acid) could impact on tomont division, but complete inactivation was only achieved at concentrations above 500 ppm, equivalent to 25 ppm of PAA for 48 h exposure. Studies using a similar fish parasite, *Ichthyophthirius multifiliis*, showed that a treatment with 8 to 15 ppm of PAA for 1 h was effective at inactivating the different developmental stages and could kill cysts of the parasite (Meinelt *et al.*, 2009; Picon Camacho, 2010). Similarly, the inactivation of the cysts of several dinoflagellates (i.e. *Gymnodinium catenatum*, *Alexandrium catenella* and *Protoceratium reticulatum*) was achieved using 400 ppm of Peraclean® Ocean, a commercial solution containing PAA at 14% (Gregg & Hallegraeff, 2007).

The mechanism of action of H_2O_2 and PAA are similar and involves the production of hydroxyl radicals that oxidize enzymes and proteins, and increase the permeability of cell walls by destruction of sulfhydryl and sulfur compounds (Lieke *et al.*, 2020). PAA is however more potent than H_2O_2 because of its fat solubility (Straus *et al.*, 2018). This non-specific mode of action prevents an adaptation of the microorganisms to the H_2O_2 and PAA, thus prevents the development of resistance (Lieke *et al.*, 2020). In addition, the molecules produced when H_2O_2 and PAA are degraded have little or no toxicity, which is a clear advantage in relation to other conventional disinfectants like copper sulphate and formalin (Straus *et al.*, 2018). Indeed, H_2O_2 degrades to water and oxygen, and PAA degrades to water, oxygen and acetic acid, which is quickly metabolized by microorganisms (Lieke *et al.*, 2020). Both chemicals could be safely implemented in therapeutic measures against *A. ocellatum*; however, according to our data,

higher concentrations are needed to completely eliminate the parasite. An alternative to the use of higher concentrations could be the implementation of sequential treatments at lower concentrations, since dinospores and trophonts are susceptible to treatment (Montgomery-Block *et al*, 2001). Information on PAA toxicity for marine species is scarce, but safe values were established bellow 5 ppm (Straus *et al.*, 2018). Studies with Atlantic salmon (*Salmo salar*) smolts at 2.4 ppm PAA for 30 min only caused marginal morphomolecular changes on the skin (Lazado *et al.*, 2020). Possible therapeutic concentration identified in this work – 250 ppm OX-Virin®, equivalent to 12.5 ppm of PAA – seems high in comparison with these safe values, although *in vivo* studies should be performed in the future to precisely determine safe concentrations of PAA for common Mediterranean marine aquaculture species such as European seabass and gilthead seabream.

Virkon®S is a mixture of peroxygens, surfactants, organic acids and inorganic salts. Used in the farming industry for the disinfection of equipment and facilities, the product is described as effective against a wide range of viruses, bacteria and fungi (Tedesco *et al.*, 2019). In this work, Virkon S was effective in inactivating tomont division at 1000 ppm and dinospore production at 100 ppm. Levels are much lower than the 5000 ppm used to kill the eggs of the parasite *Toxascaris leonina* (El-Dakhly *et al*., 2017). In aquaculture, Virkon S is used at a concentration of 10000 ppm for the disinfection of ponds and farm equipment (Sudova *et al*., 2007). Exposure of salmonids to this concentration for 15 min was effective in controlling *Gyrodactylus salaris* infection (Koski *et al.*, 2016). It has also been reported that 10 days of treatment with 4 and 10 ppm Virkon S resulted in the complete reversal of artificially-induced saprolegniasis in the common carp *Cyprinus carpio* without harmful effects to the fish, but concentrations higher than 20 ppm were lethal to fish (Rahman & Choi, 2018). Exposure of the gilthead seabream to 10000 ppm resulted in 50 % mortality within 24 h (Acosta *et al*., 2021). Fingerlings of the rainbow trout *Oncorhynchus mykiss* exposed to 30 ppm for 3.5 h showed no observable toxic effects, but smaller fry only survived when exposed to concentrations bellow 10 ppm (Stockton-Fiti & Moffitt, 2017). More studies are needed to better evaluate the potential effects of this product on marine fish. Disinfection of tanks and equipment of *A. ocellatum* is an option with lower concentrations than described for other pathogens (1000 ppm vs 10000 ppm).

