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Toxicity and histological alterations in mussels exposed to lithium

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ABSTRACT

The increased use of lithium in lots of industrial processes and devices has produced a great environmental impact on aquatic environment. Lithium concentrations is higher in places where mining processes occur and in places where Li-ion batteries are disposed of. There is only scarce information concerning the toxicity of lithium in marine organisms. Therefore, the objective of this article is to determine the toxicity and histological alterations of lithium (Li), using the mussel *Mytilus galloprovincialis* as a model organism. For this purpose, an acute toxicity test was headed, thus, mussels were exposed for 9 days to a range of seven different acute concentrations of lithium (2, 5, 13, 34, 89, 233 and 610 mg / L Li). Then, mussels were exposed to four different sublethal concentrations of lithium (0, 1, 50, 100 mg / L Li) for 17 days. Results showed that *M. galloprovincialis* had a LC50 value lower than concentrations found in ambient waters, but also showed that lithium induces toxicity and mortality in a time-dependent manner. Although condition index was not significantly affected by the exposure, samples were collected for further histopathological and histochemical studies. These findings open new perspectives for the understanding of the toxic effects of lithium on marine organisms, and will make industries aware of possible recycling and recovering methods for lithium.

El uso del litio en una gran cantidad de procesos industriales y dispositivos ha producido un gran impacto ambiental en el medio acuático. Las concentraciones de litio son más altas en los lugares donde se producen procesos de minería y se desechan las baterías de iones de litio. Hay escasa información sobre la toxicidad del litio en organismos marinos. Por lo tanto, el objetivo de este artículo es determinar la toxicidad y las alteraciones histológicas del litio (Li), utilizando el mejillón *Mytilus galloprovincialis* como organismo modelo. Para ello, se llevó a cabo un ensayo de toxicidad aguda, exponiendo los mejillones a un rango de siete concentraciones agudas diferentes de litio (2, 5, 13, 34, 89, 233 y 610 mg / L Li) durante 9 días. Posteriormente, los mejillones fueron expuestos a cuatro concentraciones subletales diferentes de litio (0, 1, 50, 100 mg / L Li) durante 17 días. Los resultados mostraron que *M. galloprovincialis* tenía un valor LC50 inferior a las concentraciones encontradas en aguas ambientales, pero también mostraron que el litio induce toxicidad y mortalidad a diferentes concentraciones en función del tiempo. Aunque el índice de condición no se vio afectado significativamente por la exposición, se cogieron muestras para estudios histopatológicos e histoquímicos. Estos hallazgos abren nuevas perspectivas para la comprensión de los efectos tóxicos del litio en los organismos marinos, y harán que las industrias apuesten por los posibles métodos de reciclaje y recuperación de dicho metal.

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1. INTRODUCTION

New ways of transport (electric vehicles, e-bikes, drones), connectivity applications (smartphones, tablets, laptops, small home appliances) and stationary applications (energy storage systems) lead to scale up the use of batteries (Comission 2006/66/EC). Since the amount of applications of batteries is increasing, the use of them has changed in the last decade, being lithium-ion batteries the main storage technology (IEA, 2017). Lithium (Li) converts chemical energy into electric energy very efficiently, reducing costs and increasing manufacturing capacities (IEA, 2017). In this contest, demand for lithium has already risen dramatically and the extraction of this element has significant environmental impact. In the same way, the amount of lithium batteries placed on the market has increased in a high rate (Comission 2006/66/EC), while lithium waste battery collection rate is still very low (Comission 2006/66/EC; Swarnakar & Choubey, 2014; Zeng et al., 2014). In most cases, as they are classified as "other batteries" and the European Parliament do not set strong collection targets or reporting obligations to promote the recovery of the chemicals within lithium batteries, they end up in landfills with municipal solid waste. Even though some Li-ion batteries are processed, this is headed with a view to recovering another metals it contains (nickel, cobalt…) and discarding elements like lithium (Comission 2006/66/EC; Zeng et al., 2014).

Unfortunately, chemicals in those batteries could cause loads of effects on the environment and human (Moore, 1995; Aral & Vecchio-Sadus, 2008; 2011). The increasing use of lithium, in particular, brings up new concerns about possible environmental and health impacts (Lebedeva & Brett, 2016). Therefore, the assessment of the toxicity of this metal in different organisms under different exposure doses and time intervals is of great importance.

Lithium is a soft, grey alkaline metal. It was discovered in 1817 and is the lightest naturally occurred metal with a 0.534 g / cm^3 density. It has the atomic weight 6.941 and the atomic number 3. It is the 33rd most abundant element in nature and about 145 minerals on earth's crust are made up of lithium (0.002 – 0.006 wt%) (Aral & Vecchio-Sadus, 2011; Giraldo, 2016). Lithium is widely spread in trace amounts in soils rocks, and surface, sea and ground waters (Aral & Vecchio-Sadus, 2011).

Biogeochemically, lithium is a highly mobile element. Consequently, it can be transported long distances from the source, increasing the levels of lithium in aquatic environments. Moreover, the environmental impact can be intensified due to the increased mining process and use of lithium and its compounds.

The pure metal never occurs in nature, it is present forming salts like phosphates, silicates and micas (Aral & Vecchio-Sadus, 2008; 2011). The lithium in these salts, as a result of

weathering processes, will reach soils, where it will be taken up by plants (Lenntech, 2007). As plants have presence in the food chain, animals and humans may also get exposed to lithium by oral intake (Kabata-Pendias & Mukerjee, 2007). Median values of lithium in soils are 3 – 350 mg / kg (Aral & Vecchio-Sadus, 2008). It is also present in aquatic environments, primarily in ionic form. When this metal reacts with water, lithium hydroxide and hydrogen are formed. According to literature, in fresh water Li concentration varies between 0.001 and 0.01 mg / L and seawater contains lithium at levels between 0.17 – 0.19 mg / L (Reiman & Caritat, 2012), while ground water concentrations may reach 0.5 mg / L (Aral & Vecchio-Sadus, 2008). Nevertheless, to date, there is scarce information about the concentration of lithium in marine environment and even less about the effects it causes.

