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Bioactivity of extracts from extremophile microalgae *Coccomyxa* sp. on chloride cells in operculum tissue of *Fundulus heteroclitus*

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RIASSUNTO

Le microalghe *Coccomyxa* sp., studiate in questa tesi, sono organismi poliestremofili isolati dalle acque di drenaggio della miniera di S. Domingo, ambiente caratterizzato da bassi valori di pH (< 3) e alta concentrazione di metalli disciolti come rame e ferro.

OBIETTIVI

Valutazione del potenziale bioattivo di estratti cellulari ottenuti da colture di *Coccomyxa* sp. coltivate a pH 7 con o senza l'esposizione a rame (0.6mM Cu^{2+}), nel modello animale *ex-vivo* (*Fundulus heteroclitus*), e nell'espressione dei geni selezionati.

Il lavoro è stato sviluppato in tre sezioni principali:

- Confermare la cultura uni-algale di *Coccomyxa* sp. ceppo VAL007;
- Caratterizzazione funzionale degli estratti di *Coccomyxa* sp. sul modello animale *ex vivo Fundulus heteroclitus* attraverso la tecnica elettrofisiologia (Ussing chamber);
- Coltura *in vitro* del tessuto opercolare di *Fundulus heteroclitus* alla presenza di estratti di microalghe e analisi dell'espressione genica (NKCC1, NKCC2 e CFTR) successiva a essa.

MATERIALI E METODI

Prima di iniziare la sperimentazione sul modello animale *ex vivo Fundulus heteroclitus,* è stata accertata la sola presenza di *Coccomyxa* sp. ceppo VAL007 nella cultura algale da cui sono stati ottenuti successivamente gli estratti. Per verificare lo stato di partenza della cultura di *Coccomyxa* sp., attraverso amplificazione, clonaggio e sequenziamento del frammento di DNA, sono stati utilizzati due set di primers: la prima coppia di primers è universale per il gene 18S, avente come target un frammento del genoma nucleare, la seconda è una coppia primers specifica per il gene 23S presente nel genoma dei cloroplasti. In seguito, la coltura iniziale è stata suddivisa in due colture distinte, in una è stato aggiunto rame (0.6 mM Cu^{2+}), mentre l'altra è rimasta invariata. 8 giorni dopo l'aggiunta di rame, a una delle due colture, è stato effettuato il conteggio delle cellule con camera di Bürker e sono stati preparati gli estratti attraverso omogeneizzazione meccanica.

Gli estratti sono stati somministrati all'epitelio opercolare di *Fundulus heteroclitus,* un pesce caratterizzato da un epitelio opercolare ricco di cellule cloruro-secernenti, in cui sono presenti tre proteine di trasporto ionico: il cotrasportatore basolaterale Na^+-K^+-$2Cl^-$ (NKCC1), il regolatore apicale di conduttanza trans membrana della fibrosi cistica (CFTR) e il cotrasportatore apicale NKCC2.

Le potenzialità bioattive degli estratti di *Coccomyxa* sp. sono state testate, con la tecnica dell'elettrofisiologia attraverso l'utilizzo del Ussing chamber, sul tessuto epiteliale di *Fundulus heteroclitus.* Utilizzando questa tecnica è possibile misurare il flusso ionico del tessuto attraverso "voltage clamp to 0"

by "short circuit current" (intensità di corto circuito, Isc), che consente di mantenere il tessuto a un potenziale zero, e di misurare quindi la corrente necessaria per mantenerla tale; questa corrente (Isc) è direttamente correlata al flusso di Cl^-, quindi più il valore di Isc si avvicina a zero, minore è il flusso di Cl^- e viceversa. Per testare l'effetto degli estratti di *Coccomyxa* sp., con l'aggiunta di rame e non, sulla variazione dell'Isc, sono stati somministrati in concentrazioni crescenti (millioncell/ml) e in lati differenti, basolaterale e apicaledel tessuto.

L'esperimento successivo valuta l'effetto degli estratti di *Coccomyxa* sp., sulla regolazione dell'espressione genica di cotrasportatori specifici (NKCC1, NKCC2 e CFTR) sulla coltura *in vitro* del tessuto opercolare di *Fundulus heteroclitus* attraverso Real-Time PCR. Prima di estrarre l'RNA, ogni epitelio di *Fundulus heteroclitus* è stato diviso in tre parti, una parte incubata con estratti di *Coccomyxa* sp.($16x10^6$ cells/ml), un'altra con estratti con rame e un'altra come controllo, per un periodo di 24 ore. Dopo l'incubazione dei tessuti è stato estratto l'RNA, rimosso il DNA genomico e sintetizzato il cDNA, utilizzato nella Real-Time PCR. Come gene di referenza è stato scelto 18S.

RISULTATI

Dai risultati ottenuti dall'analisi della sequenza di DNA è stata confermato lo stato di cultura uni-algale *Coccomyxa* sp. ceppo VAL007.

I valori basali di Isc (-211.3 ± 24.8 μA/cm²) confermano che in queste condizioni sperimentali Isc è una diretta misura del flusso di Cl^-.

Per quanto riguarda l'effetto di estratti di *Coccomyxa* sp., somministrati al modello animale *ex-vivo Fundulus heteroclitus* attraverso la tecnica elettrofisiologia, solo la combinazione dei fattori, lato basolaterale ed estratti con aggiunta di rame, hanno causato un decremento dei valori di Isc fino a raggiungere il 100% di inibizione (significatività statistica p=0,0003), con meno di 64×10^6 cell/ml; mentre per le altre combinazioni di fattori, la stessa concentrazione, non è stata sufficiente a raggiungere il 50% di inibizione.

In contrapposizione con i risultati ottenuti con la tecnica dell'elettrofisiologia, non si è verificata nessuna variazione statisticamente significativa per quanto riguarda l'effetto degli estratti di *Coccomyxa* sp., sulla regolazione dell'espressione genica di co-trasportatori specifici (NKCC1, NKCC2 e CFTR) sulla coltura *in vitro* del tessuto opercolare di *Fundulus heteroclitus.*

DISCUSSIONE E CONCLUSIONI

L'effetto degli estratti di *Coccomyxa* sp., somministrati al modello animale *ex vivo Fundulus heteroclitus,* attraverso la tecnica elettrofisiologia, evidenzia l'importanza del lato in cui vengono somministrati e il pretrattamento che subiscono le microalghe e avendo come target il cotrasportatore NKCC1, questo è stato dimostrato con l'uso del diuretico bumetanide, un inibitore specifico del NKCC1, in quanto addizionando sullo stesso tessuto, in tempi differenti, sia bumetanide che estratti con rame, si instaurava una sorta di competizione tra i due composti. In studi precedenti è stato dimostrato che anche la sola aggiunta di rame al tessuto opercolare causa una diminuzione

dei valori di Isc, ma con danni irreversibili, invece i tessuti trattati con estratti di microalghe con rame, una volta lavati, riprendevano le funzionalità al 100%. Questo può essere dovuto alla capacità delle microalghe di produrre composti che eliminano la tossicità del metallo, a volte traendone vantaggio.
Per quanto riguarda il mancato effetto degli estratti di *Coccomyxa* sp., sulla regolazione dell'espressione genica di cotrasportatori specifici (NKCC1, NKCC2 e CFTR) sulla coltura *in vitro* del tessuto opercolare di *Fundulus heteroclitus*, si possono formulare tre ipotesi: i) gli estratti di *Coccomyxa* sp., con o senza rame, non hanno influenza sulla regolazione dell'espressione genica. ii) La concentrazione degli estratti di *Coccomyxa* sp. non era sufficientemente elevata. iii) Il periodo di incubazione del tessuto opercolare di *Fundulus heteroclitus* non era sufficiente per conseguire a cambiamenti. Queste tre ipotesi meritano successivi studi futuri, visto le potenzialità degli estratti di *Coccomyxa* sp., riscontrate in questo studio.

ABSTRACT

Microalgae have been studied because of their great potential as a source of new compounds with important value for biotechnology and to understand their strategies of survival in extreme environments. The microalgae *Coccomyxa* sp., studied in this thesis, is a poly-extremophile witch was isolated from the acid mine drainage of S. Domingos mine. This environment is characterized by low pH (<3) and high concentration of metals, such as copper and iron. The main purpose of the present work was to evaluate the potential bioactivity in an *ex-vivo* animal model (*Fundulus heteroclitus*), and expression on selected genes, of cellular extracts obtained from cultures of *Coccomyxa* sp. at pH 7 without or with exposure to copper (0.6mM Cu^{2+}). The extracts of *Coccomyxa* sp. cultured at pH 7 exposed to copper show a great potential to be used as epithelial NKCC inhibitors, revealing their potential use as diuretics, but did not show significant effects on gene expression. *Coccomyxa* sp. could be a good source of cellular extracts with a great potential to be used in pharmaceutical and biotechnology industries.

Keywords: *Coccomyxa,* polyextremophile, microalgae, NKCC, Ussing chamber, qPCR, short circuit current

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I. INTRODUCTION

1. Microalgae

The prokaryotes are the most studied extremophile organisms (Madigan and Oren, 1999; Satyanarayana *et al*., 2005), however the unicellular eukaryotes, for example microalgae, were also reported to inhabit extreme environments (Chong *et al*., 2011; Vaquero *et al., 2012;* Gross*, 2000).* Microalgae have an important role in different ecosystems, they are responsible for 30-50% of the photosynthetic production of the planet (Field *et al.,* 1998). Recently, microalgae have been the subject of many studies because they can absorb sunlight and carbon dioxide to produce energetic compounds (lipids and carbohydrates) which can be converted into bio fuels (Hu *et al.,* 2008; Ridolfi *et al.,* 2009) and protein supplements (Becker, 2007). Moreover, recent studies have been conducted to understand how microalgae survive in extreme environments and to discover new compounds of biotechnological interest (Pulz and Gross, 2004; Vaquero *et al.,* 2012).

1.1. Aquaculture

A range of microalgae species is produced in hatcheries and is used in a variety of ways for commercial purposes. Previous studies have estimated the main factors in the success of a microalgae hatchery system as the dimensions of the container/bioreactor where microalgae are cultured, exposure to light/irradiation and concentration of cells within the reactor (Tredici and Materassi, 1992)

1.2. Extremophiles

An extremophile is an organism that thrives in physically or geochemically extreme conditions that are detrimental to most life forms on Earth (Rampelotto, 2010; Rothschild and Mancinelli, 2001), adapting to unusual limits of one or more abiotic factors in the environment. Some of the extreme conditions are temperature, pH, high salinity, high levels of radiation and high pressure. Most extremophiles are microorganisms such as bacteria and Achaea, since higher organisms generally are less adaptive to wide variations from the norm in environmental conditions. Upper limits of existence for

carbon-based life forms appear to be about 150 degrees Celsius, based upon inherent thermal stabilities of amino acids and polypeptides essential to DNA manufacture. In some cases extremophile metabolism thrives on the exotic variation in environmental conditions; in other situations, there is adaptive behavior such as metabolic diapause (cryptobiosis) or desiccation (endospore

Table 1: unicellular extremophiles classified in several groups based on the type of environment and relative examples.

CLASSIFICATIONS	AMBIENTAL CONDITIONS	EXAMPLES
Psychrophile	Low temperatures (-20°C to 10°C)	*Arthrobacter* sp.
Thermophile	High temperatures (41°C to 121°C)	*Thermus aquaticus*
Acidophile	pH acid (<3)	*Coccomyxa* sp.
Alkaliphile	pH basic (8,5 to 11)	*Natronobacterium pharaonis*
Barophile	High atmospheric pressure (>38 MPa)	*Halomonas salria*
Halophile	High salinity (1-3,5 M of NaCl)	*Dunaliella acidophila*
Oligotroph	Low quantity of dissolved carbon (0,2-16,8-16,8 mg/l)	*Sphingomonas* sp.
Metallotolerant	High concentration of dissolved metal	*Coccomyxa* sp.

formation). Extremophiles are believed to have been some of the earliest life forms on earth, since such early organisms would have to be adapted to harsh conditions, at least in comparison to present day environments. Some microorganism extremophiles have shown industrial potential, such as the ability to remove sulphure compounds from crude oil at high temperatures (Fred *et al.,* 2006).

1.2.1. Classifications

There are many classes of extremophiles that thrive all around the globe; each corresponding to the way its environmental niche differs from mesophilic

conditions. These classifications are not exclusive. Many extremophiles fall under multiple categories and are classified as polyextremophiles. For example, organisms living inside hot rocks deep under Earth's surface are thermophilic and barophilic such as *Thermococcus barophilus* (Marteinsson *et al.*, 1999). A polyextremophile living at the summit of a mountain in the Atacama Desert might be a radioresistant xerophile, a psychrophile, and an oligotroph. Polyextremophiles are well known for their ability to tolerate both high and low pH levels. The unicellular extremophiles can be classified in several groups based on the type of environment (Madigan and Oren, 1999; Satyanarayana *et al*., 2005) **(tab. 1)**.

1.2.2. Adaptation to low pH and high concentrations of metals

The microalgae living in acidic environments have had to adapt to this extreme condition. One of the main factors is the low pH due to a high concentration of protons (H+). The plasma membrane of microalgae is not very permeable to the passage of H+ from the external environment to the intracellular fluid, so the cell does not consume a lot of energy to prevent proton entry, the impermeability of the membrane may be due to the presence of sterols, fatty acids and proteins (Gross, 2000; Brake and Hasiotis, 2010). The microalgae also have the capacity to regulate intracellular pH through ionic transporters present in the cell membrane, such as for example, H+ pump (Messelerli *et al.,* 2005). The mine acid waters possess a low concentration of dissolved phosphate due to the presence of aluminum, phosphate is an essential element for the functioning of each type of microalgae, so they have developed the ability to produce phosphatases resistant to an acid environment (Gross*,* 2000).

Another important factor that microalgae must overcome is the presence of metals such as copper, lead, zinc, aluminum. These metals induce the oxidation of the plasma membrane and possible rupture, in addition to the denaturation of the proteins, blocking of functional groups or decompositions of essential metabolites (Brake and Hasiotis, 2010).

In order to tolerate the high concentration of metals in the water, microalgae must adopt different strategies such as:

- Incorporate lipophilic antioxidants into the membrane (Gross*,* 2000).

- Reduction of the entry of metals or increasing their flow.
- Mechanism of complexation of metals, with precipitation on the extracellular side.
- Grouping in essential biofilms (Brake and Hasiotis, 2010).

1.2.3. Coccomyxa

The microalgae *Coccomyxa* sp. studied in this work, belongs to the family of *Coccomyxaceae* (*Eucaryota; Viridiplantae; Clorophyta; Trebouxiophyceae*), it is a polyextremophile isolated in the acid waters of mine S. Domingos (*Valente et al*., 2013). It includes more than 30 species found in both marine and fresh water, are epiphytic species, symbiotic with lichen, bark and even protozoa parasites. They are unicellular polyextremophiles (acidophilic, metallotolerant) microalgae, able to tolerate low pH levels and high concentration of dissolved metals.

2. The mine of S. Domingos

On Earth there are many acidic environments, generated by geological and anthropogenic activities through the leakage of acid water from mines, which combine low pH to high concentrations of metals. The intense mining activity produced considerable amount of residues, which have caused the environmental deterioration of the zone in all its aspects: soil degradation, water resources pollution, biodiversity decrease, and even, atmospheric pollution in some moments of the history.

The most important environmental problem derives from the sulphide (mainly

Pyrite) oxidation contained in the aforementioned residues. This process produces an extremely acid leachate with high contents of sulphate, metals and metalloids known as acid mine

Figure 1: geographic position of the Iberian Pyrite Belt and related main mining district.

drainage (Lowson, 1982; Parker and Robertson, 1999; Younger *et al.,* 2002). The mine of S. Domingos is located in the northern sector of the Iberian Pyrite Belt, about 5 km from the Spanish border **(fig. 1)**. It's one of the most emblematic Portuguese massive sulphide deposits and one of the world's largest reserves of mineral sulfur. Although mining activity has ceased at present, the large-scale exploitation of this deposit between 1857 and 1966 favored the production of enormous waste dumps, where oxidation of pyrite and associated sulphide is resulting in the acid mine drainage production. The final acid discharge with high contents of metals, as copper, lead, zinc, with amounts of copper which can reach a maximum of 1468 mg/Kg, and low pH (<3), from S. Domingos reaches the Chanza river, main effluent of Guadiana river, causing its partial pollution (Pinho *et al*., 2014).

The field-bearing secondary minerals within waste deposits indicate that these materials are reactive. Jarosite and other secondary low crystalline minerals as oxides, ox-hydroxides, and iron hydroxyl-sulphates are important for the environment as they play a crucial role in the solubility of the potentially toxic chemical elements (Alvarez-Valero *et al.,* 2007).

