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Article

Biological effects of non-ionizing electromagnetic fields at 27 GHz on sperm quality of *Mytilus galloprovincialis*

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Abstract: Recently, a rising use of wireless internet technologies has been demonstrated. The devices which use these technologies emit in new spectral regions an electromagnetic radiation (EMFs) which could interact with the male reproductive system. The aim of this study was to investigate *in vitro* influence of electromagnetic fields at 27 GHz on sperm quality in *Mytilus galloprovincialis*. Sperm samples, were collected from sexually mature males of *M. galloprovincialis* and placed in seawater. Once evaluated the number and quality of spermatozoa, sperm cells were exposed to electromagnetic fields radiated by a pyramidal horn antenna. The effect of exposure was evaluated after 10, 20, 30, 40 and 60 minutes with light microscope and using Eosin test. Ten replications were performed for each time series, and statistical analysis was carried out byt-test. A significative decrease in sperm motility was observed after 10 minutes of exposure and after 30 minutes most of sperms were immobile and not vital. This study provides useful data on potential ecological impact of the 5G high-band on animal fertility, whose effect is currently under investigation.

Keywords: Mussel; vitality; motility; millimeter waves; SAR

1. Introduction

With the roll-out of 5G mobile networks, significantly higher mobile broadband speeds and increasingly wider use of mobile data will be ensured. This is made possible also by the use of additional higher frequency bands [1]. 5G aims to greatly enhance the potentiality of communications, from virtual reality to autonomous vehicles to the industrial internet and smart cities. Furthermore, 5G is considered to be the core technology for the Internet of Things (IoT), where machines communicate with machines [1]. 5G networks will work in different frequency bands and are divided into two different groups [2]. The first group, called Frequency Range 1 (FR1), includes the frequency bands below 6 GHz, some of which have already been used by previous standards but extended to cover new portions of the spectrum between 410 MHz and 7125 MHz. The second group, called Frequency Range 2 (FR2),includes the frequency range between 24.25 GHz and 52.6 GHz (millimeter waves or mmWave) and has a lower range but allows a wider available bandwidth than the bands of the FR1 group. At the same time, a change in exposure to electromagnetic fields (EMF) of humans and the environment is expected. The

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introduction of this new technology that operates in different frequency bands has attracted a significant amount of toxicity studies [3-5]. To date, however, only a few studies have been conducted on the high frequencies that will be used by 5G [2] and the data are not sufficient to conclude on the existence or not of health effects related to exposure to electromagnetic fields in the band of frequencies around 26 GHz [6]. Tissue heating is the main mechanism of interaction between radiofrequency fields and the human body. The levels of radiofrequency exposure of current technologies cause a negligible increase in temperature in the human body. As the frequency increases, there is less penetration into the body's tissues and the absorption of energy becomes more limited to the surface of the body (skin and eyes). As long as the overall exposure remains below levels fixed by international guidelines, there are no consequences for public health [7]. WHO is conducting a health risk assessment from radio frequency exposure, covering the full range of radio frequencies, including 5G, reviewing literature data on potential health risks from exposure to 5G. Unfortunately, little is known about the effect this new technology could have on coastal marine species. Due to increasing pressure on the environment by humans, biodiversity loss has become one of the greatest environmental concerns. Habitat destruction and overexploitation represent the greatest stressors to marine biodiversity, but excessive anthropization, including the installation of antennas or of repeaters, can also be a threat especially for reproduction of many species [8]. Consequently, artificial electromagnetic fields could impact on the ecological processes in sensitive species, such as spawning or feeding migrations, homing, predation and detection of sexual mates [9-11]. In particular, aquatic invertebrates seems to be sensitive to external factors, and their gametes may be involved at different levels of biological organization [12]. It has been observed an alteration on release of gametes (both spermatozoa and eggs) in seawater, crucial aspect which decreases the reproductive success for the survival of species. Even if some parameters identified as targets of environmental stress are useful biomarkers for the evaluation of exposure to conventional pollutants (pesticides, heavy metals and chemicals substances) [13-18], the effects of electromagnetic fields have not yet been studied. Some evidences suggest that the global environment conditions are changing rapidly and contaminated conditions could interfere with reproductive mechanisms. In this view, some literature studies have shown biological effect on reproductivity at different frequencies band below 6 GHz [2, 5], in this paper we have investigated the effect of electromagnetic fields at 27 GHz on sperm quality of bivalve mollusk *Mytilus galloprovincialis* by *in vitro* assays. The experiments were conducted with a commercial pyramidal horn antenna with an incident density power not exceeding the international limits stated by the ICNIRP for frequency above 6 GHz; spermatozoa were exposed to electromagnetic fields up to 1 hour and during the exposure we have evaluated motility and vitality, important parameters that reflect the health status of the spermatozoa and consequently the reproductive success of species.

