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EFFECT OF SUBMARINE ACOUSTIC NOISE IN JUVENILE SEA BREAM (SPARUS AURATA) AND MUSSELS (*MYTILUS GALLOPROVINCIALIS*)

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Palabras Clave:
Deep Sea Mining; noise pollution; stress; biochemical effects; invertebrates; fish;molecular effects

ABSTRACT
The earth' resources are running out, the population will increase and further sources will be needed. These, were found in the deep ocean. To date the mining activities (Deep Sea Mining, DSM) they have not started. One impact of DSM could be underwater noise. Considering the noise frequencies of anthropic activities in the ocean, and the European directive on underwater noise control, we stressed the animals with four acoustic 1/3 band noises around: 63 Hz, 125 Hz, 500 Hz, 1kHz. We study the effects on Sparus aurata juveniles, and on Mytilus galloprovincialis. We will analyse behavioural., molecular and biochemical responses.

RESUMEN
Los recursos de la tierra se están agotando, la población aumentará y se necesitarán más fuentes. Esaos fueron encontradas en el océano profundo. Hasta ahora, las actividades mineras (Deep Sea Mining, DSM) no han empezado. Un impacto del DSM podría ser el ruido subacuático. Teniendo en cuenta las frecuencias de ruido de las actividades antrópicas en el océano, y la directiva europea sobre el control del ruido submarino, molestamos los animales con cuatro ruidos acústicos de 1/3 de banda alrededor: 63 Hz, 125 Hz, 500 Hz, 1kHz. Estudiamos los efectos en juveniles de *Sparus aurata* y en *Mytilus galloprovincialis*. Analizaremos respuestas comportamiental., moleculares y bioquímicas.

INTRODUCTION

A century and a half ago, Swedish explorers discovered for the first time the oceanic minerals deposits in the Kara Sea (Peacock & Alford,2018). In the seventies of the nineteenth century, the HMS Challenger expedition confirmed the presence of these deposits. Since these years, the interest for these sites has grown, but commercial exploitation has been disheartened by low mineral prices and technological difficulties to reach these sites (Peacock & Alford,2018). However, the world population growth, the consumption and technological development are stimulating the future commercial exploitation of these sites. The statistical data say that the demand for nickel, for example, will increase about 50% by 2030. The most important resources are found in the nodules at the bottom of the Clarion-Clipperton site (CCFZ), in the Mexico's fracture (Petersen et al.,2016). The deposits that seem to be most useful for commercial exploitation are three: non-active hydrothermal vents, cobalt crusts and manganese polymetallic nodules. The first are a fractures that pour hot material into the ocean depths. They are rich in copper, zinc, lead and gold. The second are formed by the precipitation of the metals contained in the sea water and grow at a very slow pace. The third are formed due to the precipitation of metals around debris. The management of exploration and exploitation licenses for these sites concerns the International Seabed Authority (ISA) founded in 1994 by the United Nations Convention (UNCLOS, 1982; Mengerink et al.,2014). The parties that have joined the convention are 167 States with also the European Union. For this reason, the companies or organizations that want to extract minerals must be sponsored by a country that has joined the UNCLOS. These company performs the recognition of the minerals and divides the site into two parts. The ISA decides which part to donate to a developing country for exploitation(Peacock & Alford,2018). The ISA is obliged to guarantee a fair distribution of benefits, protection and conservation of biodiversity. Every state, from its shores, has decision-making and jurisdictional power within a stretch of sea. Beyond this stretch of sea we enter in the international waters that names "Area". They are the seas that belong to everyone and they are not of anybody in which ISA has power. To date, the ISA has granted 28 exploration permits in 20 countries for the collection of minerals samples from the seabed (Petersen et al.,2016). Sea submarine mining is becoming a reality.The ISA requires that the rules and laws be implemented by 2020 and that each country should develop its own rules and laws before the start of the activities. Today, many companies are interested in this activity: Global Marine Mineral Resources (GSR), UK Seabed Resources, Nautilus minerals, Neptune minerals, etc. (Peacock & Alford,2018). However, the effects on the environment and on biodiversity are not known, mainly because the extraction activities have not started. According to some authors, for example, a site should be exploited if the concentration of the nodules exceeds 10 kg per square meter with a slope of less than 10%(Peacock & Alford,2018).. Even the tools that will be used are still little known, they should be collectible vehicles powered by a boat with an electric cable that move forward and backward.This would sdraw and separate the nodules collecting useful material and expelling unwanted sediments in the sea. In these deposits lives a wide range of bacterias or other organisms at least 50 micrometers, which will probably die during the exploration phases or which will be smother, for example, by the sedimentary cloud that are formed after extraction(Peacock & Alford,2018). The nodules take millions of years to form again and their biological communities will develop slowly. This leaves us to think that communities risk not recovering after long periods of time. Evaluating the environmental and ecological impact of these activities is difficult. It is necessary to establish the extent of these effects and their impact on biological systems and communities. It is important to evaluate the positive and negative aspects of this DSM activity. It is essential to reduce the impact of extractions and this also depends on the standards and readiness with which these will be adopted by ISA.