3. *Fundulus heteroclitus*

The *Fundulus heteroclitus*, is an exceptionally wide ranging cyprinodont fish. It naturally occurs along the east coast of North America from southwestern Newfoundland to northeastern Florida. This species is ubiquitous in North American East Coast salt marshes, being mostly found in sheltered coastal waters. Although occasionally it inhabits freshwater habitats, the species is best known from the tidal salt marsh, a fluctuating physical environment for which they are well adapted due to considerable plasticity in their ecological requirements (Kneib, 1986).

As adults, *Fundulus heteroclitus* range between 12.7 and 17.8 centimeters in length, the females growing larger than the males. They have flattened heads and the mouth is turned upward, clearly an adaptation to feeding at the surface of the water. This attractive fish is dimorphic, males are darker in color than females and exhibit blue or orange markings during the breeding season, are dark olive green on the dorsal side and lighter yellow on the ventral side. They also display vertical stripes along their sides. Females are silvery yellow

on the ventral side and that color gradually fades to a more distinct yellow on the dorsal side. All *Fundulus heteroclitus* have a single soft dorsal fin and their pelvic fins are located close to the rear fin(National Oceanic and Atmospheric Administration Coastal Services Center, 2001).

Fundulus heteroclitus is the premier model for environmental biology (Burnett et al., 2007). The inner opercular epithelium of the killifish *Fundulus heteroclitus* is rich in chloride cells and provides a proxy model system to study chloride cell function (Karnaky et al., 1977). When the membrane is removed and mounted in Ussing chambers, the short circuit current is equivalent to Cl^- secretion rates (Degnan et al., 1977)

4. Objectives

The main objectives of the present work was to evaluate the potential bioactivity in an *ex-vivo* animal model (*Fundulus heteroclitus*), and expression on selected genes, of cellular extracts obtained from cultures of *Coccomyxa* sp.at pH 7 without or with exposure to copper (0.6mM Cu^{2+}). The work was developed into three main parts:

- Establishment of unialgal culture of *Coccomyxa* sp. strain VAL007 (chapter I);
- Functional characterization of *Coccomyxa* sp. extracts on animal model *ex-vivo Fundulus heteroclitus* through epithelial electrophysiology techniques (chapter II);
- Opercular tissue of *Fundulus* was cultured *in vitro* in the presence of microalgae and the expression of NKCC1, NKCC2 and CFTR was analyzed (chapter III).

II. ESTABLISHMENT OF UNIALGAL CULTURES OF *Coccomyxa* sp. STRAIN VAL007

1. *Coccomyxa* sp. strain VAL 007

The microalgae *Coccomyxa* sp. strain VAL007 studied in this work, belongs to the family *Coccomyxaceae* (*Eucaryota; Viridiplantae; Clorophyta; Trebouxiophyceae*). It is a unicellular polyextremophile isolated from the acid waters of the S. Domingos mine (*Valente et al*., 2013). The genus *Coccomyxa* includes more than 30 species found in both marine and fresh water. These can be epiphytic species, symbiotic with lichen and tree bark and even protozoa parasites. Some of the species are polyextremophiles (acidophile, metallotolerant) microalgae, able to tolerate low pH levels and high concentration of dissolved metal (Vaquero *et al*., 2012; Leonardo *et al*., 2016). Most of the published studies have focused on the characterization of the physiological adaptations to these conditions, the optimization of culture conditions for the production of metabolites of interest and use in bioremediation technology of environments contaminated with metals or radioisotopes (Leonardo *et al*., 2016). As a way to test the possible application in human medicine, some studies have used extracts of these microorganisms and analyzed their effects *in vitro* and *in vivo*. A recent study by Komatsu *et al*. (2013) evaluated the antiviral activity of *Coccomyxa gloeobotrydiformi* on cells infected by the human influenza virus A and another study by Sun *et al*., (2013), using rats have as the classic model of myocardial occlusion of the middle cerebral artery, observed the therapeutic effects of *Coccomyxa gloeobotrydiformis* on the recovery from ischemic stroke.

2. Materials and Methods

2.1. Verification of unialgal cultures of *Coccomyxa* sp.

To verify that the starting cultures consisted only of *Coccomyxa* sp., isolated and maintained in the Laboratory of Microbial Molecular Ecology (UAlg-CIMA), the following steps were performed **(fig. 2)**. Three samples of 2 ml each were collected from each starting culture and the cells recovered after

centrifugation at 3500 g for 1 minute. The culture medium was discarded and the pellet was used for DNA extraction.

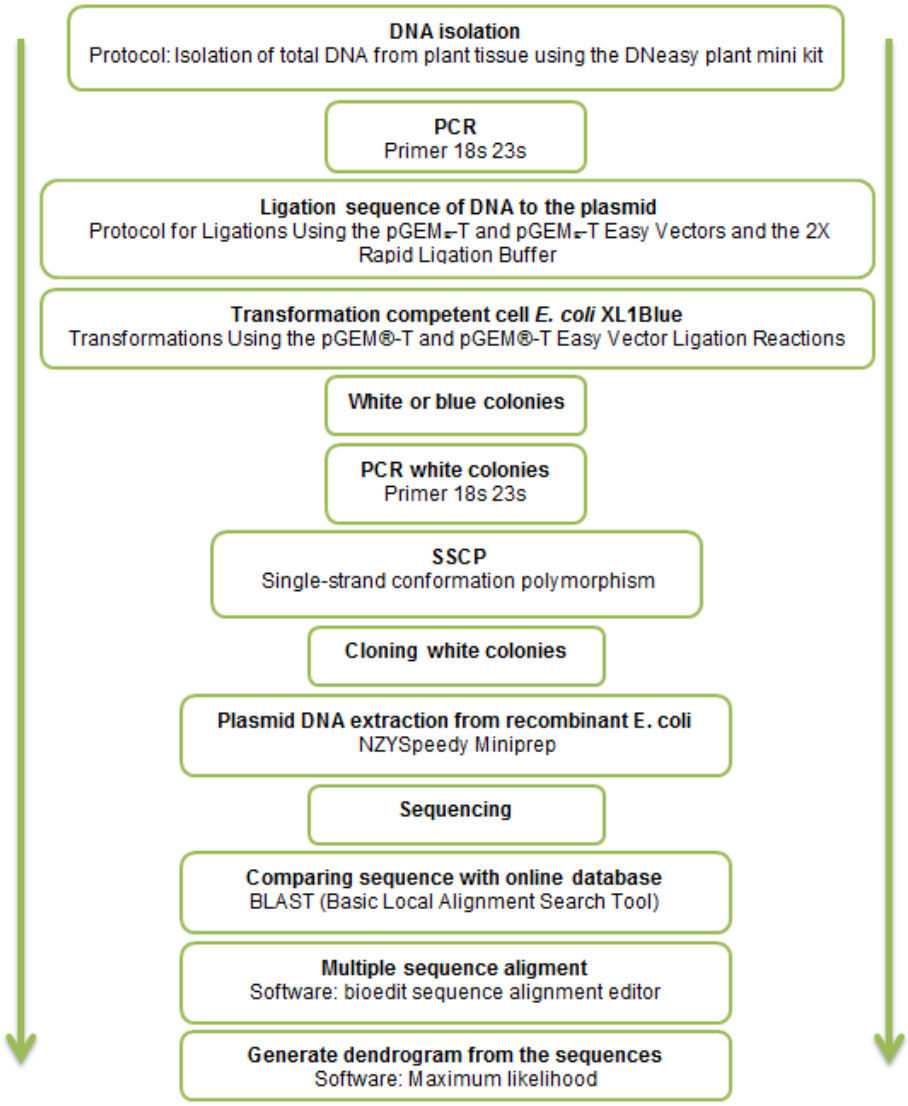


Figure 2: sequence of steps to verify the unialgal state of the starting cultures of *Coccomyxa* sp.

2.2. DNA isolation

Protocol: Isolation of total DNA from plant tissue using the DNeasy plant mini kit. (QUIAGEN Companies, DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit Handbook 07/2003).

1. Grind plant tissue under liquid nitrogen to a fine powder using a mortar and pestle. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Continue immediately with step 2.

2. Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) to a maximum of 100 mg disrupted plant and vortex vigorously.

3. Incubate the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation by inverting tube.

4. Add 130 µl Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.

Recommended: Centrifuge the lysate for 5 min at 14,000 rpm.

5. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 14,000 rpm.

6. Transfer the flow-through fraction from step 5 into a new tube (not supplied) without disturbing the cell-debris pellet.

7. Add 1.5 volumes of Buffer AP3/E to the cleared lysate, and mix by pipetting.

8. Pipet 650 µl of the mixture from step 7, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at ≥8000 rpm, and discard the flow-through. Reuse the collection tube in step 9.

9. Repeat step 8 with remaining sample. Discard flow-through and collection tube.

10. Place the DNeasy Mini spin column into a new 2 ml collection tube (supplied), add 500 µl Buffer AW2, and centrifuge for 1 min at ≥8000 rpm. Discard the flow-through and reuse the collection tube in step 11.

11. Add 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 14,000 rpm to dry the membrane.

12. Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied), and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25°C), and then centrifuge for 1 min at ≥8000 rpm to elute.

13. Repeat step 12 once.

2.3. PCR

The DNA samples obtained were subjected to PCR (polymerase chain reaction) amplification, using universal primers for the 18S gene (nuclear genome) and the 23S gene (chloroplastidial genome). The PCR is a technique for amplification of DNA *in vitro* using thermostable DNA

polymerases. It involves repetitive cycling of three steps: denaturation, annealing and elongation **(fig.3)**. The entire cycling process of PCR is automated and can be completed in just a few hours using a thermal cycler **(tab. 2).**

Table 2: PCR program used for the PCR amplification of a fragment of the 18S gene or the 23S gene.

PCR program	
94 °C	3"
94 °C	30'
48 °C	30'
72 °C	45'
72 °C	10'

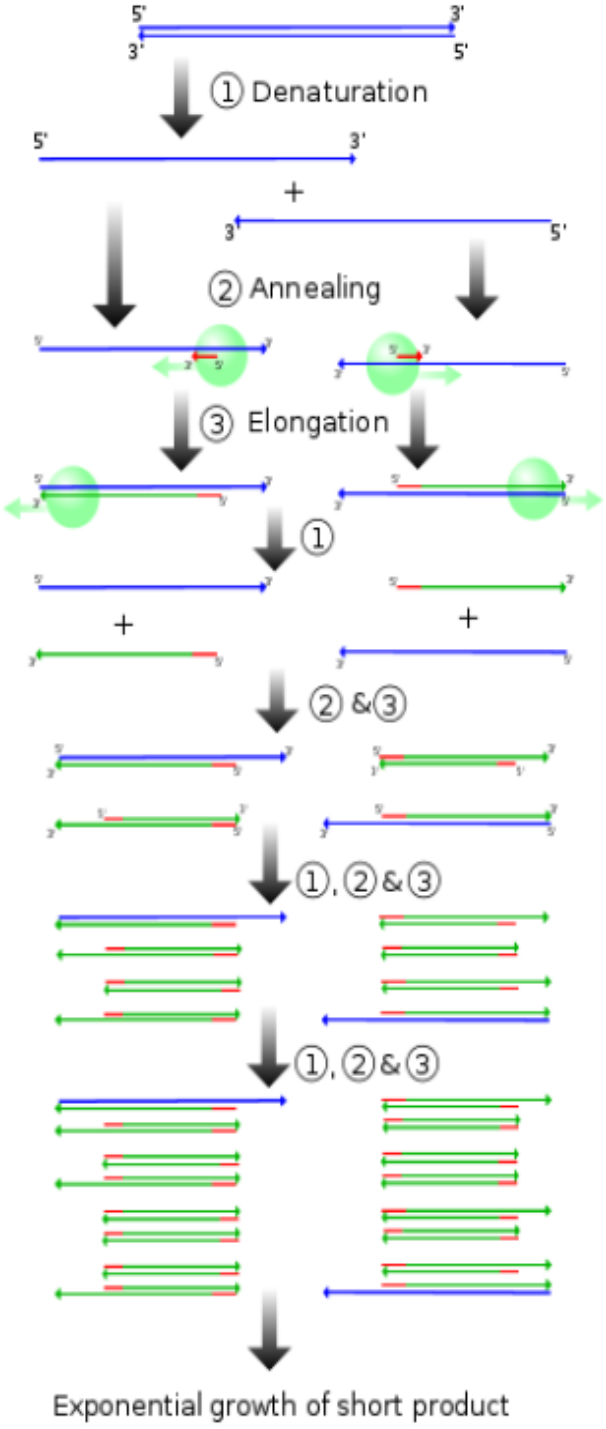


Figure 3: sequence of PCR steps:
1- Denaturation step: This step consists of heating the reaction to 90–95 °C. It causes DNA separation by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA.
2- Annealing step: The reaction temperature is lowered to 50–65 °C allowing hybridization of the primers to the single-stranded DNA template.
3- Elongation step: At this step, the Taq polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction.

The PCR technique requires the following components and reagents:

- Primers (Forward & Reverse) **(tab. 3)**: short pieces of artificially prepared DNA that will target the gene fragment of interest in the entire genome. They are complementary to the 3' ends of each strand of the double stranded target gene i.e. DNA.

Table 3: Primer pairs used in this work, selected to amplify two regions with phylogenetic information to molecularly identify phototrophic eukaryotes: 18S gene for 18SrRNA (nuclear DNA) and 23S gene for 23S rRNA (chloroplastidial DNA).

Gene	Primer	Sequence 5'→3'	bp	References
18s	18s_f	GTCAGGGTGAAATTCTTGGATTTA	700	Rasoul-Amini *et al*., 2009
	18s_r	AGGGCAGGGACGTAATCAACG		
23SrV	23SrV	GGACAGAAAGACCCTATGAA	410	Sherwood and Presting, 2007
	23SrV	TCAGCCTGTTATCCCTAGAG		

- Taq polymerase (DNA polymerase): It's an enzyme whose function is to extend the new DNA strand. *Taq* polymerase attaches near the 3' end of the primer and starts adding nucleotides. It requires double stranded DNA to become functional. The DNA polymerase in our bodies breaks down at temperatures below 95 °C, the temperature necessary to separate two complementary strands of DNA in a test tube.

- Deoxynucleotide triphosphate (dNTP's): They are the building blocks from which the DNA polymerases synthesizes a new DNA strand, Taq polymerase grabs nucleotides that are floating in the liquid around it and attaches them to the 3' end of a primer.

PCR reactions were done in a final volume of 50 µl (45 µl of PCR reaction components and 5 µl of DNA sample) **(tab. 4)**.

Table 4: final concentration of PCR reaction components in a final volume of 50 µl (45 µl of PCR reaction components and 5 µl of DNA sample).	
PCR reaction components	
H_2O $_{sterile}$(DNase and RNase free, Sigma-Aldrich, USA)	
Taq Buffer	
$MgCl_2$	0,2 mM
dNTPs	0,2 µM
Primer Fw	0,2 µM
Primer Rv	0,2 µM
Taq	1U

2.4. Ligation of DNA fragments to the plasmid-vector

The results of each PCR amplification were visualized under UV light, after electrophoresis on agarose gels (1% in buffer TAE) stained with ethidium bromide. The fragments of the expected size (700 bp for 18S and 410 bp for 23S) were ligated to the plasmid-vector following the Protocol for Ligations Using the pGEM®-T Easy Vector and the 2X Rapid Ligation Buffer **(tab. 5).**

Table 5: Reaction Component for final volume of 10 µl (3 µl of PCR product and 1 µl of the vector).	
Reaction Component	
2X Rapid Ligation Buffer, T4 DNA Ligase	1 µl
pGEM$_R$-T or pGEM$_R$-T Easy Vector (50ng)	1 µl
PCR product	3 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
nuclease-free water to a final volume of 10 µl	

The reactions were mixed by pipetting and incubated for 1 hour at room temperature.

2.5. Transformation of competent cells - *E. coli* XL1Blue

The constructs obtained were used to transform *E. coli* strain XL1Blue competent cells, prepared in the lab and kept at -80°C.

Transformation protocol:

1. Prepare two LB-AGAR/ampicillin/IPTG/X-Gal plates for each transformation,
2. Add 2µl of each ligation reaction to an aliquot of 100 µl of competent cells defrosted and kept on ice. Gently flick the tubes to mix and place them on ice for 30 minutes.
3. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C (do not shake).
4. Immediately return the tubes to ice for 2 minutes.
5. Add 250µl room temperature SOC medium (LB liquid with glucose 20 mM) to the tubes and incubate for 45 minutes at 37°C with shaking (~200rpm).
6. Plate 150µl of each transformation culture onto duplicate LB-AGAR/ampicillin/IPTG/X-Gal plates.
7. Incubate the plates overnight (16–24 hours) at 37°C. White colonies will contain the desired inserts.