The disinfection of pond bottoms can be achieved with different methodologies. Classical methods involve drying and ploughing, addition of lime and chlorination (OIE, 2009). The present work investigated the effects of each method on the inactivation of *A. ocellatum*

tomonts. Drying tomonts for 30 min was enough for a full inactivation. Previous studies also reported an inability of the tomonts of *A. ocellatum* to survive desiccation challenges (Paperna, 1984b; Massimo, 2019). Other parasites can also be successfully inactivated following desiccation. For example, development of the eggs of the pathogenic nematode *Pseudocapillaria tomentosa* were inactivated following desiccation for 2 h (Kent *et al.*, 2019) and hatching of the eggs of the monogenean parasite *Benedenia seriolae* was prevented following desiccation for 3 min (Ernst *et al.*, 2005). Drying and ploughing are commonly used methods of treating a pond bottom to reduce its organic content, improve nutrient recycling, buffer pH, eliminate pests, and achieve disinfection through a combination of microbial degradation, exposure to sunlight, aeration, and desiccation (OIE, 2009). The results from this work demonstrate that a prolonged drying period of earth ponds between production cycles may potentiate the inactivation of *A. ocellatum* tomonts and by so help prevent the resurgence of the disease in the tank.

The ability of chlorine, in the form of sodium hypochlorite, to totally inactivate *A. ocellatum* tomonts was demonstrated at 250 ppm following a 1 h treatment. Chlorination may be used for routine treatment of ponds between crops or when disease eradication is the goal. For this purpose, a concentration of 10 ppm has been recommended (OIE, 2009). This concentration is however much lower than the effective dose for *A. ocellatum* tomont inactivation. Total inactivation of the cysts of the marine dinoflagellate *Gymnodinium catenatum* was achieved with free chlorine at 500 ppm (Bolch & Hallegraeff, 1993). Other parasites with resting stages have variable resistance to chlorine (Kent *et al.*, 2019; Adeyemo *et al.*, 2019; Briancesco *et al.*, 2005). Cysts of *Giardia* were highly sensitive to chlorine with effective chlorine concentration of 0.5 ppm for 60 min, while for *Cryptosporidium* cysts a dose of 5 ppm for 120 min achieved 90% inactivation. Environmental risks associated with the application of chlorine on ponds are very high, and caution should be taken to prevent damages to fish gills, which can impair the oxygen uptake (Davis *et al.*, 2021). Neutralization of chlorine with sodium thiosulphate is recommended, and water should be tested for residual chlorine before discharge (OIE, 2009).

Lime, in the form of hydrated lime ($Ca(OH)_2$) or burnt lime (calcium oxide, CaO), has traditionally been used to sterilize solid waste or waste water (Boyd, 2003; OIE, 2009; Yanong, 2013). Its strong antiviral, antimicrobial and antiparasitic properties are thought to be due to its effect of increasing the pH to approximately 12 and, in solid waste, also to the reduction in moisture content to achieve desiccation (Zintl *et al*., 2010; OIE, 2009). Quicklime also causes

a sharp increase in temperature (between 50°C and 100°C) owing to the exothermic reaction of CaO with water. In this study only high concentrations (10000 ppm) were effective in completely inactivating tomont division and dinospores production. The pH registered at 1000 ppm and 10000 ppm of CaO (12.2 and 12.5) were in the range described to have a detrimental effect on resting stages of parasites i.e. pH 12 (Boyd, 2003). Despite that only on 10000 ppm there was evidence of inactivation of tomonts, which could be explained by the higher resistance of this parasite to pH challenge. Liming (using either hydrated lime or quicklime) has been used effectively against adenovirus, rotavirus, bacteriophages (Hansen *et al.,* 2007), poliovirus, faecal coliforms, and *Salmonella* and *Giardia* species (Bean *et al.*, 2007, Graczyk *et al.*, 2008, Kristula *et al.*, 2008). Graczyk *et al.* (2008) also reported that quicklime was highly effective against *Cryptosporidium* oocysts, although Rimhanen-Finne *et al.* (2004) observed no effect. Adding lime to earth ponds at high doses seems impractical due to the size of the ponds and the quantities needed to achieve the concentration of tomont inactivation. As an example, to achieve the concentration required for tomont inactivation in an earth pond of 1500 m^2 and 10 cm water it would be required 1.5 tonnes of CaO. Desiccation effect of CaO on tomonts was not tested in this work. According to the literature, quantity around 4-5 tonnes per hectare of CaO should be needed to achieve the desiccation effect (Boyd, 2003; OIE, 2009).