Lithium is widely used industrially: as a cell additive in electrolytic aluminum production, as a catalyst in chemical reactors, as a component of fluxes and brazing alloys together with other metals like magnesium and aluminum in air and car industry, as an ingredient in glass and ceramic production, as an additive in cement and in industrial air-conditioning systems (Moore, 1995; Kouga et al., 2013). But as I have already mentioned, one of the most important use of lithium is in batteries. For example, Zeng and Li (2013) estimated that the total weight of discarded Li-ion batteries in Chine will surpass 500.000 tons by this year, 2020.

The industrial use of lithium contributes to environmental pollution (Aral & Vecchio-Sadus, 2008; 2011). Lithium used in batteries is obtained through extracting activities, which become closely associated with negative environmental impacts depending on the site and the technology applied (Comission 2006/66/EC). During the extraction of lithium streams can be polluted with hazardous substances (Comission 2006/66/EC).

There is scarce understanding of toxicity of metals used in high technology production. Clearly, they are not essential elements but most of them may be harmful at high levels of exposure. Moreover, harmful effects may occur in different exposure concentrations in organism-dependent manner. In fact, lithium aluminum hydrides and lithium methanolate can be found in the Danish list of dangerous substances (Kjølholt et al., 2003). Although lithium is not an essential element for life and does not have a known biological use (Lenntech, 2007), presents interesting properties (Nielsen, 1988). Therefore, it is therapeutically used to treat psychiatric diseases such as manic depression (bipolarity) and other affective disorders (Moore, 1995). Teratogenic effects of lithium have been reported while there is no evidence for mutagenic or carcinogenic effects (Aral & Vecchio-Sadus, 2008; 2011). The central nervous system is the primary target organ, but it also affects metabolism, neuronal communication, and cell proliferation in a lot of organisms, including humans (Kjølholt et al., 2003; Aral & Vecchio-Sadus, 2008). In 1949, this metal was banned from use in the US because of its toxicity, and did not get US FDA approval until 1970

for acute mania (Strobusch & Jefferson, 1980) and in 1974 for maintenance therapy (Moore, 1995). Nowadays, the therapeutic window between toxicity and effective dosing is still really narrow.

In terrestrial environment, it has been reported that lithium has loads of effects in humans and other organisms (Moore et al., 1995; Aral & Vecchio-Sadus, 2008; Aral & Vecchio-Sadus, 2011). For example, rats show ulcerative rhinitis sometimes accompanied by squamous metaplasia, necrotic laryngitis and respiratory difficulties when exposed to aerosols containing 80% lithium carbonate. Signs of anorexia and dehydration were also reported (Greenspan et al., 1986).

In the contrary, to date, the increased use of lithium has not generated many studies on its effect on aquatic environment. In fact, there are no abundant and clear results on the concentration of lithium in marine environment which difficult toxicity evaluation. Some studies have performed toxicity tests of lithium for different organisms but lethal concentrations resulted in much higher concentration levels than the ones found in aquatic environment (Lenntech, 2007; US EPA, 2008). The acute environmental effect concentration (EC50) on *Daphnia magna* was determined to be 33 – 197 mg / L, and a lethal concentration (LC50) of 185 – 232 mg / L was reported for *Dreissena polymorpha* (US EPA, 2008). Valle (2019) have identified a LC50 of 324 mg / L for *Littorina littorea*.

Ecotoxicology has become a fundamental tool for monitoring the effect of pollutants on the aquatic organisms so as to protect the environment. Toxicity tests help in the generation of protection of the aquatic ecosystem. Acute toxicity testing is widely used to establish the impact of lithium in the exposed water bodies (US EPA, 2008; Khangarot & Das, 2009; Valle, 2019), since represented acute responses provide information on bivalve thresholds and measurable sensitivities to pollutants. This test allows to estimate the lethal concentration (LC50) and the lethal response time (LT50) (Crane et al., 1995). However, the acute toxicity test is a limited value because the tested population may be exposed to the pollutant for a longer time than for the period set in the test. Moreover, concentrations below the acute toxicity threshold limit are also interesting to study by means of sublethal and long-term effects. Therefore, effects and stress should be studied at lower biological organization levels (organ and sub-organ levels) administering sublethal concentrations. Sublethal toxicity test provides understanding of the interactions of metals with molecular and cellular targets which may be reflected in histochemical, histological and biometric alterations. Besides, it is useful to predict possible long-term effects at population levels.

In pollution monitoring programs and toxicological experiments mussels are popularly used as sentinel organisms due to their biological and ecological characteristics (Goldberg, 1975; Moore et al., 2004; Davies & Vethaak, 2012; Beyer et al., 2017). They are sessile filter-feeders and due to their low metabolic activity, the acute and sublethal toxicity effects exhibited will be the most accurate reflection of the environmental pollution. They also have a relative tolerance to environmental stress and a high accumulation capacity of a wide range of contaminants (Goldberg, 1975). Moreover, due to their easy collection and maintenance in laboratory, they have a really extensive use in experimentation. Therefore, lithium exposure effects were investigated using *Mytilus galloprovincialis* as a model organism.

Pollution and other environmental stressors induce integrated response in mussels, which involves effects at tissue, cellular and molecular levels. These include biological responses such as impaired health condition or histopathological alterations (Marigómez & Baybay-Villacorta, 2003; Izagirre & Marigómez, 2009; Izagirre et al., 2009; Lekube, 2014).