2.6. Verification of inserts in white colonies

After the cultivation of the transformed cells was carried out, white colonies on each plate were subjected to a PCR using the same primer pair **(tab. 3)** with which the inserted fragment had been obtained, and the same reaction components **(tab. 4)**. Size of the obtained fragments was verified after electrophoresis on agarose gels (1% agarose in TAE), stained with ethidium bromide and under UV light.

2.7. SSCP (single strand conformation polymorphism) analysis

In order to select recombinant clones for sequencing a single strand conformation polymorphism analysis (SSCP, Orita *et al*., 1989) was conducted. This analysis allows the detection of a SNP (single nucleotide polymorphism) in single strand sequences of up to 700 nt. For this the double stranded DNA is initially denatured and subjected to electrophoresis in

polyacrylamide gels. Polymorphisms between sequences of the same size are detected as different band patterns on the gels after staining, usually with silver nitrate.

For this analysis, 1 µl of the PCR product was added to 9 µl of denaturing buffer (95% formamide, 20 mM EDTA pH 8.0, stained with bromophenol blue), denaturated at 90 °C for 5 min and placed on ice. The denatured DNA was subjected to electrophoresis in 8% polyacrylamide gels (1X TBE buffer), conducted at 4 °C and 200 V, during 2 hours. After electrophoresis, the polyacrylamide gels were stained with silver nitrate. For this the gels were initially placed in a solution of 10% glacial acid acetic during 20 minutes (fixative) after which they were washed 3 times for 3 minutes with distilled water. Next, the gels were passed into a 1% nitric acid solution for 3 minutes, followed by 3 times 3 minute washes with distilled water. The gels were then immersed in a silver nitrate solution (1g/L $AgNO_3$, 1,5 ml/L formaldehyde) during 30 minutes, after which they were washed with distilled water for 3 times and revealed with a sodium carbonate solution (30 g/L Na_2CO_3, 1,5 ml/L formaldehyde, 2 mg/ml NaS_2O_3 + $5H_2O$). The reaction was stopped placing the gels in the initial acetic solution.

Recombinant clones evidencing different SSCP patterns, were selected for plasmid extraction.

2.8. Plasmid DNA extraction from recombinant *E. coli* colonies

The colonies of interest were grown over night in LB liquid with ampicillin.

Plasmid DNA extraction was carried out using the NZYMiniprep kit (NZYTech, Portugal).

Protocol for plasmid DNA purification (miniprep) from *E. coli* cells

All centrifugations were carried out at room temperature in a tabletop microcentrifuge at 10000-15000 rpm.

1. Harvest bacterial cells: Pellet 1-5 ml of an *E. coli* LB culture for 30 s. Discard supernatant. Remove as much media as possible.

2. Cell lysis: Re-suspend cell pellet completely in 150 µl of Buffer A1 by vigorous vortexing/pipetting. Add 250 µl of Buffer A2 and mix gently by inverting the tube for 5 times. Incubate at room temperature (10-25 ºC) for up

to 2 min. Do not vortex. Add 300 µl of Buffer A3. Mix gently by inverting the tube for 6-8 times. Do not vortex.

3. Clarification of lysate: Centrifuge for 3 min at room temperature to pellet precipitate.

4. Bind DNA: Place NZYTech spin column in a 2 ml collecting tube and load the supernatant from step 3 onto the column. Centrifuge for 30 s. Discard flow-through.

5. Wash silica membrane: Add 600 µl of Buffer AS (make sure ethanol was previously added). Centrifuge for 1 min. Discard flow-through.

6. Dry silica membrane: Re-insert the NZYTech spin column into the empty 2 ml collecting tube and centrifuge for 1 min.

7. Elute highly pure DNA: Place the dried NZYTech spin column into a clean 1.5 ml microcentrifuge tube and add 50 µl of Buffer AE. Incubate for 1 min at room temperature (10-25 ºC). Centrifuge for 1 minute and store the purified DNA at -20 °C.

2.9. Sequencing

Sequences were obtained through commercial sequencing (CCMar, Faro, Portugal) by the Sanger Method.

2.10. BLAST analysis and phylogenetic inference

The sequences obtained were subjected to a BLAST analysis at the NCBI (National Center for Biotechnology Information) site (blast.ncbi.nlm.nih.gov). This software makes a search per similarity searches and compares the submitted sequences with sequences from cultured organisms present in the database, thus allowing the identification of the organisms present in the starting cultures.

The homologous sequences (at least 98% homology) were retrieved from the database and aligned with the sequences from this study, using BioEdit (Hall, 1999) and ClustalW (Thompson et al., 1994) softwares. The dendrograms for phylogenetic inference were constructed in MEGA5 (Tamura et al., 2011) and the Maximum Likelihood Method with 1000 bootstraps.

3. Results

From the SSCP analysis 7 different patterns were obtained for the cloned fragments of the 18S gene. The dendrogram obtained with the corresponding sequences and homologous sequences retrieved from the Genbank database (NCBI), including sequences from *Chlorella* as anoutgroup **(fig. 4)**, revealed that all sequences from this study are closely related to sequences obtained from Coccomyxa species. No sequence from another organism was obtained. The same was observed with the 8 sequences obtained for the 23S gene fragment **(fig. 5)**.

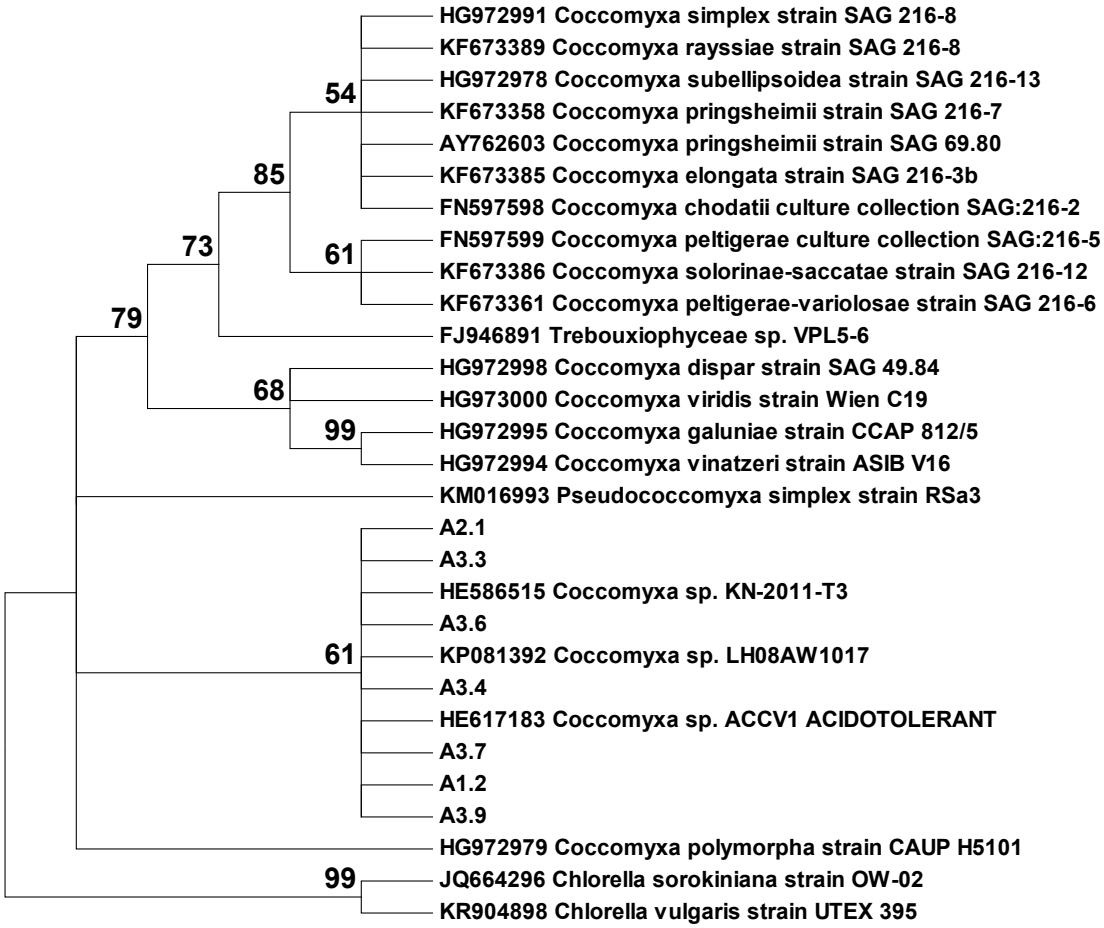


Figure 4 : Dendrogram (Maximum Likelihood method) obtained based on the alignment of partial 18S sequences obtained in this work (A1, A2, A3) and sequences retrieved from GenBank, showing at least 95% homology in a BLAST analysis. Bootstrap values are shown next to each node.

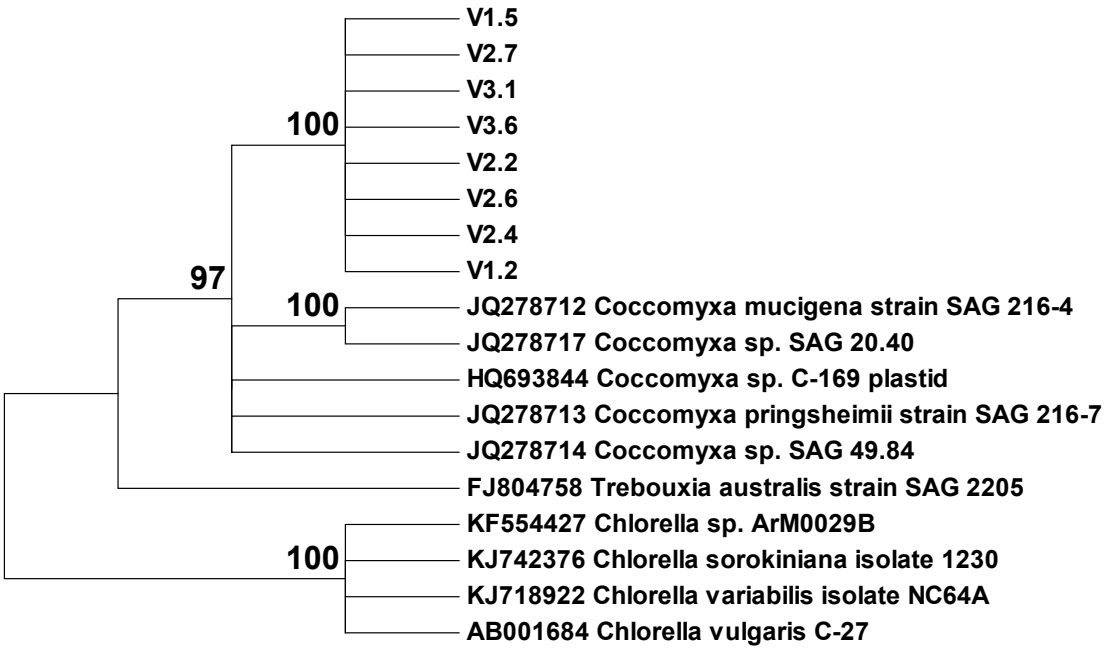


Figure 5: Dendrogram (Maximum Likelihood method) obtained based on the alignment of partial 23S sequences obtained in this work (V1, V2, V3) and sequences retrieved from GenBank, showing at least 95% homology in a BLAST analysis. Bootstrap values are shown next to each node.

III. FUNCTIONAL CHARACTERIZATION OF *Coccomyxa sp.* EXTRACTS

1. Material and Methods

1.1. Preparation of *Coccomyxa* sp. cultures

When the study began, the starting cultures of *Coccomyxa* sp. were being maintained in growth medium K9 **(tab. 6)** at pH 2,5 in cell culture flasks with a lid fitted with a 0.2 µm filter (to promote the exchange of gases and prevent contamination with microorganisms) at 24 °C temperature and 50 µmol/m^2/s light intensity at 12 hours daily. This microalgae is not mobile in the liquid column and forms a film at the bottom of the culture flasks.

Table 6: Growth medium K9 composition	
$CaCl_2 + 2H_2O$	0,1 mM
$MgCl_2 + 6H_2O$	2mM
KCl	1mM
K_3PO_4	2mM
KNO_3	20mM
K_2SO_4	20 mM
Solution of the following elements	5 ml/l
H_3BO_3	70 mM
$C_{10}H_{16}N_2O_8$	30 mM
$CoCl_2 + 6H_2O$	3mM
$MnCl_2 \cdot 2H_2O$	10mM
$(NH_4)_6Mo_7O_{24} + 4H_2O$	0,4mM
$CuSO_4 + 5H_2O$	3 mM
$FeSO_4 + 7H_2O$	10mM
$ZnSO_4 + 5H_2O$	30 mM

According to results of previous studies (Bras, 2015), the experiment was set up to obtain extracts of this microalgae from cultures at pH 7 without addition of copper (Cu^{2+}) and with addition of Cu^{2+} at a final concentration of 0.6 mM. At the beginning of this work the growth medium of the starting culture was removed and new growth medium at pH 2.5 was added. The cells were gently resuspended and samples were taken for cell counting with the Bürker

chamber. After 5 days the growth medium was removed and new medium was added at pH 7. The culture was left to stabilize for 5 days, after which time samples were taken for cell counting and the final volume of growth medium was adjusted to approx. 100 x 10^6 cells/ml. Following this, two times 75 ml of culture were taken into separate clean cell culture flasks, to one of which 0,375 ml of copper sulphate ($CuSO_4$) at 120Mm was added, to obtain a final concentration of 0.6 mM in the growth medium. Microalgae samples were taken 8 days after, for extract preparation. The following figure **(fig. 6)** shows the scheme of the experimental procedure for obtaining each type of culture.

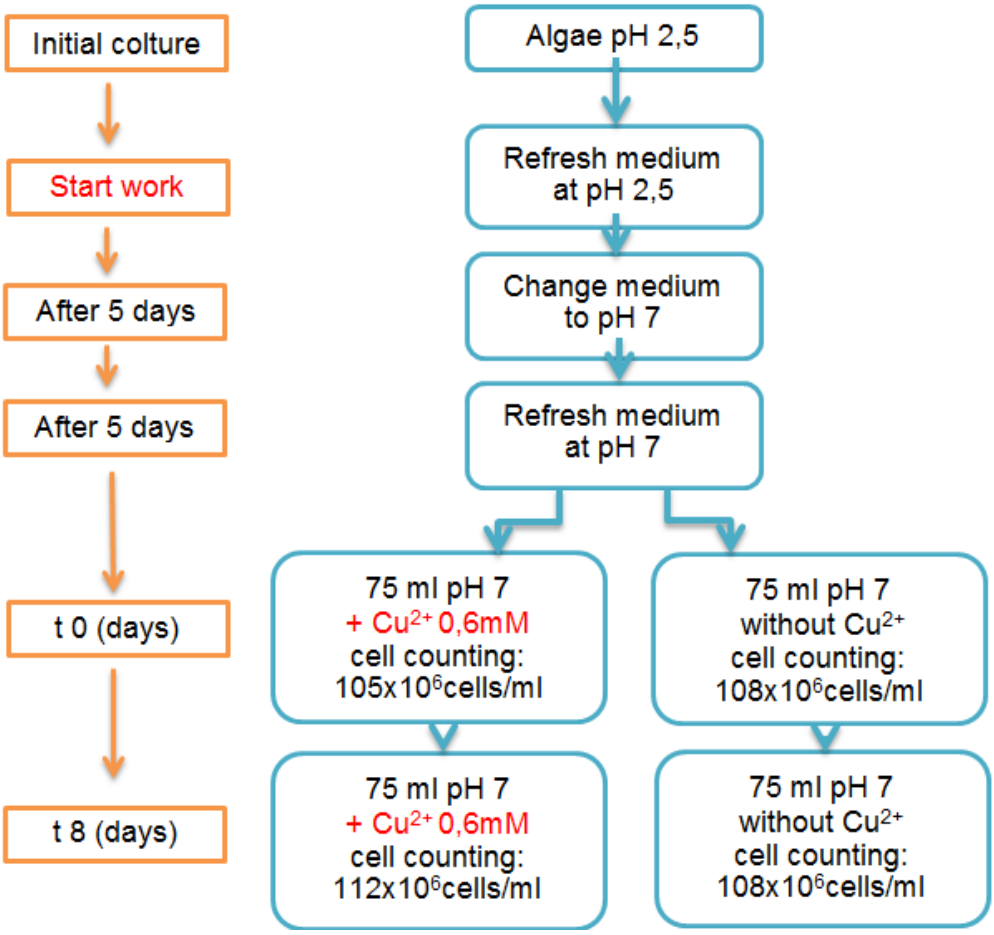


Figure 6: scheme of the experimental procedure for obtaining the extracts after 8 days (t8) of the addition of Cu^{2+}

1.2. Determination of culture cell density

The cell density was determined using the Bürker chamber **(fig. 7)** with the use of an optical microscope with incorporated camera (Leica DFC480, Leica

Microsystem, Wetzalar, Germany). Prior to counting the algal samples in were diluted 1:100. From this dilution 10 µl were taken and loaded into the Bürker chamber. Each sample was counted twice.