2. Materials and Methods

2.1 *Study design*

Focus of this study was to investigate *in vitro* influence of electromagnetic fields at 27 GHz on sperm quality in *Mytilus galloprovincialis*. First of all, the number and morphology of spermatozoa were evaluated to ensure the good quality of the samples to use; then sperm cells were exposed to electromagnetic fields radiated by a pyramidal horn antenna. The effect of exposure was evaluated after 10, 20, 30, 40 and 60 minutes by optical microscope. Vitality was assessed by Eosin test. Ten replications were performed and statistical analysis was carried out by t-test.

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2.2 27GHz antenna and exposure setup

The experiments at 27 GHz were conducted by using a commercial pyramidal horn antenna by XiBao Electronic Tech (model XB-HA28-20) at 27 GHz. The dimension of the antenna aperture are 4.4 x 3.51 cm and the maximum gain reported in antenna datasheet is 19.23 dB at 26.50 GHz. The antenna is fed by a RF signal generation (R&S SMB100A) with an output power of +23dBm that reduces to +20dBm due to insertion loss of the coaxial cable linking RF generator and antenna. The distance between the antenna aperture plane and the 6-well microplates has been fixed to 15 cm (Fig. 1) as trade-off between maximum available output power of the RF signal generator and the need to ensure an incident power density comparable with the ICNIRP international guidelines restrictions, that is comparable or, anyway, not exceeding 10 mW/cm^2 [7].

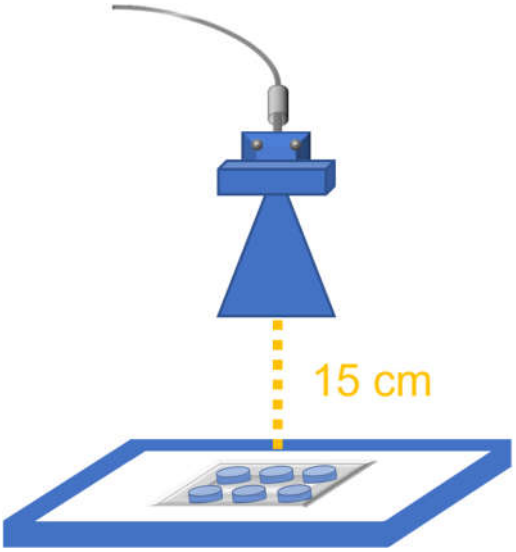

Figure 1. Experimental exposure setup.

232 Experimental Exposure Procedure

Mussels (*Mytilus galloprovincialis*) were purchased from a mussel farm in Sicily, placed in plastic bags with sea water and immediately transported to the Catania University's Laboratories. We discarded the individuals with obvious signs of breakage of the shell from the sample. Once verified their maturity, the mussels were cleaned from debris and epibionts by manual scraping of the shell, rinsed quickly with water and stabilized for 1 hour before experiments. A number of 20 individuals were selected to induce spawning eggs and sperm by applying a protocol of thermal stimulation (heat shock). Mussels were placed at temperature of 4°C for 3-4 hours and then transferred into a tank containing water heated at 28°C. The water used for the stimulation had a salinity of 30 ± 1‰ and pH 8.3 and it had been filtered with a 0.22 μm filter. After thirty minutes most of the specimens had opened the valve and resumed filtration and they were transferred to a second tank containing sea water at a temperature of 18°C. Once initiated the spawning, the specimens were immediately removed and placed into an aquarium. Spermatozoa were placed in 6-well microplates in 5ml seawater/well. Control samples (negative control) were incubated only with seawater. The effect of exposure was evaluated up to 1h (with observations intervals at 10, 20, 30, 40, 60 minutes). A controlled room temperature has allowed to maintain 22 ± 1°C in wells. Ten replications for each time series (10, 20, 30, 40 and 60) were performed.