The acoustic impact

One of the possible impacts on biodiversity could be the acoustic impact. The human activities that contribute to increasing the level of noise in the ocean depths are different, such as transport, seismic, sonar, navigation, recreation, mining, transport, offshore development, urbanization, mining of resources, transport and energy production (Bart et al., 2001; Engas et al., 1996; Myrberg, 1980; Popper et al., 2005; Sandström et al., 2005; Schwarz & Greer, 1984; Smith et al. , 2004; Richardson et al., 1995; Radford et al., 2014 Slabbekoorn et al., 2010; Kunc et al., 2016; Hawkins and Popper, 2017). Now, underwater noise is classified as pollution (Marine Strategy Framework Directive of EU Directive 2008/56 / EC of 17 June 2008) and is a real global pollutant for the World Health Organization (Kunc et al., 2016) . However, anthropogenic noise is one of the least studied sources of pollution (Hawkins et al., 2015). In water, the sound attenuates less than in air (Wartzok et al., 1999) and spreads almost 4.5 times faster than in air (Urick, 1983). The sound travels farther and faster than in the air (Williams et al., 2015). The frequency of anthropological sound in water overlaps with the biologically important sounds produced by animals for their vital functions (Hastings & Popper, 2005; Slabbekoorn et al., 2010). For this reason, human sound becomes a real threat to the life of deep ecosystems. Most of the studies are related to mammals and few to fish. In particular, juvenile organisms and invertebrates seem to be less studied (Maragos et al., 1993). Only a few studies have focused on the effects on juvenile fish behavior (Spiga et al., 2017; Buscaino et al., 2010; Neo et al., 2015), in terms of mortality or growth rates (Debusschere et al., 2014; Filiciotto et al., 2013) or physiological level (Santulli et al., 1999; Filiciotto et al., 2017; Debusschere et al., 2016). Noise pollution can cause different behavioral and physiological effects (reviewed by Weilgart, 2017; Carroll et al., 2017; Sarà et al., 2007), such as high levels of stress hormones (Wysocki et al.,2006) , increased heart rate, changes in oxygen consumption or metabolic cardiac output, parasites, irritation, distress and mortality (sometimes due to illness and cannibalism). Other effects may include a worse state of the body, less growth, change in weight, less food consumption and immune response or reproductive speed, reduction of DNA integrity with irreversible damage (Kight & Swaddle, 2011). A behavioral level can influence: the provision of food (Magnhagen et al., 2017; Wale et al., 2013; Voellmy et al., 2014), parental care (Bruintjes, et al., 2013; Nedelec et al., 2017), predatory avoidance (Simpson et al. al., 2016; Morris-Drake et al., 2016; Simpson et al., 2015), reproduction (Kight et al., 2011; Morley et al., 2014; Billard et al., 1981; Bonga, 1997), acoustic communication (Shannon et al., 2016; Kunc et al., 2016; Myrberg and Lugli, 2006), or perform the correct selection of habitat (Holles et al., 2013; Simpson et al., 2016), hearing loss (Halvorsen et al., 2012; Popper et al., 2005; Sverdrup et al., 1994; McCauley et al., 2003; Codarin et al., 2009; Popper et Fay, 2011) or masking that compromises the ability to communicate (Slabbekoorn et al., 2008; Lampe et al., 2012; Pollack, 1975; Brungart, 2001; McDonald et al., 2006; Normandeau Inc., 2012) and changes in school behavior (Busc aino et al., 2010). Also for invertebrates there are physiological and molecular responses to acoustic stress. Despite everything, the studies are few and reviewed by Roberts & Elliot, (2017). Noise levels created by the DSM are not known, but in this work we have tested by subjecting the animals to four different probable frequencies (63 Hz, 125 Hz, 500 Hz and 1 kHz), the most likely produced and likely to have a significant impact. Our interest was for the juvenile individuals of *Sparus aurata* and invertebrates such as *Mytilus galloprovincialis*, commercially important species for which very little is still known. The short-term effects (30' min and 1 hour) and long-term effects (24 hours) were analyzed to estimate the time during which the animals are accustomed to stress. For *S.aurata* physiological, physical and behavioral effects are observed, while for *M.galloprovincialis* physiological and molecular effects are observed. This study highlights the importance of the knowledge of the effects of continuous and short-term noise both in juvenile individuals (for whom studies are less) and in invertebrates. Invertebrates, despite their expectations, are essential because they perform some ecological services, such as water filtration, and play an important role in the biology of deep environments. The goal of this work is to contribute to the knowledge of the effects of this new type of human activity by comparing the effects on invertebrates and vertebrates. If the different parts work together, the underwater mining could become a global reference, and fortunately we have for the first time the opportunity to speak and write the rules / laws before activities start. We have the opportunity to act before being too late and we must do it.