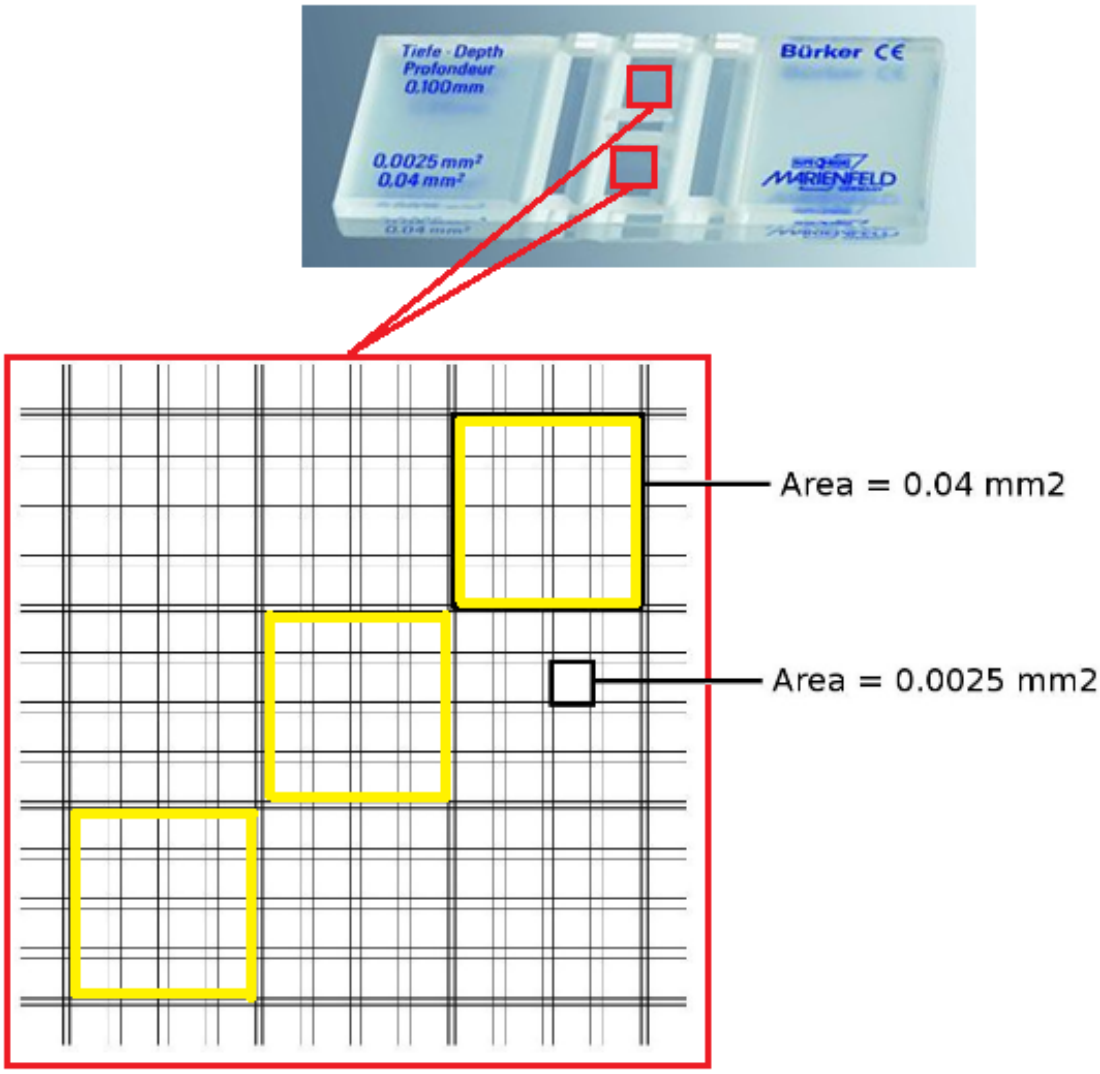


Figure 7: Representation of the Bürker chamber. The chamber depth is 0.1 mm. The ruling shows 9 large squares of 1 mm^2 each. The large squares are subdivided into 16 group squares with 0.2 mm sides. In the central large square, each group square is subdivided into 16 mini squares with 0.05 mm sides (= 0.0025 mm^2). To perform the counting were used the squares highlighted in yellow.

After counting the number of cells the following formula was applied to obtain the number of cells per milliliter.

$$N^{\circ}\,cells/_{ml} = \left(\frac{n^{\circ}\,cells}{deep\ (\mu m)\ \times area\ (\mu m^2)\ \times\ n^{\circ}\,squars}\right) \times dilution \times 10^{12}$$

1.3. Preparation of cell extracts

For the preparation of the microalgae extracts, from the culture at pH 7 and the culture at pH7 with 0.6 mM of Cu^{2+}, samples of 1.8 ml were collected into 2ml Eppendorf tubes and centrifuged at 3500 x g for 1 minute. The growth medium was discarded and 1 ml of phosphate buffered saline (PBS 1 x) was added to wash the cells, followed by centrifugation at 3500 x g for 1 minute. The PBS was discarded and the precipitated cells were stored at -20°C. A total of 48 vials, 24 with microalgae from the culture with addition of Cu^{2+} and 24 with microalgae from the culture without Cu^{2+} were obtained. Prior to testing, crude cell extracts were prepared by mechanical homogenization according to the method described below.

Procedure to obtain an extract from cells precipitated from in 1.8 ml of culture: add 600 microliters of *Fundulus* saline **(tab. 7)**, vortex, take all the contents and put into a 1,5 ml vial with 6 ceramic beads. Insert the tube into the homogenizer at 6800 rpm for 3 cycles of 30 seconds each, freeze the extract at -20 °C. The extracts prepared this way were subsequently used for electrophysiology and molecular biology studies with the opercular epithelium of *Fundulus heteroclitus*. The precipitates homogenized with *Fundulus* saline had a final concentration of 10^6 cell/mL^{-1}.

1.4. Animal model *Fundulus heteroclitus*

The *Fundulus heteroclitus*, is an exceptionally wide ranging cyprinodontid fish. It naturally occurs along the east coast of North America from southwestern Newfoundland to northeastern Florida. This species is ubiquitous in North American East Coast salt marshes, being mostly found in sheltered coastal waters. Although occasionally it inhabits freshwater habitats, the species is best known from the tidal salt marsh, a fluctuating physical environment for which they are well adapted due to considerable plasticity in their ecological requirements (Kneib, 1986). As adults, *Fundulus heteroclitus* range between 12.7 and 17.8 centimeters in length, the females growing larger than the males. They have flattened heads and the mouth is turned upward, clearly an adaptation to feeding at the surface of the water. This attractive fish is

dimorphic, males are darker in color than the females and exhibit blue or orange markings during the breeding season,are dark olive green on the dorsal side and lighter yellow on the ventral side. They also display vertical stripes along their sides. Females are silvery yellow on the ventral side and that color gradually fades to a more distinct yellow on the dorsal side.All *Fundulus heteroclitus* have a single soft dorsal fin and their pelvic fins are located close to the rear fin(National Oceanic and Atmospheric Administration Coastal Services Center, 2001). The first records of *Fundulus heteroclitus* in the Iberian Peninsula are dated between 1973-1976 (Hernando, 1975; Coelho *et al*.,1976). However, the precise date and location of the species' introduction still remains unclear. Although there have been studies describing its life-history pattern (Arias & Drake, 1986; Drake *et al*., 1987; Fernández-Delgado, 1989) and density (Arias & Drake, 1987, 1989) little is known about the role of *F. heteroclitus* in this new European habitat. The introduction of exotic species can have a negative effect on the functioning of native ecosystems (Dowling & Childs, 1992; Barlow *et al*., 1987; Richardson & Whoriskey, 1992). It is suspected that *F. heteroclitus* may have already negatively affected some native endemic species like the endangered *Lebias ibera.* This introduced species was more or less continuously distributed along the Atlantic coast of Spain, being more abundant in sites near the coastline (usually < 10 km inland), mainly in extensive marshes. The species preferred marsh related meso-habitats, such as salt lagoons, salt marsh fishponds and marsh channels, both natural and man modified. *F. heteroclitus* was mostly found at salinities > 25.

1.4.1. Osmoregulation

In the gills of *Fundulus heteroclitus*, as in all seawater teleost, the basis for ion secretion is attributable, in large part, to the concerted effort of three major ion-transport proteins: the basolateral Na^+-K^+-ATPase (NKA), the basolateral Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) and the apical cystic fibrosis transmembrane conductance regulator (CFTR) **(fig. 8)**. The primary driving force for ion secretion in ionocytes is the NKA, which maintains the low intracellular Na^+ required for secondary import of Na^+, K^+ and two Cl^- ions by the NKCC1. Elevated intracellular Cl^- is subsequently extruded through an

apical anion channel, CFTR. Intracellular Na^+ is cycled back out of the cell by the basolateral NKA; Na^+ ions are then secreted down an apical electrical gradient generated by CFTR, into the environment though shallow tight junctions between ionocytes and accessory cells (McCormick, 1995; Evans *et al*., 2005; Marshall and Grosell, 2006; Hwang and Lee, 2007).

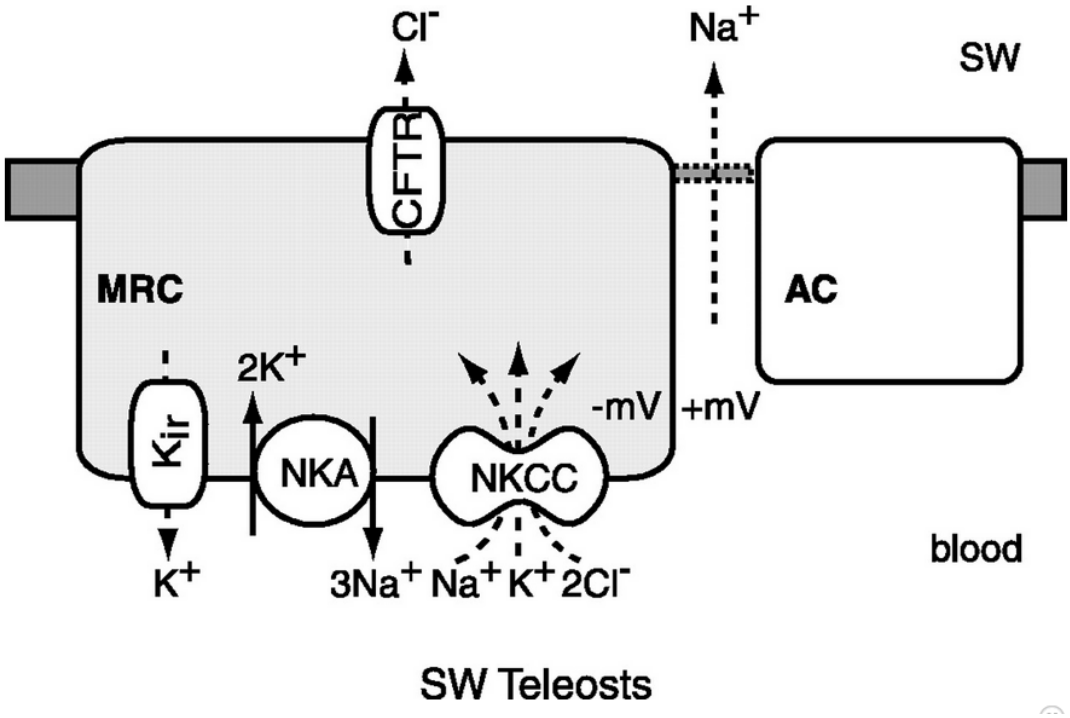


Figure 8: Working model for the extrusion of NaCl by the marine teleost gill epithelium. Plasma Na+, K+, and Cl− enter the cell via basolateral NKCC; Na+ is recycled back to the plasma via Na+-K+-ATPase and K+ via a K+ channel (Kir). Cl− is extruded across the apical membrane via a Cl− channel (CFTR). The transepithelial electrical potential across the gill epithelium (plasma positive to seawater) drives Na+ across the leaky tight junctions between the MRC (mitochondria-rich cells) and the AC (Accessory Cells)

1.4.2. Opercular membrane

As in gill tissue in the opercular membrane numerous mitochondria-rich chloride cells are present. The opercular membrane is the inner (bucal) lining of the opercular bone, it is of special interest to fish physiologists because it is an area of scale less skin and unlike the gill tissue, the opercular membrane is a flat epithelium, making the chloride cells more accessible for morphological examination and the study of their ion transport properties. The presence of chloride cells in the opercular epithelium was first observed by Burns and Copeland (1950) in seawater-adapted *Fundulus heteroclitus*. Degnan *et al.*

(1997) and Karnaky *et al.* (1977) mounted the *Fundulus heteroclitus* opercular membrane in an Ussing chamber and found net chloride secretion; since then the opercular membrane has been widely used to examine the mechanisms and control of ion secretion in teleosts.

1.4.3. Experimental procedures

The *Fundulus heteroclitus* were collected with fishing nets in the Estero La Leocadia (Cadiz Bay, Spain) were kept in the aquarium with water at a temperature of 21 °C, salinity of 35 ‰ and exposed to a period of 14h of artificial light per day with a luminous intensity of 50 µmol/m²/s. To obtain the opercular tissue the fish were immersed in a beaker with anesthetic 2-Phenoxyethanol (1: 2000) and later beheaded. The head was placed in a beaker with saline solution **(tab: 7)** and insufflation gas with ratio 99.7:0.3 of O_2/CO_2, to maintain the tissues alive. All animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. All animal protocols were performed under a Group-C license from the Direcção-Geral de Veterinária, Ministerio da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal.

Table 7: constituents of Ussing chamber saline solution (pH=8)	
NaCl	160 mM
$MgS0_4$	0.93 mM
Na_2HPO_4	3 mM
$CaCl_2$	1.5 mM
$NaHCO_3$	5 mM
KCl	3 mM
Glucose	5.5 Mm

The opercular membrane is removed using tweezers, scalpel, and scissors, under the microscope and stretched over a 0.1-0.3 cm hole in the center of a rectangular piece of Plexiglas (mounting plate). Small pins several mm

outside the hole are used to maintain the membrane flat and stretch it to its original (undissected) dimensions. A circular mounting disc with a central hole (0,126 cm^2, same size as that of the mounting plate) and small holes to accommodate the mounting pins is placed over the membrane. Modest amounts of silicone grease are placed on the inner edges of the mounting plate and disk that contact the membrane, prior to placement of the membrane. The mounting plate and disc are then placed into receiving grooves to separate the two halves of the Ussing chamber **(fig: 9)** which contain 2 ml of saline solution **(tab: 7)**. The salt solutions present in the two halves are separated from each other only by the opercular epithelium. Each chamber is gently bubbled with a gas mixture (O_2/CO_2 = 99.7:0.3) to maintain oxygen tension and fluid mixture. The chambers are mounted in supports in contact with a liquid coolant which keeps the temperature constant at 21-22 °C. Within this system the tissue can remain alive for 4-5 hours.

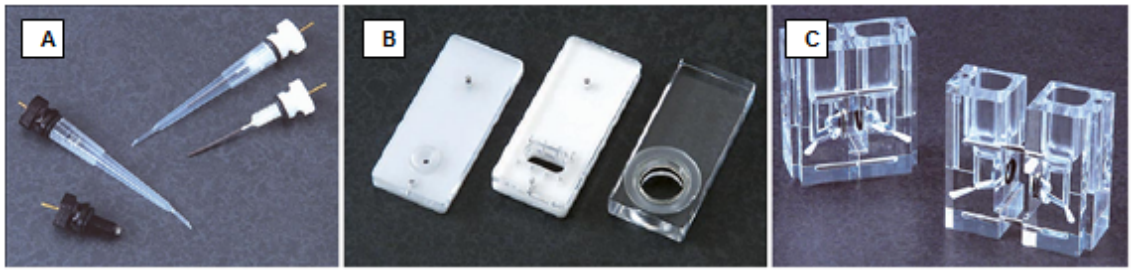


Figure 9: example of electrode (A), and insert (B) used in the Ussing chamber (C). www.warneronline.com/product_info.cfm?id=550.