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2.4 Motility analysis

We measured the motility dividing spermatozoa into two categories: motility and no motility. For each replicate of the time series exposure, we measured the motility by placing 10 µl of sperms sample on a slide and observing under an optical microscope at x400 magnification. We counted 100 spermatozoa at least.

2.5 Vitality analysis

For each replicate of the time series exposure, we measured the vitality of sperm samples. The procedure involves the positioning of 10 µl of sperms sample on a slide in which we added 10 µl of Eosin Y (0.5%, Bio-Optica). The observations were made under an optical microscope (Leica DMLB) at x400 magnification. At least 100 spermatozoa were counted. Dead spermatozoa appeared in pink due to the loss of membrane integrity, compared to live spermatozoa that maintain their original coloring.

2.6 Statistical analyses

The data obtained were processed for statistical analysis. The vitality and motility rates between spermatozoa exposed to the electromagnetic fields and the control, were compared by the t-test. Paired (Tables S1 and S2) and Unpaired (Tables S3 and S4) using GraphPad Prism software (version 9.3.1) (Table S5). Bar chart graphs of motility and vitality rate were realized using GraphPad Prism software (version 9.3.1).

3. Results

3.1 Numerical dosimetry analyses

To establish electromagnetic exposure conditions, numerical simulation has been performed by means of the commercial software CST Microwave Studio. In particular, in the CAD model we have considered the 6-well microplates, the horn antenna and a metallic plane behind the sample holder to take into account agitator for the oxygenation of samples placed at 1 cm below the 6-well microplates (Fig. 2A). In the experimental setup a polystyrene foam slab has been inserted between the metallic plane and the sample holder to prevent reflecting plane to be very close to aqueous samples, however it has not been considered in the simulation as dielectric parameters of foam can be neglected. The dielectric parameters of the materials adopted for the dosimetric analysis at working frequency of 27 GHz are reported in Table 1.

Table 1. Dielectric parameters adopted for the numerical dosimetry.

Component	Material	Dielectric Constant	Loss Tangent	Mass density
Horn antenna	*Perfect electric conductor (good metal)*	-	∞	-
Aqueous sample	*Salt water (30‰ salinity)*	*23.64*	*1.27*	*1029 [Kg/m³]*
6-weel microplates	*Polystyrene*	*2.5*	*0*	-
Ground plane	*Perfect electric conductor (good metal)*	-	∞	-

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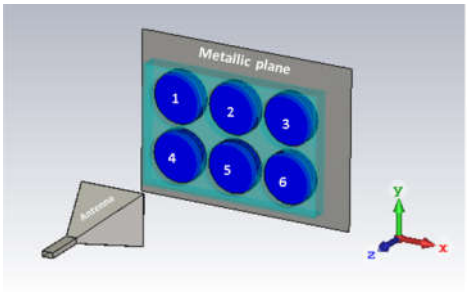
In order to evaluate exposure condition, three complementary metrics have been considered [7]:

- density power of the incident field [W/m^2];
- the local specific absorption rate (point SAR) [W/Kg];
- power loss density (PLD) deposited into the exposed aqueous samples [W/m^3].

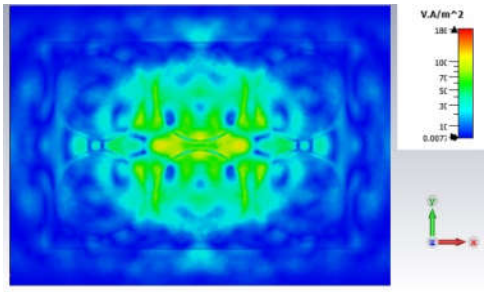
The resulting incident power density is reported in Figure 2B, as it can be seen, although the power density is not uniform above the 6-well microplates, since far field condition are not satisfied [7], it reaches values ranging from 30 to 100 W/m^2 over the antenna footprint area. Taking into account that the metallic plate of the agitator gives rise to a total reflection, these values are comparable with 10 mW/cm^2 set by the international guidelines as exposure limit above 6 GHz, see Table 2 in [7].