Biomarkers are used as warning signals of biological effects caused by environmental pollutants (Wu et al., 2005). Among these, lysosomal responses to pollutants in mussel digestive cells are widely used, being core biomarkers for biological affects assessment in marine pollution monitoring programs (UNEP/RAMOGE, 1999; Moore et al., 2006; Davies & Vethaak, 2012). The digestive gland is involved in immune-defence, detoxification and homeostatic regulation, being the main site for metabolic activity (Marigomez et al., 2002; Moore and Allen, 2002). Lysosomal responses are dependent on the time and may also be opposite at different times depending on the type and the dose of pollutant (Marigómez & Baybay-Villacorta, 2003; Izagirre & Marigómez, 2009). Exposure for weeks or months provokes lysosomal enlargement in mussel digestive cells, while exposure to low pollutant concentrations for a few days will also elicit lysosomal size reduction (Marigómez & Baybay-Villacorta, 2003; Izagirre & Marigómez, 2009). Nowadays, three main lysosomal responses to environmental stress can be noticed in mussels: reduction of destabilization of the lysosomal membrane, lysosomal enlargement and changes in lysosomal contents such as lipofuscin or neutral lipid accumulation (Marigómez & Baybay-Villacorta, 2003; Davies & Vethaak, 2012; Blanco, 2018).

Histopathological alterations in selected organs and tissues, mainly gills, digestive gland and gonad, are conceived as tissue-level biomarkers. These alterations reveal disturbances at different biological organization levels (Moore and Simpson, 1992) and are sensitive and reliable indicators to provide an overall assessment of the general health status of aquatic species (Stentiford et al., 2009). Mussel histopathology has been applied to search on the relationships between exposure to contaminants and environmental health, allowing for the detection of a wide range of alterations that may produce mortality or morbidity (Marigómez et al., 2006; Bignell et

al., 2008). Accordingly, a plenty of histopathological criteria can be applied when using mussels as model organisms: changes in the cell-type composition of the digestive gland and in the structure of the digestive diverticula, net mass loss in the digestive gland epithelium and thus, epithelial thinning and atrophy (Marigómez et al., 2006; Davies & Vethaak, 2012, Lekube et al., 2014; Blanco, 2018). Among degenerating changes in digestive cells related to pollution effects, the increase in the relative proportion of basophilic cells is well documented (Davies & Vethaak, 2012; Blanco, 2018). This is measured in terms of volume density of basophilic cells (Vv_{BAS}), which may surpass 0.12 μm^3 / μm^3 after pollutant exposure (Marigómez et al., 2006; Blanco, 2018). Epithelial thinning and atrophy can also be measured by means of luminal radio/mean epithelial thickness (MLR/MET), which may surpass 0.7 µm/µm in spring and 1.2 µm/µm in autumn after exposure to pollutants, and connective tissue to digestive tissue (CTD) ratio (Marigómez et al., 2006; Davies & Vethaak, 2012; Blanco, 2018).

After a stress response at lower level organization, it may subsequently be evident at higher biological organization levels. In this contest, changes in allometric ratios, condition indices and growth parameters may be caused by sublethal levels of environmental toxicants (Lober & Wright, 1982; Marigomez & Ireland, 1990; Marigomez et al., 1990). Flesh weight and condition indices have been demonstrated to change as a result of depressed growth in the presence of metals (Marigomez & Ireland, 1990; Cajaraville et al., 1992; Soto & Marigomez, 2000).

The aim of the present work is to assess the acute and sublethal effects of Li in mussels to help to determine the risk of lithium exposure to aquatic organisms. This may also raise awareness within the society in order to recycle the waste batteries of their gadgets and within the industries in order to promote the recovery of the lithium within them.

2. HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

The hypothesis of this work is that environmental pollutants such as Li, besides causing acute effects in high concentrations, also provoke sublethal effects in mussels when exposed to lower concentrations.

2.2. Objectives

In order to demonstrate the hypothesis, the following objectives are to be achieved:

To stablish the lethal concentration of Li for *Mytilus galloprovincialis*.

To study alterations in condition index, histological features and lysosomal responses in terms of sublethal toxicity in *Mytilus galloprovincialis*.

To provide data for determining risk of lithium exposure to *Mytilus galloprovincialis*.

3. MATERIALS AND METHODS

3.1. Test organisms and acclimatization of the samples

Intertidal mussels (*Mytilus galloprovincialis*) of 2.5 – 3.5 cm shell length were used in toxicity testing. Mussels for both experiments were collected from the low tide-mark level in Plentzia (Basque Coast; 43º24'N, 2º55'W) at different times, in October 2019 for the acute toxicity test and in January 2019 for the sublethal toxicity test.

All mussels on arrival were transported to the laboratory and maintained in 20 L polyethylene containers with running seawater for 14 days to acclimatize. They were kept under the following laboratory conditions: water temperature – 18-19ºC, pH – 7.8, conductivity – 48.000-51.000 Ω, salinity – 33 PSU and ambient light from overhead laboratory lights was used with a 12h photoperiod. Mussels were fed every three days a commercial marine microalgae mixture of *Isochrysis spp., Tetraselmis spp., Pavlova spp., Nannochloropsis spp. and Spirulina spp.* (1:10; 3.5 mL; Acuinuga, A Coruña, Spain). After acclimatization, the animals were used for the exposure experiments (acute toxicity test and sublethal toxicity test).

3.2. Experimental design

3.2.1. Acute toxicity test

The lithium was applied as LiCl (>99%, ACROS, USA). A mother solution (5 g Li / L) was prepared diluting 31.25 g LiCl in 1 L seawater. Contaminant was provided together with water change.

Fibbonacci Scale numbers were used to select the 8 exposure concentrations for this chemical in the acute experiment. The Fibbonacci sequence consists of summating the two previous numbers to obtain resulting number, starting with 0 and 1. Those exposure concentrations where chosen based on the ones selected in the acute toxicity test of *Littorina Littorea* (Valle, 2019): 0 (control), 2, 5, 13, 34, 89, 233, 610 mg / L Li. Besides, LC50 value of 185-232 mg / L obtained for *Dreissena polymorpha* (Zebra mussel) (US. EPA, 2008) was taken into account.