The Cl^- transport from basolateral to the apical side can be measured through "voltage clamp" to 0 mV, by “short circuit current”, Isc (Hoffmann *et al*., 2002). The short-circuit current is measured with paired polyethylene 3% agar-3M KCl bridges (two electrodes, **fig. 9)** each halve) placed 2-3 mm from each side of the membrane, which terminate in 3 M KCl and are in turn connected to a voltmeter by Ag/AgCl electrodes. Current may be passed across the membrane via agar bridges in each chamber that are connected to Ag/AgCl electrodes and a current clamping device (Fuentes *et al.,* 2006). The "voltage clamp" method has its basis on the Ohm law (R=V/I, where R is the Resistance, V the Voltage and I the Current) considering constant the resistance. Thus it was obtained the value of current required to be injected

so as to clamp the voltage to 0 mV. Isc is directly proportional measurement of the ionic transport, as well as, the secretory tissue activity. The voltage clamp to 0 mV and the current injection were generated by voltage current clamp amplifiers with automatic correction for fluid resistance and voltage asymmetry. DVC-1000 (World Precision Instrument, Sarasota, USA) or VCC600 (Physiologic Instruments, San Diego, USA)

1.4.4. Bioactivity test

When the Isc of the tissue inside the insert was stable, injecting the extracts (previously prepared and stored at -20 °C, section 2.4.) in the saline solution. Each application was performed after the stabilization of the tissue.

The biometric parameters were recorded and saved to a computer via a data acquisition system Lab-Trax-4 (World Precision Instrument, Sarasota, USA).

All data obtained were subjected to statistical analysis using the software Prism 5 (GraphPad Software, La Jolla, California, USA). The statistical significance was determined as adequate by two- way ANOVA or Student t-test, considering p values < 0,05.

2. Results

2.1. Basal values

Before adding the cell extracts into the Ussing chamber the basal values of the tissues were recorded **(tab. 8)**. The results presented in Table 8 confirm that in these experimental conditions the Isc is a direct measure of the Cl^- ion flow through the opercular epithelium.

Table 8: basal values (average ± standard error) of the biometric parameters of *Fundulus heteroclitus* epithelium mounted into the Ussing chamber in voltage clamp. Vt is voltage, Isc is the short circuit current, R is the epithelium resistance.	
Vt *(mV)	16.1 ± 2.7
Isc (µA/cm²)	-211.3 ± 24.8
R (Ω.cm²)	70.4 ± 8.3
*Measured in open circuit, before voltage clamp	

2.2. Extracts added to the basolateral side

The extracts of microalgae, with the addition of Cu^{2+} **(Fig. 10, Fig. 12A)** and without **(Fig. 11, Fig. 12B)**, were added in increasing concentrations (4×10^6 cell/ml; 8×10^6 cell/ml; 16×10^6 cell/ml; 32×10^6 cell/ml; 64×10^6 cell/ml) in the basolateral side in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. The addition of extracts of *Coccomyxa* exposed to copper caused a pronounced decrease of Isc coming to inhibit almost completely. In contrast, the addition of copper-free extracts, induced a change in Isc values, but much lower compared to extracts with copper, with values that never exceed the Isc threshold of -100 µA / cm².

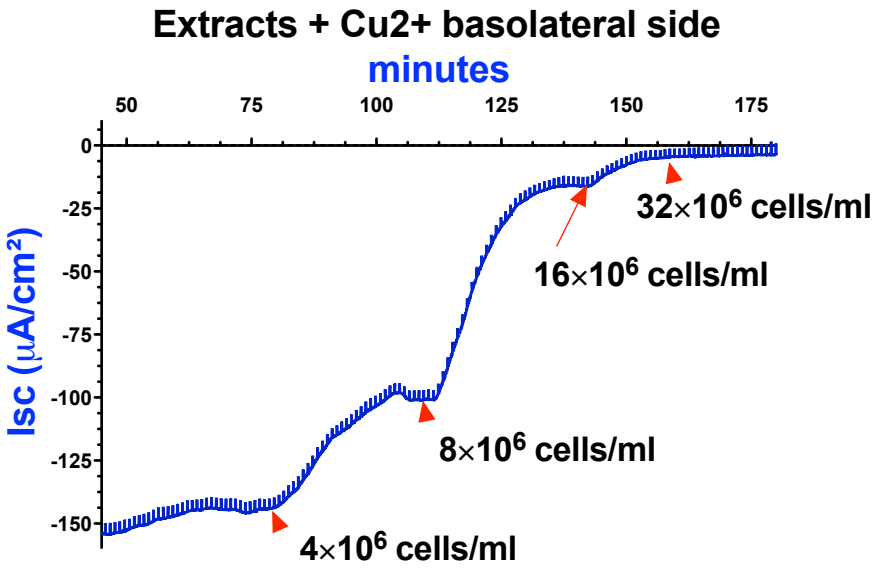


Figure 10: Original recording of short circuit current (µA/cm²) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. cultured in the presence of Cu^{2+}, applied in increasing concentrations (4×10^6 cell/ml; 8×10^6 cell/ml; 16×10^6 cell/ml; 32×10^6 cell/ml) in the basolateral side of the opercular epithelium. The current vertical lines with a regular period correspond to the voltage pulse of 1 mV, used to calculate the resistance of the epithelial tissue (Rt, Ωcm^2) by Ohm's law.

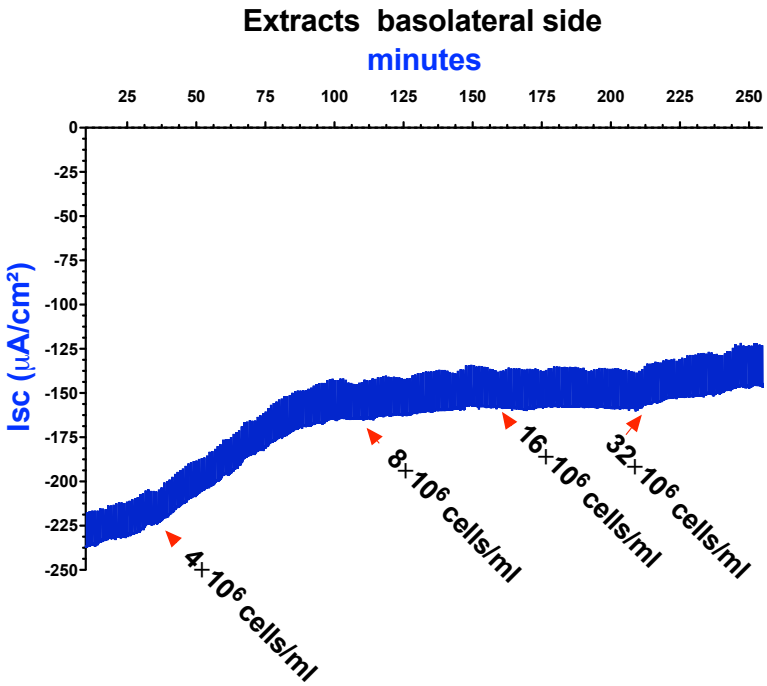


Figure 11: Original recording of short circuit current (µA/cm²) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. culture, applied in different concentrations (4×10^6 cell/ml; 8×10^6 cell/ml; 16×10^6 cell/ml; 32×10^6 cell/ml) in the basolateral side of the opercular epithelium. The current vertical lines with a regular period correspond to the voltage pulse of 1 mV, used to calculate the resistance of the epithelial tissue (Rt, Ωcm^2) by Ohm's law.

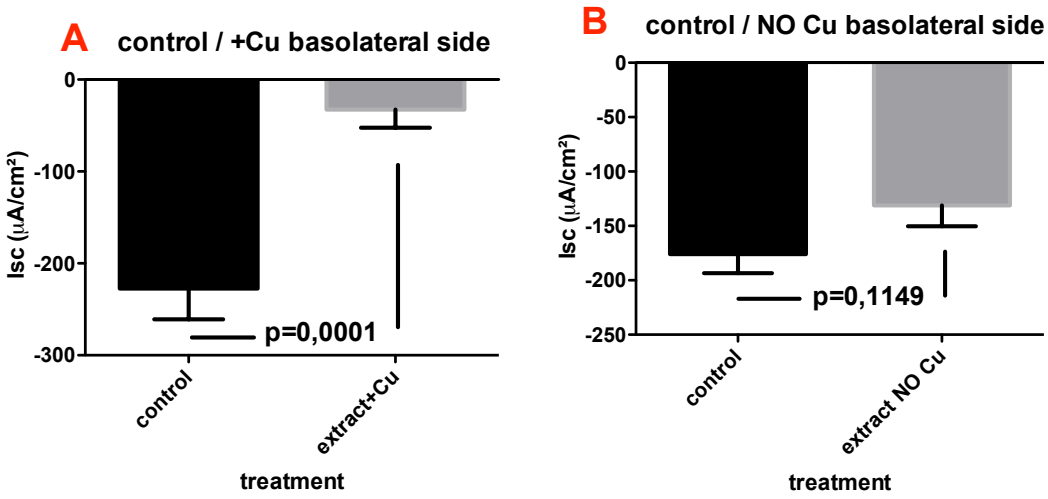


Figure 12: variation of short circuit current (Isc) in relation to respective control in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. culture after the addition of the last dose of extracts (32×10^6 cell/ml), applied in the basolateral side of the opercular epithelium. **A**: addition of extracts with Cu^{2+}, **B**: addition of extracts without Cu^{2+}. Each column represents the men + SEM of 6 individuals. Differences between treatments were assessed by Student's t-test (and considered significant for $p<0.05$ (shown in the figure).

2.3. Extracts added to the apical side

After testing the effect of the extracts on the basolateral side, the extracts were also tested on the apical side of the opercular epithelium. The extracts of microalgae, cultured in the presence of Cu^{2+} **(Fig. 13, Fig. 15A)** or in the absence of Cu^{2+} **(Fig. 14, Fig. 15B)**, were added in increasing concentrations (4×10^6 cell/ml; 8×10^6 cell/ml; 16×10^6 cell/ml; 32×10^6 cell/ml; 64×10^6 cell/ml) in the apical side in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. In both tests (with and without Cu^{2+}) the addition of extracts caused a slight decrease of the Isc with no statistically significant differences between the two treatments.

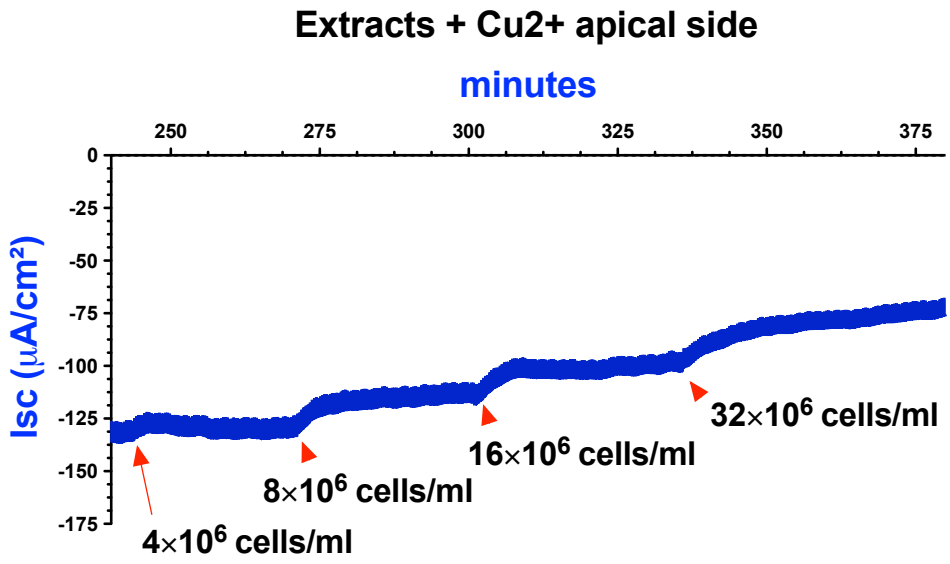


Figure 13: Original recording of short circuit current (µA/cm²) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. Culture with Cu^{2+}, applied in different concentrations (4×10^6 cell/ml; 8×10^6 cell/ml; 16×10^6 cell/ml; 32×10^6 cell/ml) in the apical side of the opercular epithelium. The current vertical lines with a regular period correspond to the voltage pulse of 1 mV, used to calculate the resistance of the epithelial tissue (Rt, Ωcm^2) by Ohm's law.

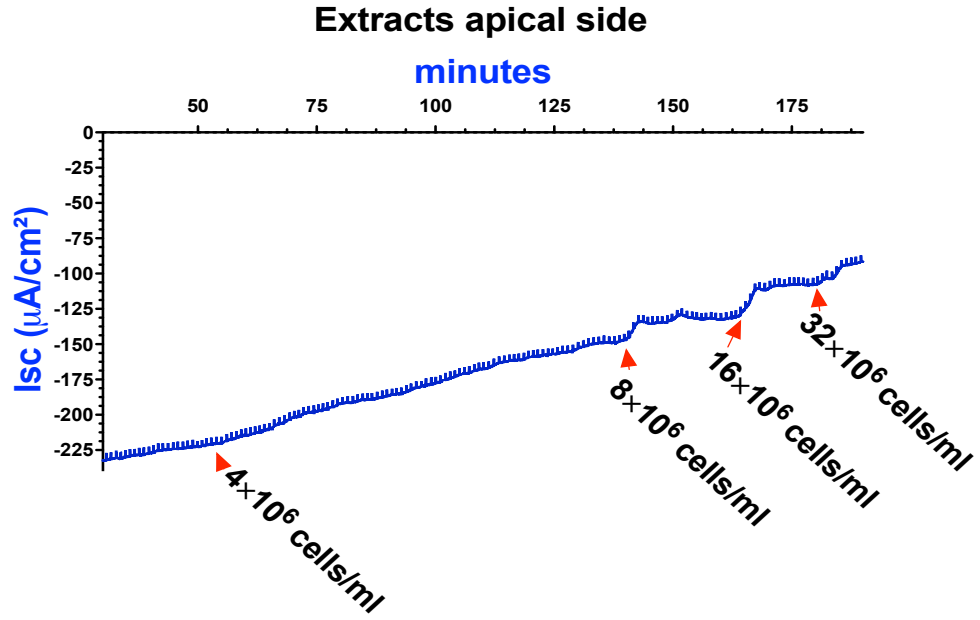


Figure 14: Original recording of short circuit current (µA/cm²) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. culture, applied in different concentrations (4×10^6 cell/ml; 8×10^6 cell/ml; 16×10^6 cell/ml; 32×10^6 cell/ml) in the apical side of the opercular epithelium. The current vertical lines with a regular period correspond to the voltage pulse of 1 mV, used to calculate the resistance of the epithelial tissue (Rt, Ωcm^2) by Ohm's law.

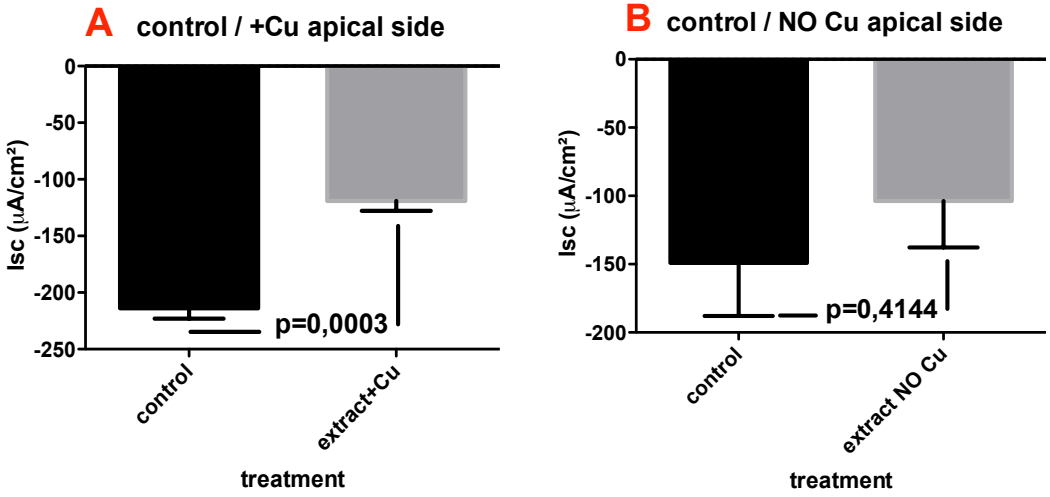


Figure 15: variation of short circuit current (Isc) in relation to respective control in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. culture after the addition of the last dose of extracts (32×10^6 cell/ml), applied in the apical side of the opercular epithelium. **A**: addition of extracts with Cu^{2+}, **B**: addition of extracts without Cu^{2+}. Each column represents the men + SEM of 6 individuals. Differences between treatments were assessed by Student's t-test (and considered significant for $p<0.05$ (shown in the figure).