As far as SAR calculation is concerned, it is worth to underline that specific absorption rate (SAR) averaged over a 10g cubic volume, considered by international guidelines for frequencies below 6 GHz, cannot be considered here, as the total mass of the samples (5g) is smaller than averaging mass considered by ICNIRP guidelines [6]. For this reason, we report the local SAR as numerically evaluated in each cell of the grid used to discretize the samples in the wells (point SAR). The computed SAR level averaged in each layer of the aqueous samples is shown in Figure 2C. The SAR distribution is non homogeneous in the different aqueous samples as larger values are reached in the central wells (2 and 5). This is due to near field condition exposure that entails a not local plane wave front of the field impinging on the samples. Moreover, as expected, top layers of the aqueous samples show larger SAR values than in depth layers, due to strong electromagnetic discontinuities and small penetration depth in salt water at 27 GHz, (0.65 mm). Finally, the PLD also shows that power deposition into the central samples are about three times larger than that in the peripheral samples. However, as spermatozoa cells move randomly inside the sample, they will experience an average PLD values of about 22 mW/cm^3 in the outermost samples (well number 1, 3, 4, 6) and about 52 mW/cm^3 in the innermost ones (well number 2 and 5), see Figure 2D. It is worth to stress that, although non homogeneous exposure are not suggested as good condition for dosimetric evaluation, in this case, as sperm vitality is observed on spermatozoa samples taken separately from each well, this may allow to understand is possible effects are associated to different power level conditions.

Last but not least, it is worth noticing that only numerical dosimetry has been performed because continuous thermal monitoring during exposure protocol is not viable due to invasiveness of most common temperature probes (thermocouple or optical fiber) at this working frequency. This notwithstanding, water temperature measurements of the aqueous sample reveal an increase of about 1°C after the exposure time with respect to the sham samples.



A



B

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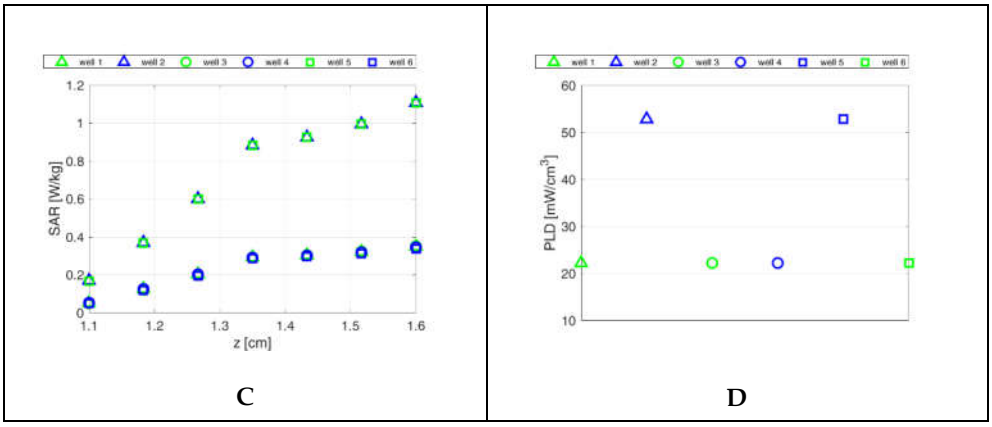

Figure 2. Experimental setup for numerical dosimetry. A) CAD model (6-well microplates numbered for understanding following figures). B) incident power density at distance 0.5 cm from the air-water interface of the aqueous samples. C) Average point SAR in each sample layer along depth z-direction. D) average PLD for each aqueous sample in the wells.

3.2 Sperms vitality and motility analyses

A significative decrement of sperm's vitality (Fig. 3) was observed already after 10 minutes of exposure at 27 GHz , compared to control samples. Eosin test showed a high mortality rate of spermatozoa in all exposed samples already after 30 minutes (Fig. 4). We observed that electromagnetic fields irradiation induced also a significant decrease in sperm motility (Fig. 5) after 10 minutes of exposure .