Our results showed that decreasing salinity to 5‰ and 10‰ had a significant negative impact on tomont division and dinospore production, but total inactivation was only achieved with continuous exposure to 0‰ salinity. These results are in accordance with previous *in vitro* challenges (Paperna, 1984b), where *A. ocellatum* could be inactivated by repeated dips in freshwater. However, unless fish are moved into a new tank after treatment, the disease will not be controlled because tomonts can complete their life cycle even after 4 h of freshwater bath. Exposure to freshwater for 1 h was effective in removing *A. ocellatum* from the red snapper *Lutjanus argentimaculatus* (Cruz-Lacierda *et al.*, 2004). A daily hyposaline bath (10‰) for two weeks could successfully reduce the cumulative mortality of infected with *A. ocellatum* European seabass from 65% to 15% (Bessat & Fadel, 2018). Infections by *A. ocellatum* were reported in wild fish of the Gulf of Mexico in estuarine waters at 2-3 ppt salinity, which suggest that different geographic isolates of the parasite may have differential salinity tolerances (Francis-Floyd & Floyd, 2011, Paperna, 1984b). Hypo-salinity would be very difficult to implement on earth ponds, due to large quantities of freshwater needed to lower salinity to effective levels, but on indoor tanks the method could be applied in fish species that tolerate low salinity levels.

The UV radiation is defined as a physical treatment, able to inactivate a wide range of bacteria, fungus and protozoa (Moreno-Andrés *et al.*, 2020). UV radiation damages nucleic acids by causing strand breaks, this produces pyrimidine dimers and pyrimidine and pyrimidone photoproducts (Barrett *et al.*, 2020). These mutations can block DNA transcription and lead to aberrant cell behaviour and/or loss of fidelity during replication. Cells have mechanisms to mitigate DNA damage, but extreme damages could lead to cell death (Barrett *et al.*, 2020). UV-C is commonly used, as its production is relatively inexpensive and it can efficiently damage DNA (Cleaver, 2006; Barrett *et al.*, 2020). No information was available on the resistance of *A. ocellatum* tomonts to UV-C light prior this work. Complete tomont inactivation was achieved at 139.2 mJ/cm^2 but a reduction of tomont division and total arrest of dinospore production was achieved at lower levels (11.6 mJ/cm^2 and 46.4 mJ/cm^2, respectively). Most of the literature data available on parasite inactivation by UV light is related to resistant parasite stages present in drinking water, including cyst from *Giardia lambia* and *Giardia intestinalis* and *Cryptosporidium parvum* (Adeyemo *et al*., 2019; Fernández-Boo *et al*., 2021). *Giardia sp* could be inactivated after exposure to UV-C at 20.8 mJ/cm^2 while for *Cryptosporidium sp.* only an exposure to 83.2 mJ/cm^2 UV-C were able to inactivate 97% of the parasite (Adeyemo *et al*., 2019). Information about the effect of UV-C light on marine protozoan parasites is very scarce and variable. Ford *et al*. (2001) showed that 30 mJ/cm^2 were enough to prevent oysters from infection with the parasites *Haplosporidium nelsoni* and *Perkinsus marinus* in hatcheries. More recent studies found that 94 mJ/cm^2 were required to inhibit the proliferation of bivalve parasites *Bonamia ostrea* and *Perkinsus olseni*, and 450 mJ/cm^2 was enough to eliminate the parasites, which is much higher than the results obtained for *A. ocellatum*(Fernández-Boo *et al.*, 2021). The results obtained from inactivation of tomonts of *A. ocellatum* with UV-C are similar to other studies with dinoflagellates. Cysts of the dinoflagellate *Scripsiella trochoidea* were inactivated under a UV-C lamp with a dose of 77.2 mJ/cm^2 (Yang *et al.*, 2015). The germination of *Chattonella* sp. cysts decreased to 6% of the control upon exposure to UV light for 2 h, whereas the germination of cysts from other species of dinoflogellates (*Alexandrium* sp. and *Gymnodinium* sp.) was decreased to 40% of the control (Montani *et al.*, 1995). UV-C radiation seem to influence the sporulation capacity, probably causing irreversible defects on DNA. Tomonts of *A. ocellatum* generally deposit in the sediments of ponds being protect from UV radiation, and may not be prepared to withstand UV radiation.

Some studies have tested the possibility of applying UV-C light directly to the fish to reduce the occurrence of external parasites. Fish infested with sea lice were exposed with UV-C light

in tanks at a cumulative dose of 100 mJ/cm^2 on each side over a 6-day period. The UV-C treatment reduced by 99% the production of copepod compared to the control, but it also negatively impacted fish welfare and was associated with early-stage cataract-like pathologies, poorer skin condition and behaviours indicative of discomfort (Barret *et al.*, 2020). Application of the UV-C technology to the disinfection of earth ponds seems unlikely due to the amount of inlet water that needs to be treated, but also direct application of UVC on fish at therapeutic dose would be harmful for fish. UV-C could, however, be used for indoor facilities and data on the effect of UV-C on *A. ocellatum* cysts are needed to set preventive measures. Cysts of *A. ocellatum* can travel through the water column (personal observation), thus facilities where inlet water comes from a source with an history of *A. ocellatum* infections should take measures for an efficient treatment of the water. Outbreaks in indoor tanks have been already reported, and water treatment with UV could prevent severe damages, especially when referring to broodstock tanks (Marques *et al*., 2019). We propose that the application of UV-C at a dose higher than 46.4 mJ/cm^2 on inlet water would prevent tomonts to sporulate and infect fish on indoor tanks.