2.4. Combined treatment/side

The comparison of Isc values after the administration of microalgae extracts cultured in the presence of copper (32×10^6 cells/ml), in the basolateral and apical side **(Fig. 16A),** shows an evident decrease in the values of Isc in the experiments carried out by adding the extracts to the basolateral side, which is not observed when the extracts were added to the apical side. A highly significant effect was observed (p=0.0003). In the case of extracts of microalgae not exposed to copper, the Isc variations after the addition of the equivalent to 32×10^6 cells/ml in the apical or in the basolateral side **(Fig. 16B)**, resulted in little difference with not statistical significance (p=0.4694) between both sides.

A summary of all results is presented in **Fig. 17**, were the results are presented normalized in relation to control values of Isc. This representation of the data shows that the treatment with extracts of microalgae cultured in the presence of Cu has a real effect in the inhibition of the short circuit current (Isc) but only if it's added in the basolateral side. This combination of factors is

the only one can reach 100% of Isc inhibition with less than 64×10^6 cell/ml **(Fig. 17)**, and shows a reduction of 50% of the current at around 8×10^6 cell/ml; for the others three groups of experiments the addition of 64×10^6 cell/ml is still not enough to reach 50% inhibition of the current. The treatment with extracts of microalgae with Cu^{2+} in the apical side can get only 40% of Isc inhibition and the two treatments with extracts of microalgae without Cu^{2+} reach about 30% of Isc inhibition **(Fig 17)**.

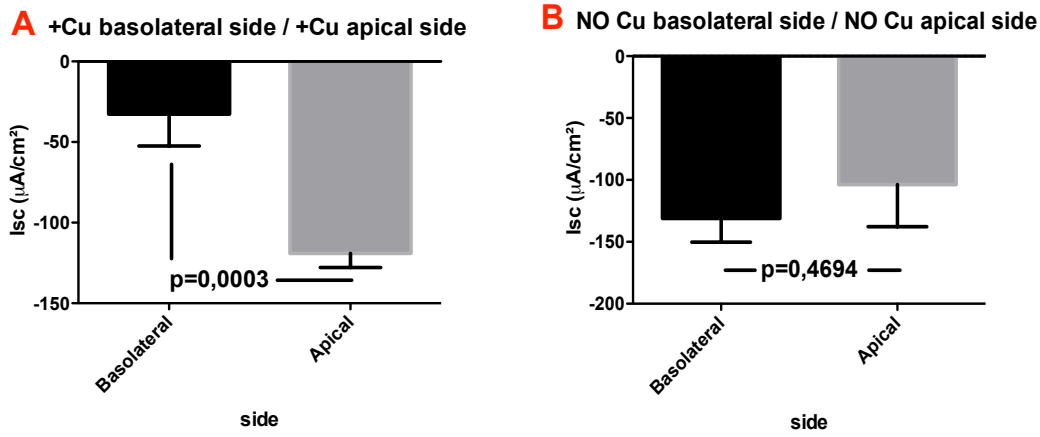


Figure 16: variation of short circuit current (Isc) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. culture after the addition of the last dose of extracts (32×10^6 cell/ml), applied in the ■basolateral and □ apical side of the opercular epithelium. **A**: addition of extracts with Cu^{2+}, **B**: addition of extracts without Cu^{2+}. Each column represents the men + SEM of 6 individuals. Differences between treatments were assessed by Student's t-test (and considered significant for $p<0.05$ (shown in the figure).

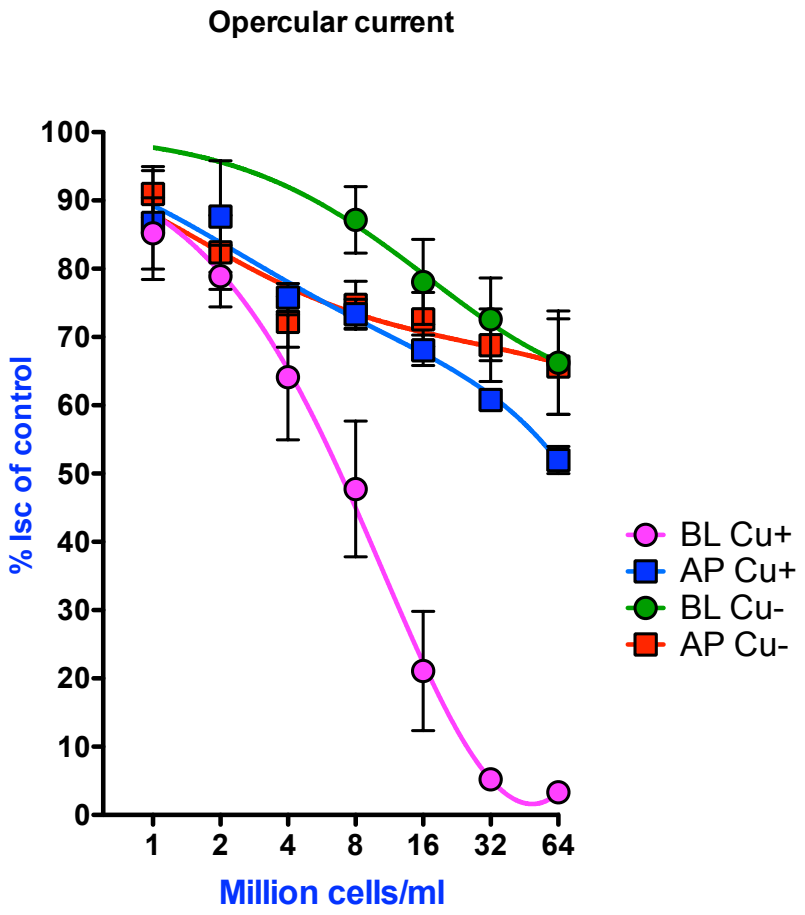


Figure 17: Percentage variation of short circuit current (Isc) in relation to respective control in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. add in different concentrations (1×10^6 cell/ml; 2×10^6 cell/ml 4×10^6 cell/ml; 8×10^6 cell/ml; 16×10^6 cell/ml; 32×10^6 cell/ml; 64×10^6 cell/ml) and in different conditions: BL Cu+: extracts with Cu^{2+} applied to the basolateral side of the opercular epithelium; AP Cu+: extracts with Cu^{2+} applied to the apical side of the opercular epithelium; BL Cu-: extracts applied to the basolateral side of the opercular epithelium; AP Cu-: extracts applied to the apical side of the opercular epithelium.

2.5. Bumetanide added to the basolateral and apical sides

Bumetanide was used in this work because it's a known diuretic and specific inhibitor of the co-transporter NKCC (Haas and Forbush III, 2000; Ju *et al.,* 2013). To confirm this, bumetanide (200µM) was added in the basolateral

side of opercular membrane preparations **(Fig. 18 and 21A)** and in the apical side **(Fig. 19 and 21B)** and this showed a huge and almost complete inhibition of the Isc when added to the basolateral side, but in the experiments where bumetanide was added in the apical side there was no variation of the Isc. The microalgae extracts were added in the same epithelial tissue before and after the addition of bumetanide **(Fig. 22 and 23)** to verify whether bumetanide and the algae extracts were competing and/or acting on the same mechanism.

It was decided to add the extracts with Cu^{2+} because in the previous experiments these were the only ones with significant inhibitory effects on Isc. The example in figure 21 shows that after the first addition of extracts of microalgae grown in the presence of Cu^{2+} (8×10^6 cell/ml) a decrease of Isc value was observed. After the addition of bumetanide the Isc value went close to zero quickly. A new extract of microalgae was added, this time at much higher concentration 32×10^6 cell/ml and resulted in no further variation of Isc. The evidence that makes clear than the extracts work through the same mechanism, as bumetanide is shown in figures 25 and 26. When adding the extracts with Cu^{2+} before the bumetanide there is a decrease of Isc, if the addition is after bumetanide there is no effect of the extracts in the current **(Fig. 22)**. A similar observation was made for bumetanide, if bumetanide was added before the extracts with Cu^{2+}, there is a big decrease of Isc, if the addition is after there is a little effect **(Fig. 23)**. This suggests a competition between the algae and bumetanide, what was added before (extract or the bumetanide) inhibits the NKCC co-transporter and the current never becomes positive.

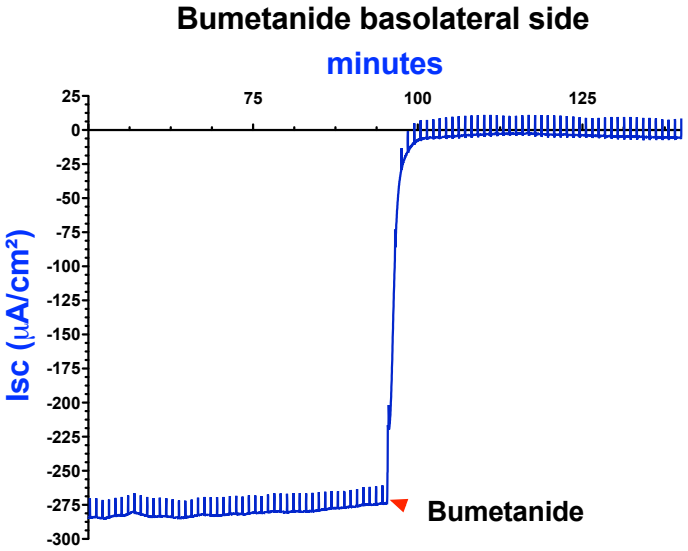


Figure 18: Original recording of short circuit current (µA/cm²) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the diuretic bumetanide (200 µM), applied to the basolateral side of the opercular epithelium. The current vertical lines with a regular period correspond to the voltage pulse of 1 mV, used to calculate the resistance of the epithelial tissue (Rt, Ωcm^2) by Ohm's law.

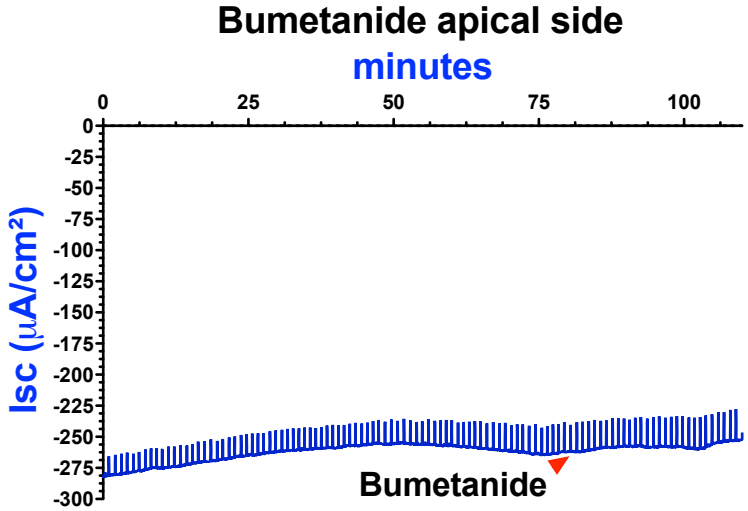


Figure 19: Original recording of short circuit current (µA/cm²) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the diuretic bumetanide (200 µM), applied to the apical side of the opercular epithelium. The current vertical lines with a regular period correspond to the voltage pulse of 1 mV, used to calculate the resistance of the epithelial tissue (Rt, Ωcm^2) by Ohm's law.

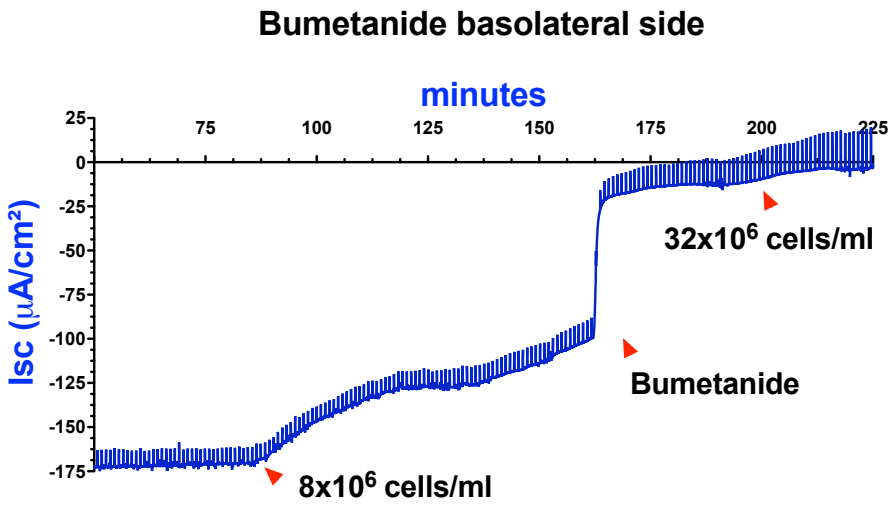


Figure 20: Original recording of short circuit current (µA/cm²) in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Combined response to the extracts of *Coccomyxa* sp. culture (8×10^6 cell/ml) with Cu^{2+}, applied to the basolateral side of the opercular epithelium before the addition of the diuretic bumetanide (200 µM), applied to the basolateral side and then extracts of *Coccomyxa* sp. Culture (32×10^6 cell/ml) with Cu^{2+} applied to the basolateral side of the opercular epithelium. The current vertical lines with a regular period correspond to the voltage pulse of 1 mV, used to calculate the resistance of the epithelial tissue (Rt, Ωcm^2) by Ohm's law.

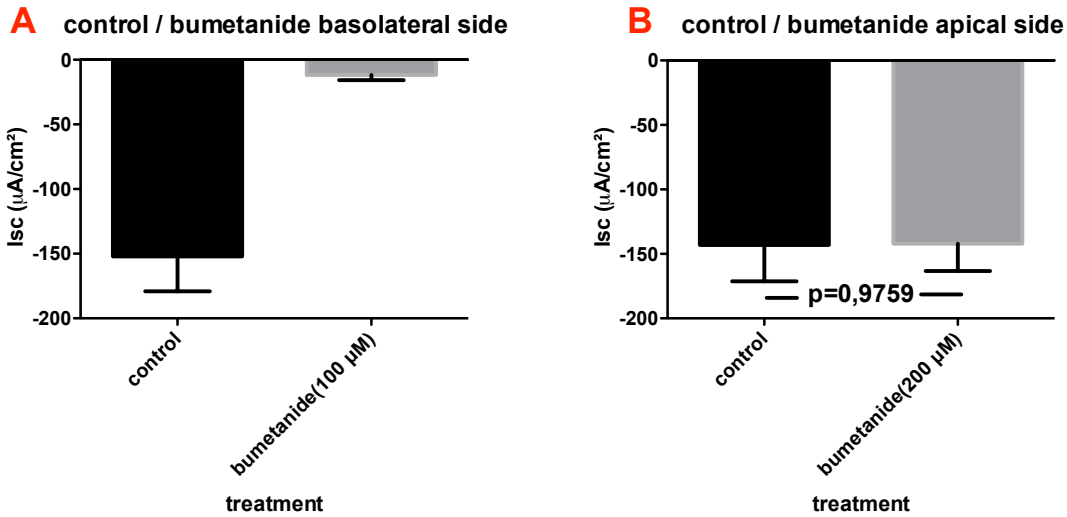


Figure 21: variation of short circuit current (Isc) in relation to respective control in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the diuretic bumetanide (200 µM), applied to the **A** basolateral side and **B** apical side of the opercular epithelium. Each column represents the men + SEM of 6 individuals. Differences between treatments were assessed by Student's t-test (and considered significant for $p<0.05$ (shown in the figure).

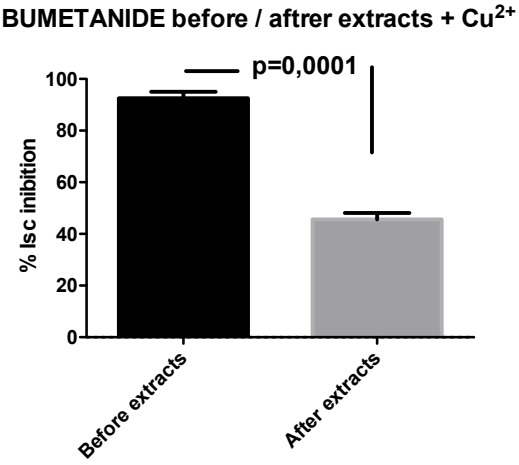


Figure 22: percentage of short circuit current (Isc) inhibition in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the diuretic bumetanide (200 µM), applied in the basolateral side of the opercular epithelium ■ before and□ after the addition of extracts of *Coccomyxa* sp. culture (8×10^6 cell/ml) with Cu^{2+}. Each column represents the men + SEM of 6 individuals. Differences between treatments were assessed by Student's t-test (and considered significant for $p<0.05$ (shown in the figure).