24 experimental groups were established (control, 2, 5, 13, 34, 89, 233, 610 mg / L) bearing in mind the 3 replicas that were set per exposure concentration. Each concentration was

tested in 5 mussels which were kept in 0.5 L polyethylene bottles under continuous aeration for 9 days (Fig. 1).

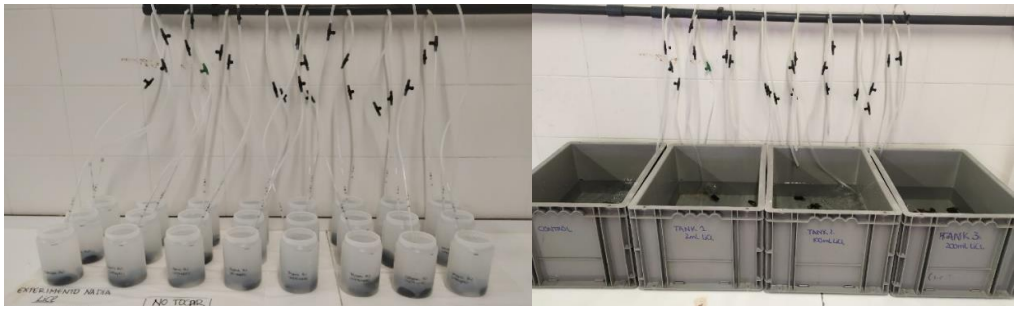

Fig 1. On the left, the assembly of the acute toxicity test's experimental design. On the right, the assembly of the sublethal toxicity test's experimental design. (PiE – Plentizako itsas Estazioa; October, 2019 and February, 2020).

The control water and the different solutions were replaced daily at 13.00 h. Mussels were fed a commercial marine microalgae mixture of *Isochrysis spp., Tetraselmis spp., Pavlova spp., Nannochloropsis spp. and Spirulina spp.* (1:10; 87.5 µL; Acuinuga, A Coruña, Spain) every day at 13:00 h, just after water change.

The experimental groups were checked every day for mortalities. Mussels were considered dead when the valves were opened or partially opened and did not close or respond to manipulation. The number of dead specimens was removed and recorded daily. Mortality data was used to calculate the median lethal concentration (LC50) ant the lethal response time (LT50).

3.2.2. Sublethal toxicity test

Once the acute lethal toxicity of lithium for *M. galloprovincialis* was established, a range of sublethal concentrations of the contaminant that could be used was identified. Moreover, a set of mussels (N=20) did not undergo exposure experiments and was sacrificed at the starting point of the experiment (T0). This provides the ability to have a general control of the experiment.

Mussels were distributed in 4 experimental groups, each made of 50 mussels in 10 L polyethylene tanks under continuous aeration (Fig. 1). Each experimental group was exposed to different sublethal concentrations of the metal: control (C), low concentration (L; 1 mg / L), intermediate concentration (I; 50 mg / L) and high concentration (H; 100 mg / L). The exposure was to be set for 7 and 21 days. Slight mortality was detected in the high concentration group on the day 15, so the second exposure period was adjusted to 17 days. During the exposure, mussels were fed daily a commercial marine microalgae mixture of *Isochrysis spp., Tetraselmis spp., Pavlova spp., Nannochloropsis spp. and Spirulina spp.* (1:10; 1.75 mL; Acuinuga, A Coruña, Spain). Water and contaminant change were headed every two days at 13:00 h.

The experimental groups were checked and observations were recorded daily. At the start point of the experiment (T0) and at end of each exposure period (7 and 17 days), 20 mussels of each experimental group were randomly chosen and sacrificed. Weight (g) and biometrical measurements (cm) were headed, using a laboratory balance and a Vernier caliper. A cross section of the whole flesh of 10 individuals was fixed in 4% formaldehyde in seawater for histological analyses. The gills, digestive glands and feet of the other 10 individuals were dissected out and placed in plastic cryovials. Those cryovials were flash-frozen with liquid nitrogen and stored at -80ºC until histochemical analysis. Besides, at the end of the 17 days exposure, 5 mussels of each experimental group were dissected and stored at -80ºC until chemical analysis.

Flesh condition index and biometry

After each exposure period, measurements of each mussels were recorded: length (L, maximum measure along the anterior-posterior axis), width (W, maximum lateral axis) and height (H, maximum dorsoventral axis). Wet flesh weight (WFW) and upper and lower valve weights (V) were measured when the mussels were opened.

Flesh condition index (FCI) was calculated as described by Lober and Wright (1982), obtained as flesh weight (mg) to shell weight (g), using the wet flesh weight and the shell weight (Marigómez et al., 2006).

Histopathological alterations

The cross-section cut of the 10 individuals of each experimental group was fixed in 4% formaldehyde in seawater (pH 7.2) for histological analyses. The cross-section cut should include as many organs as possible, so that a section with the representation of all the organs is obtained (Fig. 2).

Fixed samples were to be dehydrated in graded ethanol series and embedded in paraffin as described by Culling (1974). A Leica RM2125 rotary microtome was to be used to cut paraffin blocks in order to obtain 5 µm thick sections. The sections were to be mounted on albumin coated glass slides and dried at 37ºC for 24 hours. Finally, sections were to be stained with hematoxylin-eosin using an automated slide stainer (Table 1; Fig. 3). Histological changes in the digestive gland would have been studied under a light microscope (Nikon Optiphot microscope).