Statistically significant differences ($p < 0.0001$ and $p < 0.0004$ only for Paried t-test motility) have been obtained for all times of exposure between spermatozoa exposed and controls both for vitality and motility rate (Figs 6 and 7).

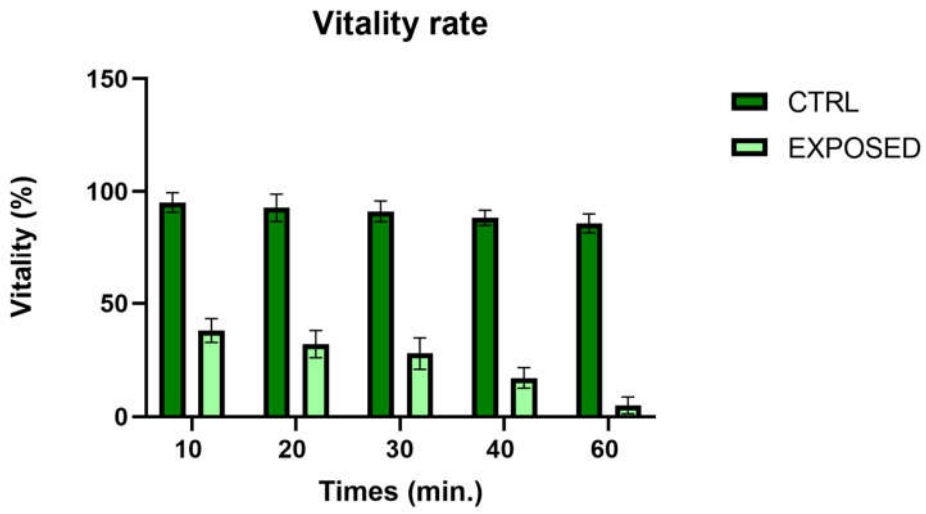

Figure 3. Vitality percentage at different times of exposure. The data represent the mean of observation performed in tenfold by same observer to avoid subjective differences in vitality evaluation.

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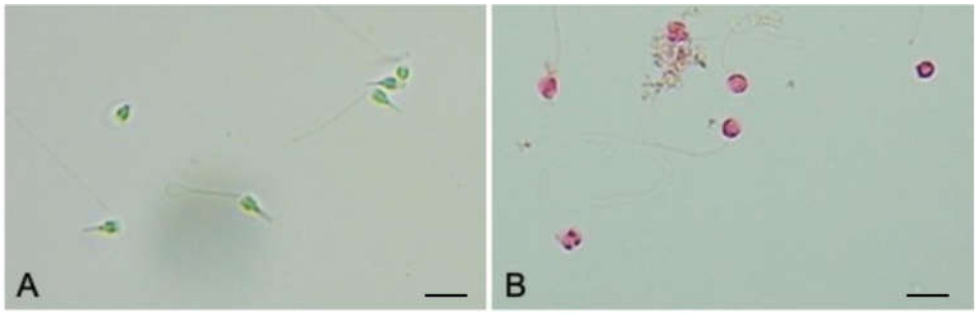

Figure 4. Vitality evaluation with Eosin Test on *Mytilus galloprovincialis* sperms. A) Untreated sample. B) After exposure to 27 GHz for 30 minutes. Scale bar: 4 μm.