An attempt was made in this work to provide a new methodology to evaluate motility of dinospores of *A. ocellatum* based on digital cell movement tracker with the software OpenCASA. Previous studies applied the same principle to evaluate the swimming behaviour of microalgae in toxicology studies (Zhao *et al.*, 2019; Zheng *et al.*, 2012). Motility analysis of dinospores provided significant information on the effect of formalin, H_2O_2, PAA and Virkon S on the flagellate. Reduction of motility parameters were observed for most of the chemicals and concentrations tested, with formalin presenting the lower values of VSL, VCL and VAP. This data may indicate that tomonts that survive flush treatments release dinospores potentially damaged thus with a lower capacity to find a host in the water column. The data provided from the analysis of dinospores also showed a great dispersion of results in some of the parameters evaluated. The method relies on an adaptation of software developed specifically for sperm motility, and by so parameters were chosen to better fit the size and shape of dinospores (Alquézar-Baeta *et al.*, 2019). Despite the difficulties, this method looks a promising alternative for toxicologic studies and further studies should be performed to optimize this methodology (Zhao *et al*., 2019).

6 Conclusions

This work has evaluated several alternatives to current treatments of amyloodiniosis with a focus on an improved efficacy in inactivating the parasite forms but also on a lesser impact on environment and fish health. A synthesis of possible preventive and treatment measures based on the data collected within the scope of this work is provided in Table 5. Because of their higher efficacy and their limited impact on the environment, we recommend the use of peroxides, e.g. H_20_2 and PAA, to inactivate *A. ocellatum* development and prevent its propagation. Future studies should aim at evaluating the toxicity of peroxides, in particular PAA, on marine aquaculture species farmed in Portugal and in the Mediterranean region such as the European seabass, *D. labrax*, and the gilthead seabream, *S. aurata*. For preventive measures on earth ponds, we confirmed that traditional drying method is highly effective and we recommend its implementation before a new production cycle. CaO can also be applied prior to a new production cycle to prevent outbreaks of *A. ocellatum*, although high quantities are needed to achieve the effective dose (10000 ppm) thus costs should be carefully evaluated. While a reduction of the salinity in earth ponds seems impractical due to freshwater requirements, it represents a suitable and cost-effective method for implementation in indoor tanks. The installation of a UV-C light disinfection system targeting inlet water is also a cost-effective method to prevent *A. ocellatum* infection of earth ponds in particular by cysts that are present in the water column. Future studies should aim at validating the recommendations proposed here but also at developing methods for the rapid diagnostic of the disease as a timely inactivation of the parasite is critical to animal welfare but also to limit economical loss due to massive death of the diseased fish. The development of new antiparasitic compounds and immunostimulants should also be investigated to provide new solutions for the prevention and treatment of *A. ocellatum* outbreaks.

Table 5. Synthesis of preventive and treatment options for tomonts of *Amyloodinium ocellatum.*

Application	Measures	Efficacy
Earth pond – with fish	Copper 0.1 ppm daily, repeated	No direct effect on tomonts. Reduction of dinospore production.
	Hydrogen peroxide (35% active H_2O_2) 250 ppm 1h flush bath, repeated	No direct effect on tomonts. Reduction of dinospore production.
Earth pond – no fish	Drying pond bottom	Total tomont inactivation.
	Calcium oxide at 10000 ppm	Total tomont inactivation.
	Chlorine 250 ppm 1h flush – must be inactivated before discharge	Total tomont inactivation.
Indoor tanks – with fish	Copper 0.1 ppm daily, repeated	No direct effect on tomonts. Reduction of dinospore production.
	Hydrogen peroxide (35% active H_2O_2) 250 ppm 1h flush bath, repeated	No direct effect on tomonts. Reduction of dinospore production.
	Formalin 250 ppm 1h flush bath, repeated	No direct effect on tomonts. Reduction of dinospore production.
	Reduction of salinity of seawater (5-10‰)	Inactivation of tomonts (25-50%). Reduction of dinospore production.
Inlet water	UV-C – 46.4 mJ/cm2	Inactivation of tomonts (71%) and no dinospore release.
Equipment/materials	Desiccation (≥ 30 min)	Total tomont inactivation.
	Chlorine ≥ 250 ppm 1h	Total tomont inactivation.
	Virkon S 1000 ppm 1h	Inactivation of tomonts (66%) and no dinospore release.

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