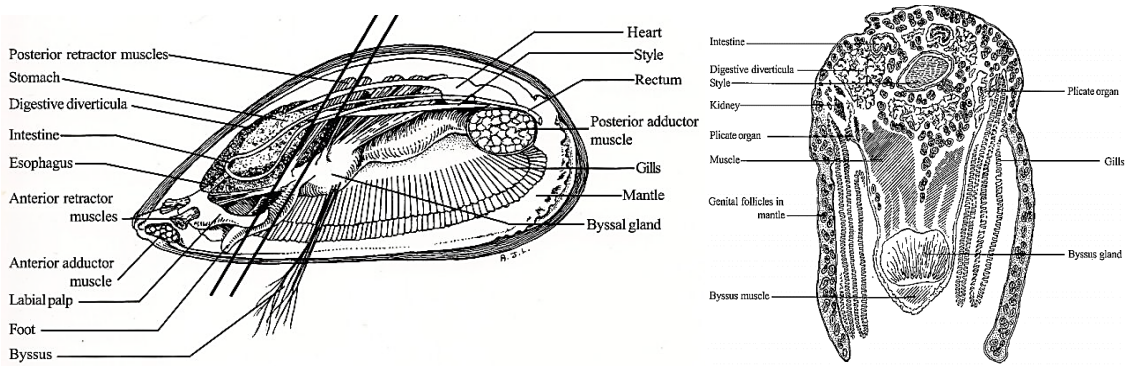

Fig 2. On the left, gross anatomy of the mussel *Mytilus edulis*. Bold parallel lines show location where cross-section should be taken. On the right, illustration of cross-section of the mussel *M. edulis* (Illustrations by Lippson, A. J.; Bozman, M.D.; Howard et al., 2004).

Table 1. Protocol for hemotoxyline and eosine staining using automed slide stainer.

STEP	STATION	TIME	REAGENT
1	1	10’	Xilol
2	2	10’	Xilol
3	3	2’	Alc. Abs
4	4	2’	Alc. Abs.
5	5	2’	96º
6	6	2’	70º
7	7	5’	Destiled H_20
8	12	4’	Hematoxyline
9	Wash 5	4’	
10	Acid alcohol	10’	
11	Wash 4	5’	
12	Lithium carbonate	10’	Eosine
13	Wash 3	1’	
14	Eosine	1’30’’	
15	Wash 2	1’’	
16	Wash 1	2’	
17	13	2’	70º
13	14	2’	96º
19	15	2’	Alc. Abs.
20	16	2’	Alc. Abs.
21	17	5’	Xilol
22	18	5’	Xilol

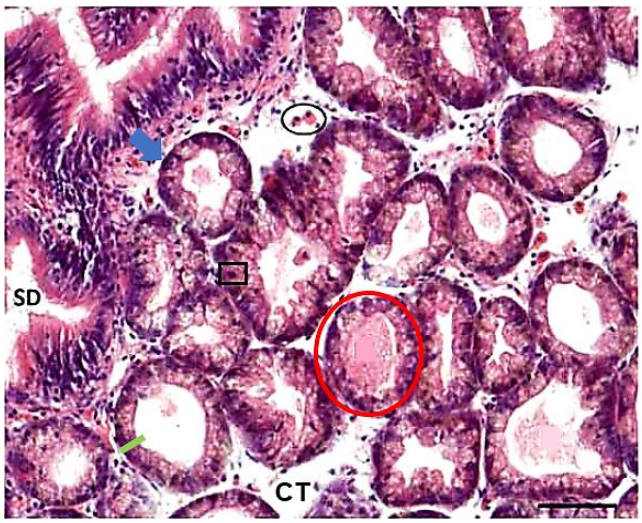

Fig 3. Micrograph of the digestive gland of mussel *M. galloprovincialis* stained wit hematoxylin-eosin. CT: conective tissue. SD: secondary duct. Black circle: haemocytes. Blue arrow: Basophilic cell. Black square: nucleus of digestive cell. Red circle: Digestive tubule. The green line indicates the height of the epithelium. Scale: 50 µm.

Histopathological alterations such as changes in cell type composition (i. e. basophilic cells), atrophy of the epithelium and loss of digestive gland histological integrity were planned to be measured. A Weibel graticule multipurpose test system M-168 was to be used in order to record hits of basophilic and digestive cells, luminal area and connective tissue. The volume density of basophilic cells (Vv_{BAS}) in digestive gland was to be determined according to Soto et al. (2002). Besides, the integrity of the digestive gland was to be obtained as connective tissue-to-diverticula (CTD) ratio, determining the extent of the interstadial connective tissue relative to the space occupied by digestive diverticula (Brooks et al., 2011; Blanco, 2018). Likewise, the mean epithelial thickness of the digestive alveoli (MET) was to be determined together with other estimates such as mean luminal radius (MLR) and the MLR-to-MET ratio (MLR/MET) (Blanco, 2018).

Lysosomal biomarkers

The determination of lysosomal membrane stability (LMS) was based on the time of acid labialization (labialization period, LP; min) required to produce the maximum staining intensity according to UNEP/RAMOGE (1999). This was to be evaluated in cryotome sections (10 µm thick; Leica CM 3000 cryotome) of digestive gland chucks after hexosaminidase (Hex) activity was histochemically demonstrated.

Another sort of cryotome sections (8 µm thick; Leica CM 3000 cryotome) of digestive gland were to be stained for the histochemical demonstration of β-glucuronidase activity according to Cajaraville et al. (1991) with a view to quantify lysosome structural changes (LSC). The following stereological parameters were planned to be determined for each mussel digestive gland with the aid of an image analysis system: lysosomal volume density ($Vv_{LYS}=V_{LYS}/V_C$), lysosomal surface density ($Sv_{LYS}=S_{LYS}/V_C$), lysosomal surface-to-volume ratio ($S/V_{LYS}=S_{LYS}/V_{LYS}$) and lysosomal numerical density ($Nv_{LYS}=N_{LYS}/V_C$); where V_{LYS}, S_{LYS} and N_{LYS} are the volume, surface and number of lysosomes, and Vc the volume of digestive cells.