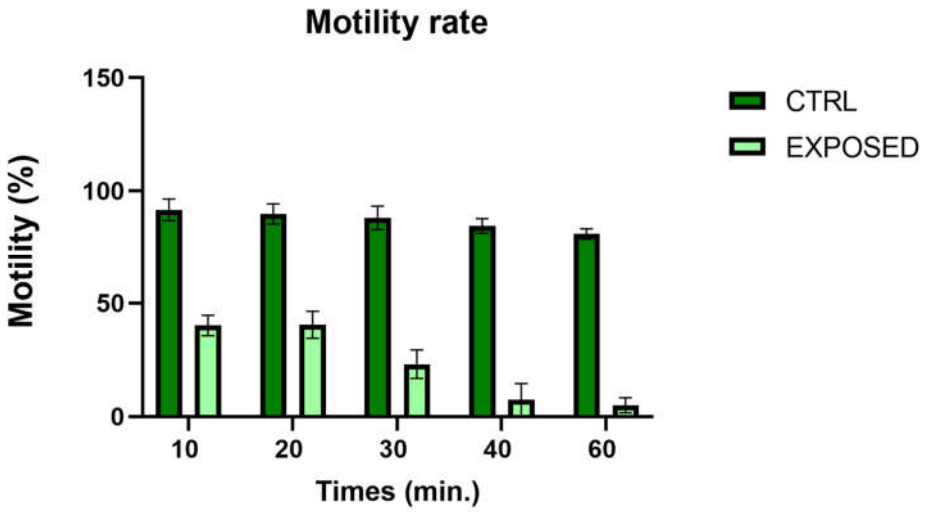

Figure 5. Motility percentage at different times of exposure. The data represent the mean of observation performed in tenfold by same observer to avoid subjective differences in motility evaluation.

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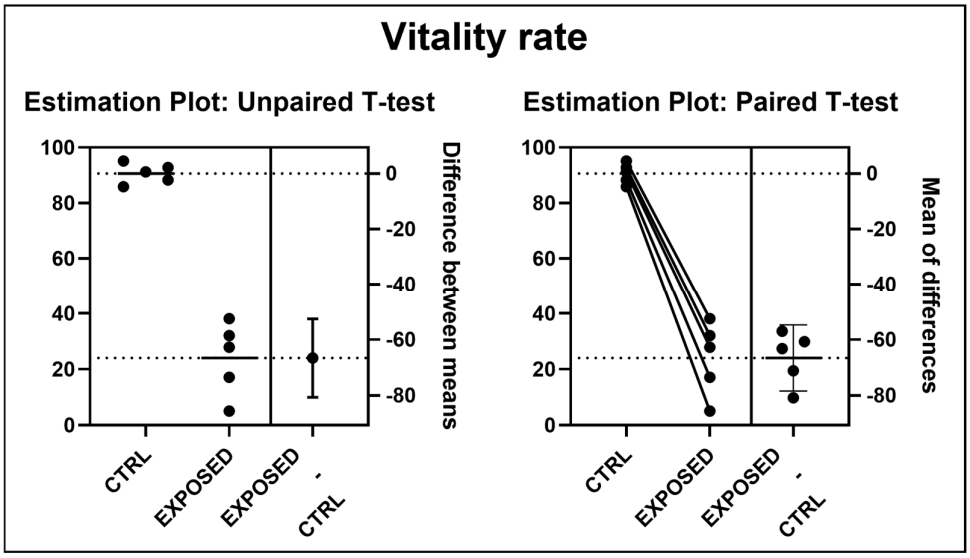

Figure 6. Line Plot comparing mean vitality rate of *M. galloprovincialis* sperm samples exposed to 27GHz than control.