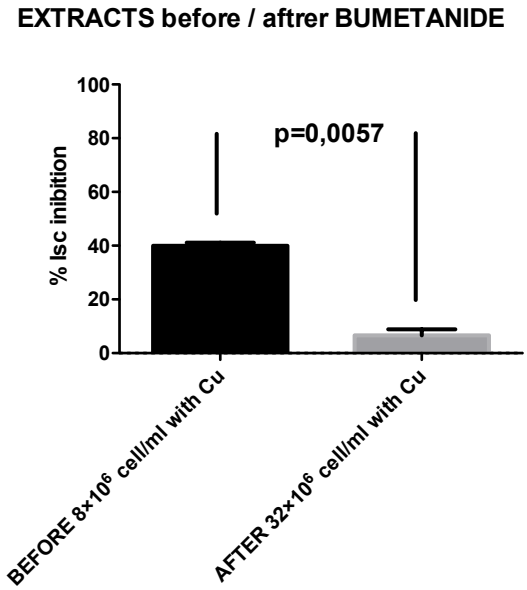


Figure 23: percentage of short circuit current (Isc) inhibition in opercular epithelium of *Fundulus heteroclitus* mounted in the Ussing chamber in voltage clamp. Response to the extracts of *Coccomyxa* sp. culture with Cu^{2+} applied in the basolateral side of the opercular epithelium ■ before (8×10^6 cell/ml) and □ after (32×10^6 cell/ml) the addition of the diuretic bumetanide (200 µM). Each column represents the men + SEM of 6 individuals. Differences between treatments were assessed by Student's t-test (and considered significant for $p<0.05$ (shown in the figure).

IV. GENETIC EXPRESSION STUDY

1. Introduction

All cells of the same organism have the same genome, however, only a percentage of genes is expressed in every cell type. Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but in non-protein coding genes such as rRNA genes or tRNA genes, the product is a structural or housekeeping RNA. In addition, small non-coding RNAs (miRNAs, piRNA) and various classes of long non-coding RNAs are involved in a variety of regulatory functions (Taft R., *et al*., 2010). When studying gene expression with real -time polymerase chain reaction (PCR), scientists usually investigate changes, increases or decreases, in the expression of a particular gene or set of genes by measuring the abundance of the gene-specific transcript. The investigation monitors the response of a gene to treatment with a compound or drug of interest, under a defined set of conditions. Gene expression studies can also involve looking at profiles or patterns of expression of several genes. Whether quantitating changes in expression levels or looking at overall patterns of expression, most scientists performing gene expression analysis use real-time PCR.

1.2. Real-time PCR technique

Real-time PCR (or qPCR – quantitative PCR) is one of the most powerful and sensitive gene analysis techniques available. It is used for a broad range of applications including quantitative gene expression analysis, genotyping, copy number, drug target validation, biomarker discovery, pathogen detection, and measuring RNA interference. Real-time PCR measures PCR amplification as it occurs, so that it is possible to determine the starting concentration of the nucleic acid under study. In traditional PCR, which is based on end-point detection, results are collected after the reaction is completed, making it impossible to determine the starting concentration of the nucleic acid. Every real-time PCR contains a fluorescent reporter molecule to monitor the accumulation of PCR product. As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore.

There are three phases in a basic PCR run: i) Exponential in which the exact doubling of product occurs at every cycle (assuming 100% reaction efficiency). Exponential amplification occurs because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon. Real-Time PCR focuses on the exponential phase, which provides the most precise and accurate data for quantitation. During the exponential phase, the real-time PCR instrument calculates two values: the Threshold, which is the level of detection at which a reaction reaches a fluorescent intensity above background and the CT, which is the PCR cycle at which the sample reaches the threshold. The Ct value is used in absolute or relative quantitation; ii) Linear: As the reaction progresses, some of the reagents are consumed as a result of amplification. The reactions start to slow down and the PCR product is no longer doubled at each cycle; iii) Plateau: The reaction has stopped, no more products are made, and if left long enough, the PCR products begin to degrade. Each tube or reaction plateaus at a different point, due to the different reaction kinetics for each sample. These differences can be seen in the plateau phase. The plateau phase is the end point, where traditional PCR takes its measurement.

There are two experimental design and analysis methodologies for relative quantitation of gene expression: the Relative Standard Curve Method and the Comparative CT Method ($\Delta\Delta$Ct). The first requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not have to be equivalent. This method requires that each reaction plate contain standard curves, and requires more reagents and more space on a reaction plate. This approach gives highly accurate quantitative results because unknown sample quantitative values are interpolated from the standard curve(s). This method should be considered when testing low numbers of targets and small numbers of samples and if the amplitude of expression changes to be expected are unknown.

Another paramount aspect when doing qPCR is that to obtain accurate relative quantitation of an mRNA target, the expression level of an endogenous control has also to be evaluated. By using an endogenous control as an active reference, one can normalize quantitation of targets for differences in the amount of total nucleic acid added to each reaction. For

example, if it is determined that a calibrator sample has a two-fold greater amount of endogenous control than a test sample it can be due to the calibrator sample being loaded with two-fold more cDNA than the test sample. Therefore, the test sample target would have to be normalized by two-fold to accurately quantify the fold-differences in target level between calibrator and test samples. The endogenous controls or housekeeping genes used in qPCR are usually genes pertaining to the primary metabolism of the cell, expected to have a stable level of expression regardless of the cell state or environmental conditions.

2. Materials and Methods

2.1. Tissue incubation with microalgae extracts

Using the same procedure described in the previous section the opercular epithelium was removed from 8 fishes. Each sample was divided in three sections with approximately the same area, thus obtaining three sets with eight replicas and each epithelium section was deposited in a well of a 24 well plate.

One set of 8 epithelium sections was incubated only with cell culture medium **(tab. 9)** as a control. Another group was incubated with cell culture medium plus microalgae extracts (16×10^6 cell/ml) from the culture without Cu^{2+}. The third group was incubated with cell culture medium and extracts of microalgae (16×10^6 cell/ml) from the culture with 0.6 mM of Cu^{2+}. The 16×10^6 cell/ml concentration was chosen because it was the closest concentration to the dose response of 50% of Isc inhibition. The plate was incubated for 24 hours under constant agitation (orbital shaker Unimax 2010 Heidolph, Instruments GmbH & Co.), in a controlled atmosphere (gas ratio 99.7: 0.3 of O2 / CO2) at room temperature. After the incubation period each epithelium section from each set was subjected to total RNA extraction.

Table 9: culture medium constituents for the incubation of the *Fundulus heteroclitus* epithelia used for expression analysis. The following components were added to the saline solution **(tab. 7)**.
10µl/ml vitamins (MEM 100X Vitamins, Sigma-Aldrich, USA)
20µl/ml essential amino acids (MEM 50X Vitamins, Sigma-Aldrich, USA)
10µl/ml non-essential amino acids (MEM 100X Vitamins, Sigma-Aldrich, USA)
10µl/ml antibiotic: Penicillin 10 000 IU/ml; streptomycin 10 000 IU/ml; Gibco™, Scotland)
20µl/ml of L- glutamine (200mM, Sigma-Aldrich, USA)

All the quantification of RNA, cDNA and pDNA, were carried out with the use of Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA).

2.2. Total RNA extraction

For total RNA extraction the commercial kit Total RNA Kit II - Animal Tissue Protocol (Total RNA Kit I - Animal Tissue Protocol - EZNA TOTAL RNA KIT I, OMEGA BIO-TEK, USA) was used, following the protocol given below:

Protocol:

Table 10: Quantity of TRK Lysis Buffer for ≤ 15 mg of tissue.	
Amount of Tissue (mg)	**Amount of TRK Lysis Buffer (µl)**
≤ 15 mg	350 µl

Disrupt the tissue with a glass mortar and pestle and homogenize the tissue in 1 ml TRK Lysis Buffer. Add 40 µl 2-mercaptoethanol. Incubate the tube containing the homogenate at room temperature for 5 minutes. Centrifuge at ≥12,000 x g at 4°C for 5 minutes to separate the aqueous and organic phase.

Transfer the upper aqueous phase into a new 1.5 ml microcentrifuge tube. Add an equal volume of 70% ethanol and vortex to mix thoroughly. Apply sample onto HiBind® RNA Mini Column inserted into a 2 ml Collection Tube. Centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow-through and re-use the collection tube in the next step. Add 500 µl RNA Wash Buffer I by pipetting directly into the spin column. Centrifuge at 10,000 x g for 1 minute at room temperature then discard the 2 ml Collection Tube and the filtrate. Place the column into a clean 2 ml Collection Tube and add 500 µl RNA Wash Buffer II. Centrifuge at 10,000 x g for 1 minute at room temperature. Discard the filtrate and reuse the collection tube in the next step. Add a second 500 µl Wash Buffer II to the HiBind® RNA Mini Column. Centrifuge at 10,000 x g for 1 minute and discard the filtrate. Then with the HiBind® RNA Mini Column and collection tube emptied, centrifuge the spin cartridge for 2 minutes at maximum speed to completely dry the iBind® matrix. Transfer the column to a clean 1.5 ml microcentrifuge tube and elute the RNA with 45-70 µl DEPC Water. Centrifuge for 2 minutes at 10,000 x g.

2.3. Removing genomic DNA from RNA samples

To remove genomic DNA contamination from the RNA samples the Ambion (Applied Bio systems) TURBO DNase kit (Ambion, Foster City, California, USA) was used, following the protocol indicated below and the reagents in **Tab. 11**.

Table 11: Reaction components in a total volume of 50 µl.

10 ug of RNA	x µl
10 x DNase buffer	5 µl
TURBO DNase	1 µl
nanopure water	to 50 µl

Protocol:

Add components in this order: water, RNA, buffer and DNase then mix gently and centrifuge briefly, incubate at 37ºC for 25 min. Add resuspended DNase inactivation Reagent and mix well. Incubate 2 min at room temperature, mixing occasionally. Centrifuge at 10,000 × g for 2 min and transfer the RNA to a fresh tube at 4°C.

2.4. cDNA synthesis

After quantification of the cleaned RNA, cDNA was synthetized using 50 ng of RNA per reaction and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, #K1621) with the following protocol:

Protocol:

Add the reagents into a sterile, nuclease-free tube on ice in the indicated order **(Tab. 12)**, mix gently, centrifuge briefly and incubate at 65°C for 5 min then chill on ice, spin down and place the vial back on ice. Add the components **(Tab. 13)** in the indicated order, mix gently and centrifuge briefly. Incubate for 5 min at 25°C followed by 60 min at 42°C then 70°C for 5 min.

Table 12: cDNA synthesis reagents I.

1-Template RNA	Total RNA	50 ng
2-Primer	Random Hexamer primer	1 µl
3-Water, nuclease - free		To 12 µl

Table 13: cDNA synthesis reagents II.

1- 5X Reaction Buffer	4 µl
2- RiboLock RNase Inhibitor (20 U/µl)	1 µl
3- 10 mM dNTP Mix	2 µl
4- RevertAid M-MuLVRT(200 U/µl)	1 µl
5- Total volume	20 µl

The cDNA obtained was stored at -20ºC.

2.5. q-PCR

For this work the 18S gene was chosen as reference gene and the genes of interest were NKCC1, NKCC2 and CFTR. Primer pairs specific of each gene were design based on the respective sequences available at GenBank for *Fundulus heteroclitus* **(Tab. 14)**.

Table 14: List of primers used in q-PCR process. Primer reference: this work.

Gene	Primer	Sequence 5'→ 3'	bp
18s	18S-Fwd	AACCAGACAAATCGCTCCAC	139
	18s-Rev	CCTGCGGCTTAATTTGACTC	
NKCC1	NKCC1-Fwd	TGCCAACATTTCTGGAGACCTT	140
	NKCC1-Rev	TTCCAGTGGCATGTCTCACAAT	
NKCC2	NKCC2-Fwd	CTGGTACCGTTGTCCGAGAT	116
	NKCC2-Rev	GCAGGAAGAGAAATCGTAGCC	
CFTR	CFTR-Fwd	CTGTCCACCTTGCAGTCATAGT	125
	CFTR-Rev	CAACGTGTGGACTAAAGGGAGA	

The primers pairs were tested at different annealing temperature (56ºC, 58ºC, 60ºC) in end-point PCR, using the cDNA already synthesized. The temperature of 58ºC was chosen because all primer pairs allowed amplification of the fragment of the expected size, at that temperature. The fragments obtained with each primer pair were cloned and sequenced; following the process already described in the chapter I paragraph 2. The identity of the sequences obtained was verified in a BLAST analysis. The corresponding minipreps were quantified and used to obtain the standard curves in the qPCR, with a dilution factor of 10× from the initial concentration of 0,1 ng/µl to 10^{-5} ng/µl.

Each q-PCR reaction was prepared according to the instructions of the PerfeCta SYBR Green SuperMix kit (Quanta, BioScience, USA) and used the reaction components show in the table 15.

Table 15: Real-Time PCR components for the respective gene: 18s, NKCC1, NKCC2, CFTR.

18s		**NKCC1**	
Quante	5µl	Quante	5µl
18s fw	0,25µl	NKCC1 fw	0,35µl
18s rv	0,25µl	NKCC1 rv	0,35µl
nanopure water	3,5 µl	nanopure water	3,3 µl
cDNA	1 µl	cDNA	1 µl
NKCC2		**CFTR**	
Quante	5µl	Quante	5µl
NKCC2 fw	0,35µl	CFTR fw	0,35µl
NKCC2 rv	0,35µl	CFTR rv	0,35µl
nanopure water	3,3 µl	nanopure water	3,3 µl
cDNA	1 µl	cDNA	1 µl

qPCR reactions were conducted in the thermocycler One-Step Plus (Applied Bio System, California, USA), with the program shown in table 16 and the results was treated with the software: One-step plus™ Systems.

Table 16: Real-Time PCR program.

96°C	30 sec	
96°C	10sec	X 40
59°C	10sec	
65°C	10sec	
59°C + 0,5°C (each 10 sec) to 95°C		

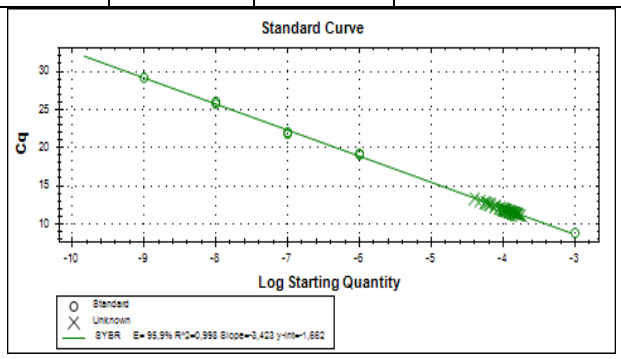
3. Results

As the following tables (**Tab. 17, 18, 19**) show, the q- PCR was solid for all the selected genes because all the describing parameters: R^2, Slope, Efficiency in the standard curve were suitable and only one gene was amplified as it's shown by single peaks in corresponding melt curves.

All genes of interest were amplified in all treatments, however there is a slight variation of the gene expression in all genes, but without statistical significance. Only in the genes tested with the extracts with the addition of copper show a solid and consistent slight decrease, but without statistical significance: NKCC1 p=0.2116, NKCC2 p =0.1687, p=0.2364 CFTR (**Fig. 24, 25 and 26)**.

Table 17: q-PCR, gene 18s with relative q-PCR parameters (R^2, Slope, Efficiency) table, standard curve where ✖ are different samples with different incubation treatment (Control +Cu^{2+}, -Cu^{2+}) and ¢ represent the standard points obtained by dilutions, and relative melt curve.

Gene	R^2	Slope	Efficiency
18s	0,998	-3,423	95,9%



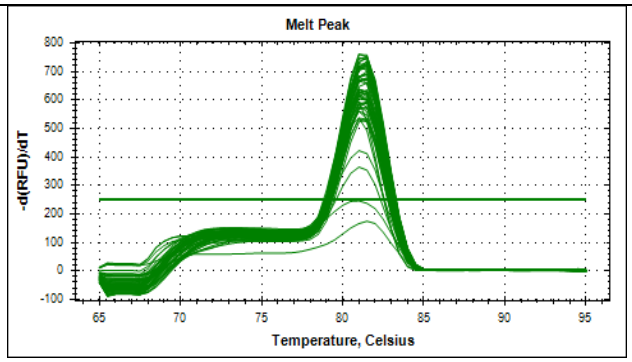
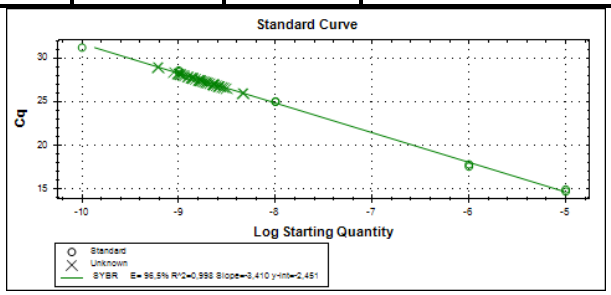
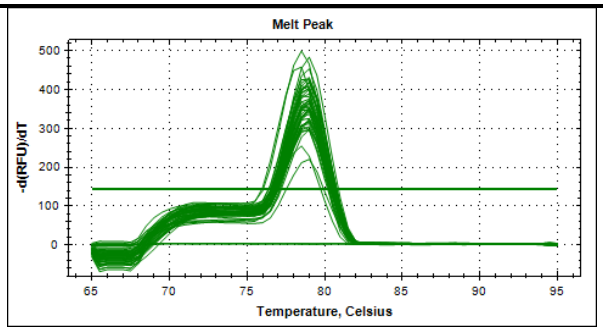


Table 18: q-PCR, gene NKCC1 with relative q-PCR parameters (R^2, Slope, Efficiency) table, standard curve where ✖ are different samples with different incubation treatment (Control +Cu^{2+}, -Cu^{2+}) and ○ represent the standard points obtained by dilutions, and relative melt curve.