Intracellular accumulation of neutral lipids (NL) were to be demonstrated histochemically by staining with Oil Red O (ORO; Culling, 1974). Cryotome sections (8 µm thick; Leica CM 3000 cryotome) of digestive gland were to be stained. The extent of Oil Red O staining in the digestive gland epithelium was to be measured by microscope and image analysis according to Marigómez and BayBay-Villacorta (2003). The volume density of neutral lipids with respect to the digestive epithelium volume (Vv_{NL}; $\mu m^3/\mu m^3$) was planned to be calculated.

Lipofuscin (LPF) accumulation of tertiary lysosomes was to be quantified using Schmorl's reaction (Pearse, 1972). Cryotome sections (8 µm thick; Leica CM 3000 cryotome) of digestive gland chucks were to be stained. Image analysis was planned to be used to measure the

extent of Schmorl positive materials (LPFs) in the digestive gland epithelium. Volume density of lipofuscins with respect to the digestive epithelium volume (Vv_{LPF}; $\mu m^3/\mu m^3$) was to be calculated.

3.3. Statistics

Statistical analyses were performed using IBM SPSS 25.0 for Microsoft. LC10 and LC50 were determined using Probit analysis at the corresponding time intervals. Differences between survival curves were estimated using the non-parametric Kaplan-Meier test. Further on, in the case of the parametric variable FCI, one-way ANOVA was applied to determine the effect of exposure period in different experimental groups. This analysis was complemented with Duncan's multiple-range test as a post-hoc, based on one-way ANOVAs. For each exposure period, differences between experimental groups were stablished according to the Student's t-test. A 95 % significance level ($p < 0.05$) was stablished for all statistical analyses carried out.

The sublethal toxicity test was not completed for reasons of the ongoing coronavirus pandemic which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The nationwide closing of the university did not let me determine differences in histological features and lysosomal responses. Therefore, I cannot show results about these sections. Anyway, I have really struggled to get this project right because of the importance it has for me.

4. RESULTS

4.1. Acute toxicity test

The mortality data obtained from the acute toxicity test performed was used to asses the acute toxicity of Li on *M. galloprovincialis*. This data was used to construct the cumulative survival curves of the different experimental groups, which is shown in Figure 4. No significant moratlity was measured in control and lower exposures throughout the testing period (< 10%). Survival was significantly ($p < 0.05$) different in the two higher concentrations (233 mg / L and 610 mg / L), while the highest concentration, 610 mg / L, resulted in 0% survival of the mussels in the last day. The longest survival times were recorded in lower concentrations than 233 mg / L (0, 2, 5, 13, 34, 89 mg / L). LT50 of the mussels exposed to 610 mg / L Li was higher (6 days) than in mussels exposed to 233 mg / L Li (4 days).

The mortality data obtained in the acute toxicity test was used to determine the different lethal concentration values (LC10 and LC50) (Table 2). A 9-day LC50 value of 153.785 mg / L Li was obtained for the mussel *M. galloprovincialis*.

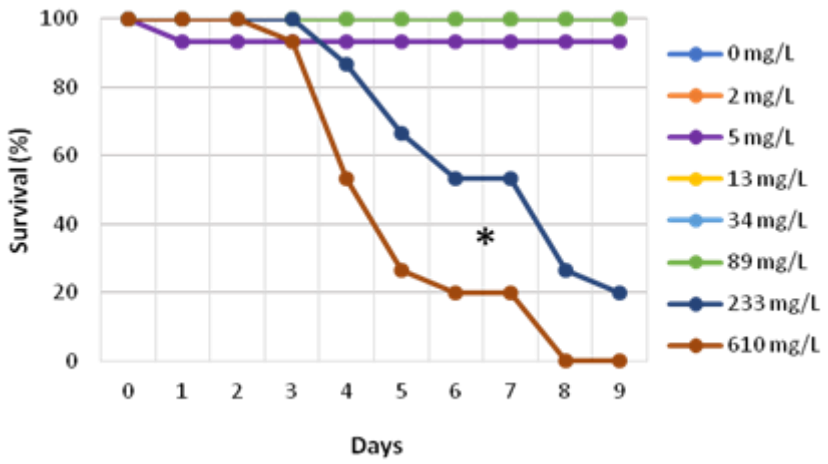

Fig 4. Cumulative survival curves of 15 individuals of mussels (*M. galloprovincialis*) when exposed to different Li cocentration exposure (0-610 ppm). Asterisk indicates significant differences among mussel populations exposed to different contaminant concentrations.

Table 2. Lethal concentration (mg / L) values (LC10 and LC50) for mussels exposed to Li.

TIME (DAYS)	LC10 (MG / L)	LC50 (MG / L)
4	9.424	1665.429
5	11.927	430.915
6	12.174	310.533
7	12.174	310.533
8	15.181	165.217
9	15.138	153.785

4.2. Sublethal toxicity test

No significant mortality was recorded in the C-Li, L-Li and I-Li groups. Mussels exposed to the highest concentration, however, were the most affected and 15 individuals died after 15 days of exposure to 100 mg / L Li. In order to carry out histochemical and condition index examination the second exposure period was set for 17 days instead of 21 days.

Flesh condition index exhibited no significant changes in relation to the exposure to Li in different periods of time according to the one-way ANOVA performed. The experimental exposures to L-Li and I-Li led to higher FCI values than those recorded in the control goup (Fig. 5). Although no significant differences were found between the experimental groups, a trend to increase FCI was observed in mussels exposed to low and intermediate concentrations while a trend to reduce FCI was observed in mussels exposed to the higher concentration (100 mg / L). Moreover, no significant differences were found between T0 and the control group, which means that the experiment is stable and the effects observed are due to the LiCl exposure and not to any external factor.