MATERIALS AND METHODS

Experimental animals

The experiments were carried out at the Polytechnic University of Valencia (UPV), in particular at the Polytechnic University of Gandia (Spain). For the experimental plan, we used 216 juvenile of *Sparus aurata* (from Sagunto, in Spain) with a weight of 14.08gr ± 0.85 and a length of 10.46 cm ± 0.16. For the acclimatization period (one week) and the maintenance period, the fish were placed in a tank of 2m*2m, with a depth of 75 cm, a volume of 4000 to 12 ± 2 °C. All with recirculated and low-density filtered sea water, 5 Kg/m^3. We have maintained water quality parameters in an appropriate range for young fish. The fish were kept with the natural photoperiod and fed with commercial dry pellets (0.5% of body weight), but they were not fed 24 hours before the experiment. We also used 216 individuals of *Myitilus galloprovincialis* taken from the locality of Galicia (Spain) weighing 20.57g ± 1.36 and a length of 6.44 cm ± 0.21. The mussels were transported in bags soaked in sea water and in thermal containers. This is to keep the humidity and temperature stable. Its maintenance in the laboratory was carried out in tanks with a ventilation system of about 8 mgl^{-1} to 28 ‰ of salinity at 12 ± 2 °C. The animals were cleaned from the epiphytes and distributed in six tanks, 70 animals were acclimated for one week and fed regularly with a monoalgal culture of *Isochrysis galbana*. The experiments were conducted under the supervision and approval of the IACUC (Institutional Committee for the Care and Use of Animals) of the UPV.

Acoustic stress
We chose disturbance frequencies following criteria: continuous low frequency sound level within the 1/3 octave 63 and 125 Hz bands according to the indications of the European Comudidad, the bands (Dekeling et al., 2014) 1/3 octave 500Hz since most fish have a range that falls within this frequency range (Popper et al., 2003) and 1/3 octave 1 kHz to cover all possible spectro emissions of these activities. The emission time was 30 minutes, 1 hour and 24 hours. The sound signals were generated by the red Pitaya emissions acquisition system and amplified with underwater speakers (Beyma-UA-UPV prototype). The transmitted signals were measured with two Reson TC4034 hydrophones in the tank to measure the emission levels at the beginning and end of the stress, about 70 cm deep and 1.5 m from the diffuser. The maximum sound pressure levels were 140 dBrms re 1 µPa.

Experimental plan
The animals were chosen randomly and divided between the control tank and the experimental tank. For each frequency, we made three replicas with 18 fish and 18 mussels. Three baskets with a height of 1.40 m and a diameter of 75 cm were placed inside the experimental tank. Six fish and six mussels were placed in each basket. The behavior of the animals was recorded with an underwater camera (Gopro HERO4) (1m away from the basket and 35cm deep) and with an external camera (Axis 1346 camera) placed 1m from the top. The arrangement of the cameras was chosen so that the entire cage was visible. The animals of the control group were placed in the same conditions but without acoustic stress, those of the test were subjected to the four experimental frequencies. The animals were sampled after 30 minutes, 1 hour and 24 hours from the beginning of the experimental plan. The video recordings allowed to be used to study the cohesion, the swimming speed and the change in the swimming height of the fish.