Gene	R^2	Slope	Efficiency
NKCC1	0,998	-3,410	96,5%





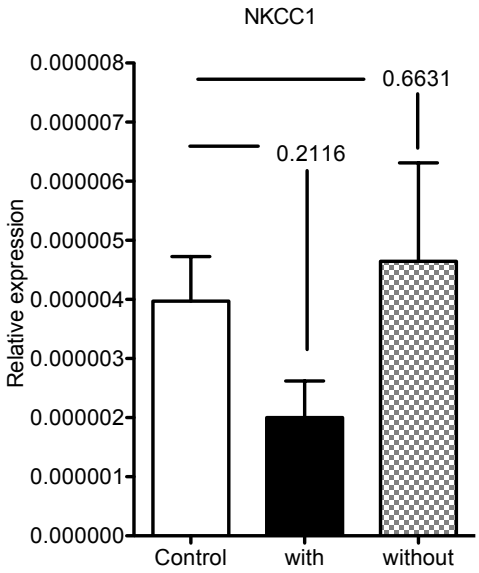
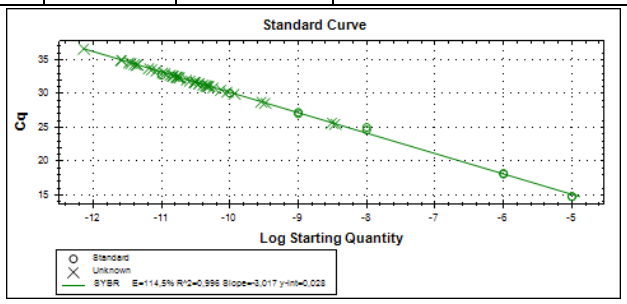
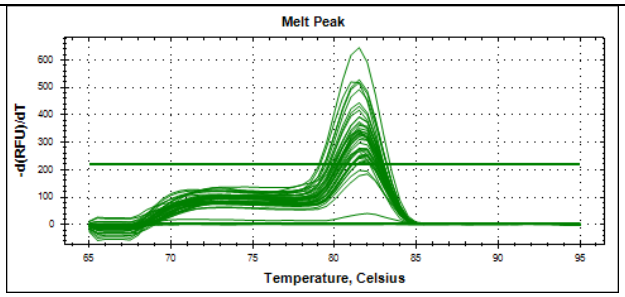


Figure 24: NKCC1 relative gene expression in different treatments with Cu^{2+} without Cu^{2+}. Each column represents the mean + SEM of 8 individuals. Differences between treatments were assessed by one-way ANOVA (and considered significant for $p<0.05$ (shown in the figure).

Table 19: q-PCR, gene NKCC2 with relative q-PCR parameters (R^2, Slope, Efficiency) table, standard curve where ✖ are different samples with different incubation treatment (Control +Cu^{2+}, -Cu^{2+}) and ○ represent the standard points obtained by dilutions, and relative melt curve.

Gene	R^2	Slope	Efficiency
NKCC2	0,996	-3,017	114,5%





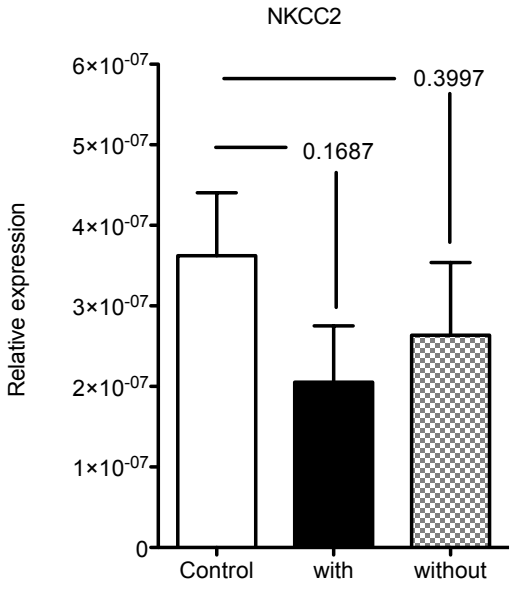
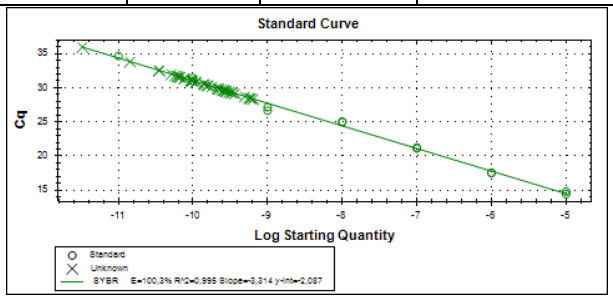
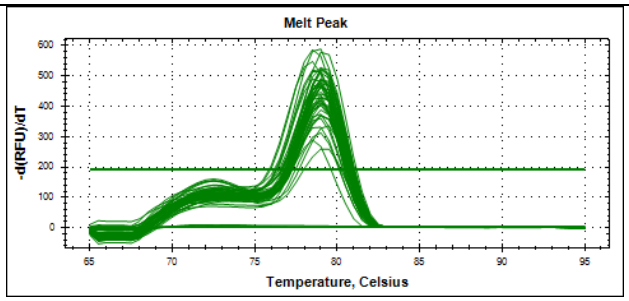


Figure 25: NKCC2 relative gene expression in different treatments with Cu^{2+} without Cu^{2+}. Each column represents the mean + SEM of 8 individuals. Differences between treatments were assessed by one-way ANOVA (and considered significant for $p<0.05$ (shown in the figure).

Table 20: q-PCR, gene CFTR with relative q-PCR parameters (R^2, Slope, Efficiency) table, standard curve where ✖ are different samples with different incubation treatment (Control, $+Cu^{2+}$, $-Cu^{2+}$) and ○ represent the standard points obtained by dilutions, and relative melt curve.

Gene	R^2	Slope	Efficiency
CFTR	0,995	-3,314	100,3%





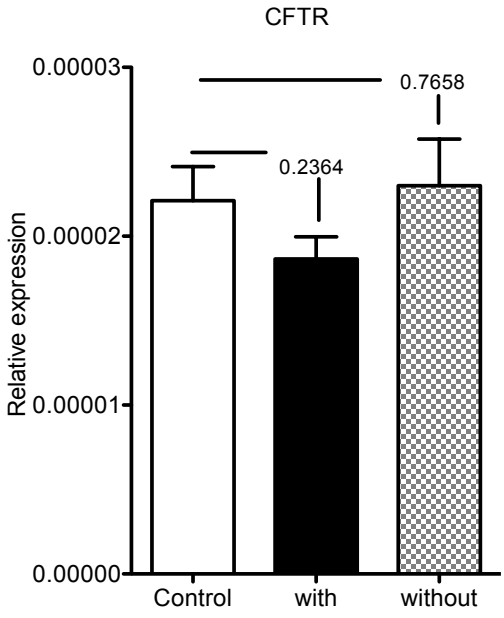


Figure 26: CFTR relative gene expression in different treatments with Cu^{2+} without Cu^{2+}. Each column represents the mean + SEM of 8 individuals. Differences between treatments were assessed by one-way ANOVA (and considered significant for $p<0.05$ (shown in the figure).

V. DISCUSSION AND CONCLUSIONS

The main goals of this work were to test the potential bioactivity of microalgae extracts (*Coccomyxa* sp.), from two different cultures, one with addition of copper (0.6mM Cu^{2+}) and the other one without, in:

-Opercular tissue of an ex-vivo animal model (*Fundulus heteroclitus*) through the epithelial electrophysiology technique in Ussing chambers,

-Quantification of the effect of *Coccomyxa* extracts in gene expression of NKCC1, NKCC2 and CFTR in opercular tissue of *Fundulus* after 24 hours of incubation.

The extracts used in this work were prepared from microextremophiles microalgae *Coccomyxa* sp. strain VAL007 that came from an acidic environment, the acid mine waters of S. Domingos (Valente *et al*., 2013). Those microalgae due to the ability to develop efficient transport mechanisms and/or the putative production of biomolecules can survive in this extreme environment (Brake and Hasiotis, 2010; Gross, 2000; Messerli *et al.,* 2005).

Before the start of experimental testing with the animal models, it was necessary to verify that the microalgae cultures consisted on a single strain of *Coccomyxa* sp. The microalgae were isolated and maintained in the Laboratory of Microbial Molecular Ecology (UAlg-CIMA), and the results from chapter 1 establish the uni-algal status of the starting cultures of *Coccomyxa*. To support this claim related to the state of the starting cultures of *Coccomyxa* sp two lines of evidence were provided. PCR for fragment amplification to assess uni-algal cultures was performed using two primers sets: the first consists on the universal primers for the 18S gene which targets a fragment of nuclear genome and the second consisted of primers specific for 23S gene which corresponds to a primer pair of the chloroplast genome. From the SSCP analysis of amplified fragments 7 different patterns were obtained for the cloned fragments of the 18S gene. A dendrogram was constructed with the corresponding 18S sequences and highly homologous sequences retrieved from the Genebank database (NCBI), including sequences from *Chlorella*, which were used as an out-group. The results revealed that all sequences obtained in this study are closely related to sequences obtained

from the *Coccomyxa* genus and cluster together in a single branch. No sequences from another organism were obtained. In the case of the 23S gene a similar result was observed with the 8 sequences cloned. It is important to note that while the cultures are exclusively *Coccomyxa,* it is impossible at this moment to identify the species. Probably because not enough sequences are deposited in the gene bank to allow identification.

The microalgae extracts were tested on the animal model *Fundulus heteroclitus*, characterized by an opercular epithelium rich in chloride-secreting cells (Karnaky 1997; Karnaky and Kinter, 1997). The presence of chloride cells in the opercular epithelium, was first observed by Burns and Copeland (1950) in seawater-adapted *Fundulus heteroclitus*. Degnan *et al.* (1997) and Karnaky *et al.* (1977) mounted the *Fundulus heteroclitus* opercular membrane in an Ussing chamber and found that the short circuit current could be directly used as an index for net chloride secretion. Since then the opercular membrane has been widely used as a model to examine the mechanisms and control of ion secretion in teleosts.

In this work, with the Ussing chamber it was possible to test the potential of the molecules present in the *Coccomyxa* extracts on the influence of the ionic transport, more specifically on the ion-transport proteins: the basolateral Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) and the apical cystic fibrosis transmembrane conductance regulator (CFTR).

It is important to note that the co-transporter NKCC2 is present in the opercular tissue of *Fundulus* as demonstrated by amplifications by qPCR, but with low expression, and its function is still uncharacterized. Through the use of electrophysiology it was impossible to establish a functional involvement in ion regulation since apical bumetanide was without effect when applied to the apical side o f opercular membranes, the putative functional location of NKCC2. However, its expression seems sensitive to algal extracts and regardless of the type of extract that is incubated epithelium there is a slight variation of gene expression, although it was never significant (Reddy and Quinton, 1999; Insering *et al*., 1998, Haas and Forbush III, 2000).

The basal values of the short circuit current in of opercular membrane of *Fundulus* (Isc = -211.3 ± 24.8 µA/cm²) confirm that in these experimental

conditions the Isc is a direct measure of the Cl^- ion flow through the opercular epithelium (Buhariwallae *et al*., 2012; Hoffmann *et al*., 2002). To test if the extracts of microalgae, with the addition of copper and without, had influence on the Isc, these were administered in increasing concentrations (millioncell/ml) to the different sides the basolateral side and the apical side of the opercular epithelium of Fundulus heteroclitus mounted in the Ussing chamber in voltage clamp.

A clear decrease in the values of Isc is evident in experiments carried out by adding the extracts of microalgae grown in the presence of copper to the basolateral side, with a highly significant effect (p=0.0003). This effect results in the nearly complete inhibition of the current at a high concentration of algae. In contrast, this effect was not observed when the extracts were added to the apical side. In the case of extracts of microalgae not exposed to copper, the Isc variations after the addition of the equivalent to 32×10^6 cells/ml to the apical or the basolateral side resulted in little difference with not statistical significance (p=0.4694) between both sides.

The treatment with extracts of microalgae cultured in the presence of Cu^{2+} had a real effect in the inhibition of the short circuit current (Isc) but only if the extract was added to the basolateral side. This combination of factors is the only one that provided 100% of Isc inhibition with less than 64×10^6 cell/ml, and shows a reduction of 50% of the current at around 8×10^6 cell/ml; in the other three groups the addition of 64×10^6 cell/ml was still not enough for 50% inhibition of the current.

These results highlight both the importance of the side in which the extracts are added and the type of pre-treatment of the algae in the final response. Considering the polarity of the epithelium and the distribution of transporters in either side we can suggest that the extracts have their main site of action at the basolateral membrane, where the NKCC1 co-transporter is functional. This distribution has been proved previously using the diuretic bumetanide because it's a known specific inhibitor of NKCC (Haas and Forbush III, 2000; Ju *et al.,* 2013). However, further evidence was required to establish that bumetanide and the algae extracts were acting on the same mechanism. Thus, extracts (microalgae extracts with copper) were added to the same epithelial tissue before and after the addition of bumetanide, and the results

were clear. Both seem to work through the same co-transporter (NKCC1) because addition of the extracts with Cu^{2+} before the bumetanide resulted in a decrease of Isc, while if the addition was after bumetanide there was no effect on the current. The same situation was observed for bumetanide, if bumetanide was added before the extracts with Cu^{2+}, there was a big decrease of Isc, if the addition was after the addition of algae extracts, there was a little further effect of bumetanide. The interpretation of these results is a competition between the algae and bumetanide to inhibit the co-transporter NKCC. It is important to consider the pretreatment that the microalgae have had, since only the extracts cultured in the presence of copper (0.6 mM) have an effect on Isc.

Crespo and Karnaky (1983) demonstrated that application of a solution of copper Cu^{2+} (40µm) to both sides of the membrane caused a decrease of 45.6% of Isc value. Bras (2015) reported that the administration of Cu^{2+} (2µM) resulted in a decrease of Isc up to 85%, using a lower concentration of copper. But notes that prolonged exposure to copper caused irreversible damage to the opercular epithelium, and notes that the epithelia treated with extracts from microalgae cultures with addition of copper recover 100% of their functionality. Vaquero *et al*., (2012) suggested that the influence of Cu^{2+}, added in the range between 0,06 and 0.4 mM, on a microalgae culture that naturally grows at low pH (*Coccomyxa onubense)*, increases algal viability, synthesis and accumulation of values of carotenoids, mostly lutein and β-carotene. Probably the microalgae in the presence of copper react with it creating some compound that inhibits its toxicity.

Once the bioactivity of the microalgae extracts was demonstrated the next natural experimental step would be to test if in addition to function, the extracts of microalgae would regulate gene expression of specific transporters. Therefore, opercular tissue of *Fundulus* was cultured *in vitro* in the presence of microalgae extracts and the expression of NKCC1, NKCC2 and CFTR was analyzed. It is important to note that only the extracts of algae grown in the presence of copper show some effect in gene expression (normalized to 18S), in relation to the control (NKCC1 p=0.2116, NKCC2 p =0.1687, p=0.2364 CFTR), but without statistical significance. The extracts of algae cultured in the absence of copper were also without effect in gene

expression. The opercular tissues were incubated with extracts of algae cultured in the presence or absence of Cu at a concentration of $16x10^6$ cells/ml for a period of 24 hours. This concentration of algae was chosen based on previous results in the short circuit current measurements, were this concentration resulted in 50% of Isc inhibition. In light of these results 3 explanations could be provided: 1) the algae do not regulate gene expression of the selected transporters; there is an effect in the function of the epithelium related with the presence of the extracts of microalgae, but there is not regulation of the gene networks; 2) the concentration of algae was not high enough to evoke an effect at the level of gene expression; 3) the time of incubation chosen conditions the final observations in gene expression. But, it was impossible to test these hypotheses within the time frame of this thesis. However, this issue would merit future studies.

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