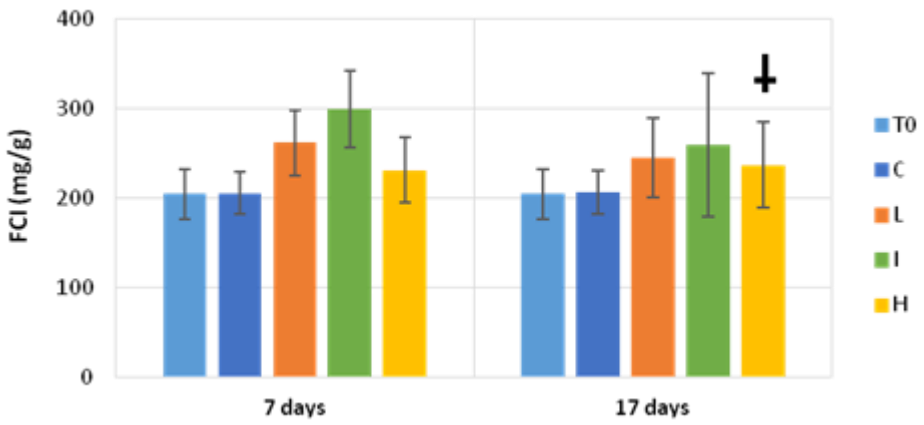

Fig 5. Flesh Condition Index (FCI) in mussels *M. galloprovincialis* exposed to different concentrations of lithium (C, control; L, low; I, Intermediate; H, high) at different exposure periods (7 and 17 days). Intervals indicate standard deviation. T0 indicates values for the group sacfrificed at the starting point of the experiment. The inverted cross indicates mortality in H-Li experimental group.

4.3. Observations

In control group and low concentration of Li, mussels remained attatched to the bottom of the tank while mussels in intermediate and high concentrations of Li were not and were easily displaced when water changes were perfomed. Tanks were covered with biofilm in control and L-Li. However, the biofilm observed in I-Li was lower, whereas no biofilm was present in H-Li.

5. DISCUSSION

In terms of environmental contamination, it has been shown that lithium use has increased due to the high demand of electronic devices such as mobile phones, laptops, tablets, photo cameras, etc. Lithium is used for the batteries in these gadgets. Moreover, the industry of electric cars has also become more popular in the last years. This could have a clear environmental impact and thus, after in the health and wellbeing of the living organisms. It is clear, therefore, the importance to research on toxicity of this metal, which is considered an emerging pollutant (Comission 2006/66/EC; Aral & Vecchio-Sadus, 2008; 2011).

In this present work an acute toxicity test has been headed in order to evaluate lithium toxicity for *Mytilus galloprovincialis*. There are several studies which have used acute toxicity testing to establish the impact of contaminants such as Li, Cu, Ag, Zn, Pb, Cd, … in the exposed aquatic organisms (Milam et al., 2005; Ferrer et al., 2006; Khangarot & Das, 2009; Valle, 2019). Fibbonacci Scale numbers were used to select the 8 exposure concentrations in this test, since several authors recommend this series (Dent & Eisenhauer, 1996; Penel & Kramar, 2012; Valle, 2019) due to the wide range of exposure concentrations it offers. This wide range of exposure concentration is of great importance because there was not available information about LC50 for

seawater bivalves exposed to Li, as far as we know. Therefore, this series was appropriate to obtain LC50 for *M. galloprovincialis*, which turned out to be 153.785 mg / L Li after 9 days of exposure.

LC50 of 185 – 232 mg / L was reported for *Dreissena polymorpha* (US EPA, 2008) after 24h of exposure, whereas Valle (2019) obtained a LC50 of 324.937 for *Littorina littorea* after 3 days of exposure with LiCl. The reported values suggest that dose effect is different between mollusks, being bivalves (*M. galloprovincialis*) more sensitive than gastropods (*L. littorea*) to lithium contamination in marine environment. Fish have been more frequently used in Li acute toxicity testing, for instance, *Pimephales promelas* (Fathead Minnow), a species commonly used in toxicity testing, had LC50 values ranging from 1.2 to 8.7 mg / L when exposed for 26 days to LiCl. Besides, *Xyrauchen texanus* (Razorback sucker) exhibited LC50 of 156 mg / L after 96 h of exposure to LiCl (Shahzad et al., 2017). These values compared to the LC50 values for *M. galloprovincialis* reported in the present study, indicates that *Xyrauchen texanus* and *M. galloprovincialis* may have similar sensitivity to Li pollution.

Besides, despite the fact that the LC50 obtained in the current study with *M. galloprovincialis* mussels is lower than the ones reported for *D. polymorpha* and *L. littorea*, it is still higher than the lithium concentrations found in seawater (0.17 – 0.19 mg / L; Reiman & Caritat, 2012). Mortality was also detected in the sublethal toxicity test in mussels exposed to H-Li (100 mg / L Li), resulting in 15 individuals died after 15 days of exposure. The resulted LC50 for *M. galloprovincialis*, however, was calculated after a 9 days-exposure. These results evidence that high concentrations of lithium in short exposure times will be toxic for mussels, but lower concentrations will also cause mussels' death when longer exposure times occur. Certainly, in view of the fact that mortality was recorded in mussels exposed to H-Li during the sublethal toxicity test, an adjusted exposure concentration for testing sublethal effect of Li is needed for future experiments. Taking the LC10 values into account, we should have set 15 mg / L Li as the highest exposure concentration, since it is the concentration at which 10% of the population will die and lower concentrations will be considered sublethal concentrations. However, the other concentrations ensure the observation of sublethal effects, which sould be easily detected. Unfortunately, the pandemic situation did not give the opportunity to observe them.