Sampling
The fish were captured at different time with a network to measure the body and collect blood and tissues. They were anesthetized with 20 mg/l of MS-222 (trianine methanesulphonate) (organic Acros). Blood samples were taken from each individual in disposable heparinized syringes through the caudal vein of each fish. After blood extraction, the fish were weighed and then measured in terms of the length of the fork to the nearest millimeter. For each sample, blood smears were made on the slide and centrifuged at 1000 g, 10 min and 4 °C. The obtained plasma was stored at

-20 °C. Blood was always collected between 10 am. and 12 am, with the power interrupted 24 h before. Cell pellets for experiment, instead, three were kept in dry conditions at -20 °C and three in RNAlater (Sigma Aldrich) at -20 °C. From each animal were taken: liver, spleen, skin, muscle and gills divided into dry and RNAlater at -20 °C for any molecular or proteic analysis. For some fish (two at a time) we have divided the fish and frozen the head and the body at -20 °C. For the other fish we put their organs in Bouin (Sigma Aldrich).
In individuals of *Mytilus galloprovincialis* hemolymph were taken from adductor muscle in 200 µl (about 800 µl of hemolymph) of anticoagulant solution (0.45 M NaCl, 30 mM sodium citrate, 26 mm citric acid and 10 mM EDTA) using a 1 ml syringe. Hemolymph was collected in a sterile Eppendorf tube. With Neubauer the total number of hemocytes per ml (THC) is counted and then the samples were centrifuged at 1000 g for 10 minutes at 4 °C. The plasmas were stored at -20 °C and the three dry pellets and three at RNAlater. For each individual, gills, mantle, digestive gland and foot were sampled and divided into RNAlater and dried and stored at both -20 °C. For two individuals of all time, the organs were kept in BOUIN and stored at room temperature .

PRELIMINAR RESULTS AND CONCLUSIONS.

The preliminar analysis of video recordings show behavioural reaction of fish when under different noise. This reactions can be summerized as:
-Change of swimming depth: fish went deeper
-Bunching: In absence of noise fish swim ocupping the wholw space of the container tank. Nevertheless, when they were affected by noise, they join in a group, as far as possible from the acoustic source.
-Habituation: after 20-30 minutes from the beginning of the acoustic emission fish seems to be habituated and they recovered the previous to the acoustic emission swimming pattern.
Figure 1

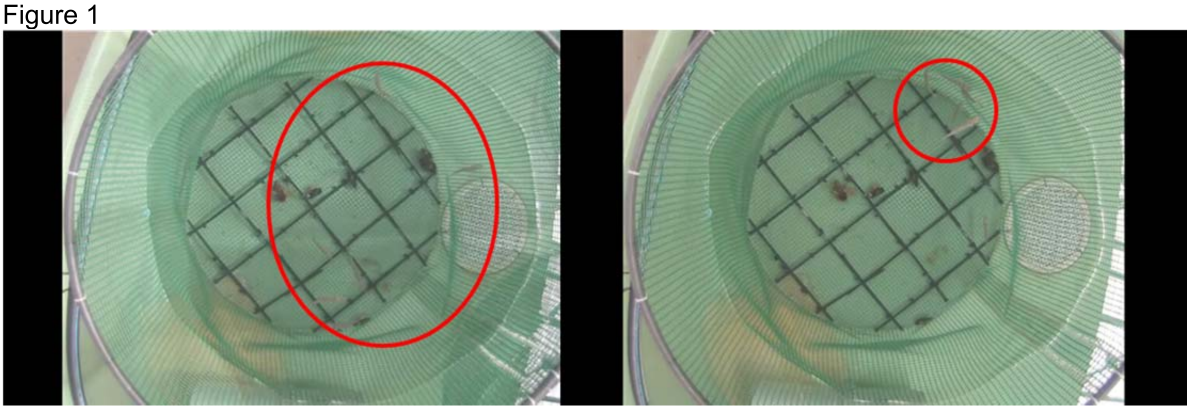

Figure 1. Red area shows the concentration area of fish before (left) and after (rigth) acoustic emission at 125 Hz.

The behavioral, biochemical and molecular variations of the two species under study will be analyzed.

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