Flesh condition index (FCI), is used as an indicator of biological effect against stress, which reflects the well-being of the organisms and their physiological status (Lobel & Wright, 1982). It has been reported that FCI is reduced on exposure to chemical pollutants (Marigomez et al., 1990; Cajaraville et al., 1992; Soto & Marigomez, 2000), since there is an increase of energy demand to face the toxic effect of pollutants and reserve material is consumed to face that energy demand. Conversely, FCI increased in mussels expose to Li, although it is a slight increase in

mussels expose to H-Li. As a result of adaptative response for mussel survival mussels will close the sell, leading to anaerobic respiration (Akcha et al., 2000). Together with the energetically expensive detoxification processes can result in energy-deficient conditions. This increasing response in the FCI may be related to a blockage of the spawning activity so as to use gonad tissue as an energy reservoir for extra demands. In this regard, mussels will have a higher FCI because they are retaining their gonads so as to have energy when energy demands get higher. Gonad resorption, which is a typical mechanism of response against environmental stress, will provide a great energy source to cope with extra metabolic demand (Cajaraville et al., 1991). The same report was given by Soto and Marigómez (2000) who get similar results in mussels exposed to cadmium, which leads to believe that Li affects similarly to mussels. In addition, and in accordance with the present results of this study, Strømgren and Nielsen (1991) found spawning reduction in mussels exposed to copper. In the long run, mussels exposed to H-Li may be exhausted due to the contaminant stress. Indeed, mortality was recorded by day 17. Therefore, histological samples were collected in order to look at the gonads. Unfortunately, it was not possible to complete the histopathological study in the current situation of the pandemic. Certainly, further research with longer period of exposure is needed to evaluate long term responses such as changes in FCI, since 17-day exposure is not enough time to see meaningful effects.

Mussels exposed to low and medium concentrations of Li were coated by biofilm, which indicates a Li associated effect in the bacterium community, which could have other implication such as alteration in bioavailability of pollutants. A similar phenomenon was observed by Scott and Major (1972) when *Mytilus edulis* was exposed to copper. These authors suggest mucus secretion from the gills as a mechanism for internal copper excretion. Bouquegneau et al. (1982) stated that mucus delimination could limit the rate of entry of the pollutant in the organisms. In agreement with this, Amiard-Triquet et al. (1986) and Naimo et al. (1992) reported similar results in their studies. Besides, no film was observed in H-Li, evidencing the toxicity if they were bacterium. Additionally, mussels exposed to I-Li and H-Li were not attached to the bottom of the tank. This weak attachment indicates intense affection, because if the mussel uses all its energy to compensate for environmental stress, there will not be energy left for the production of byssus (silky substance secreted by mollusks by which they attach to the substrate). In the same way, Pelvin (2000) indicated that mussels affected by oil pollution showed lower attachment rates than those not affected, which was also evident in the present experiment.

Despite the fact that histopathological study was not achieved due to the pandemic situation, it is expected that lithium causes effects at tissue-level. Further studies will be performed to find histopathological alterations in gills, digestive gland and gonads. Gamete

development of mussels *Mytilus spp*., in generally, starts at the beginning of winter and spring, and spawning takes place during late spring (Beyer et al., 2017). However, pollutants provoke marked changes in the reproductive cycle (Ortiz-Zarragoitia et al., 2011). Then, for assessing the course of the reproductive cycle, a histological preparation of gonad will be headed. Besides, spawning was not observed. As mussel sampling for the sublethal toxicity test was headed in January, it was not likely to see spawning in any tank since gonads are still in the early stages of gametogenesis.

The gills have respiratory and feeding role. Here, the level of oxidative processes is expected to be very high, since gills constitute an important entry site for uptake of dissolved pollutants. This is because they are the first organ which is exposed to waterborne pollutants (Vidal-Liñan & Bellas, 2013). Affected enzymatic activity by pollutants may be determined using these organs, since the physiological status of the organism weakly influence on them (Davies & Vethaak, 2012). On the other, digestive gland is one of the main target tissues in toxicology when the biomarker approach is applied. It consists of some blind-ending tubules with a digestive epithelium made up of digestive and basophilic cells. Indeed, digestive glands participates in detoxification and accumulates pollutants (Davies & Vethaak, 2012). We could expect that due to the altered health conditions of the mussels after Li exposure, the relative proportion of basophilic cells increases. In fact, under normal physiological conditions the digestive cells outnumber basophilic cells (Marigómez et al., 1992; Soto et al., 2002). Together with this, atrophy of the digestive gland and loss of digestive gland histological integrity may be observed in mussels exposed to lithium. If these histopathological effects are spotted, they may lead to a lower energetic balance and mussel death in the long term.

Although lysosomal analysis was not completed, samples were collected for future analysis. Lysosomal biomarkers are ICES core biomarkers for biological effects assessment, which are widely used in marine pollution. Future studies will deal with lysosomal biomarkers such as lysosomal membrane stability (LMS), lysosomal structural changes (LSC) and neutral lipid (NL) and lipofuscin (LPF) accumulation. In mussels exposed to L-Li we could expect no effects, whereas in I-Li concentration effects will be more significant. In mussels exposed to H-Li we could expect really aggressive effects, since many individuals died during the exposure period. We could expect enlargement of the lysosomes, which may lead to destabilization of the membrane as it has been report in mussels exposed to other metals or organic chemical compounds (Marigómez & Baybay-Villacorta, 2003; Marigómez et al., 2005; Lekube et al., 2014). On agreement with this statement, some studies found excess of enlarged lysosomes in rats exposed to lithium (Samadian, 1993; Aziz, 2015).

In conclusion, despite the fact that obtained effect data for acute toxicity of lithium still do not come close to environment concentrations, chemical influence of Li should not be underestimated. As demand for lithium is rising in order to create electronic devices and electrical vehicles, unless a recycling target for lithium metal is introduced, its environmental impact will increase. From future research perspective, Li should be extensively studied in order to understand its biological functions. Currently, some data indicates that high concentrations Li induces numerous effects in different organisms. Conversely, scarce data about sublethal toxicity effects is available, which means that more research should be done on this metal.

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