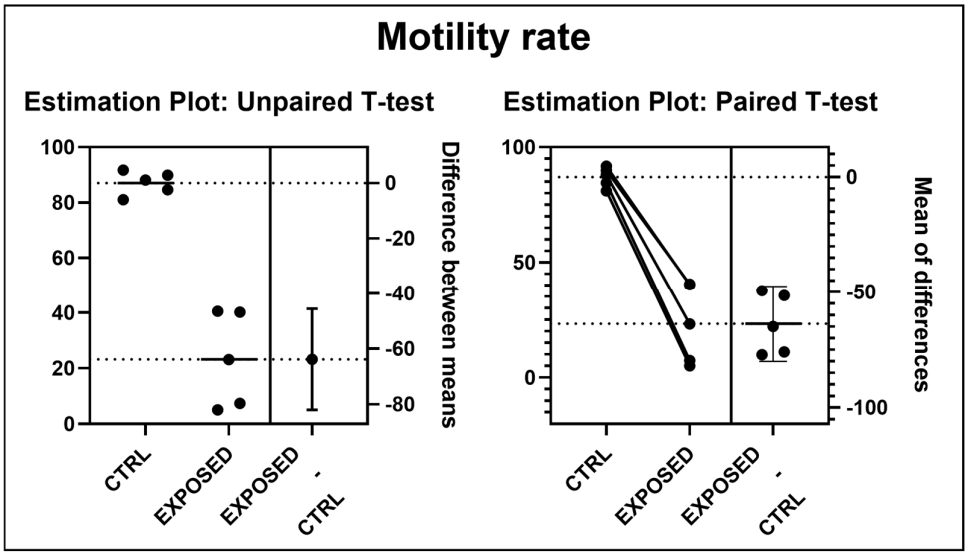

Figure 7. Line Plot comparing mean motility rate of *M. galloprovincialis* sperm samples exposed to 27GHz than control.

4. Discussion

Fifth generation of global mobile communications will bring also a new era to maritime connectivity for real-time data transmissions. The development of mobile communication technologies has requested the assessment of possible risks occurring from exposures to radiofrequency electromagnetic fields. Whereas much is present in the literature on the effect on reproduction of electromagnetic fields in terrestrial animals model [19-24], little is known about the effect in aquatic animals, except for a few data concerning zebrafish

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[16, 19]. Moreover, most of these effects were investigated at frequencies typical of previous cellular communications, mainly 2G and 3G, *i.e.* frequencies much lower than 27 GHz.

Recent evidences demonstrate that electromagnetic fields negatively affect sperm quality, sperm count, morphology and motility. In male rats 2 days old, exposed to EMF 1800 and 900 MHz for 2 hours continuously per day for 90 days. The percentage of epididymal sperm motility was significantly higher in the 1800 MHz group ($p < 0.05$). The morphologically normal spermatozoa rates were higher and the tail abnormality and total percentage abnormalities were lower in the 900 MHz group ($p < 0.05$) [9]. In another study conducted always on rats exposed to 900 MHz for 8 weeks, the Authors noticed a statistically significant decrease of epididymal sperm counts in the exposed group ($p < 0.001$), a significant decrease of sperm motility and a significant ($p < 0.001$) increase in the frequency percentage of dead spermatozoa [18]. Guo et al. [25], exposing rats for 1 month to 220 MHz demonstrated a decreased sperm count and survival rate of sperm ($p < 0.05$), increased sperm abnormalities. No differences in body weight and development among the groups were found in mice of both sexes in rats exposed to 2.45 GHz for 2 h/day, 5 days/week for 5 consecutive weeks, starting the day after birth [24]. On zebrafish exposed to 3.5 GHz RFR, specific absorption rate (SAR) ≈ 8.27 W/Kg from 6 hours post fertilization (hpf) to 48 hpf, have not been revealed significant impacts on mortality, morphology or photomotor response, but only a modest inhibition of startle response suggesting some levels of sensorimotor disruptions [25].

Several studies were conducted on evaluation of exposure by submarine power cables which produce both electric and magnetic fields. Although magnetic and electric fields' intensities decrease with distance away from the cable, marine invertebrate species are the major fauna exposed to, so they have received a greater attention [26]. A high-strength magnetic field applied during sea urchins (*Lytechinus pictus* and *Strongylocentrotus purpuratus*) fertilization delayed cell division in embryos [27, 28]. Moreover, Levin and Ernst [27] highlighted an increase in developmental abnormalities, but only in *L. pictus*. However, a 93-day exposure throughout the reproductive period of the blue mussel (*Mytilus edulis*) did not affect either its condition index or its gonad development index [29].

In our study, a notable decrease in the vitality of *M. galloprovincialis* spermatozoa after only 10 minutes of exposure at 27 GHz was shown. We observed also that electromagnetic fields irradiation induced a significant decrease in sperm motility after 10 minutes of exposure. If confirmed, possible explanation of our observation is related to a direct action of the electromagnetic field on phospholipid bilayer of cells membrane. This effect had been investigated, but for at higher frequencies (around 60 GHz), on liposomes and on giant unilamellar vesicles [30-32], where the Authors have hypothesized a change of the polarization states of water induced by the radiation, which causes a partial dehydration of the membrane and consequently a greater packing density of phospholipids.

5. Conclusions

The study indicates that electromagnetic fields at 27 GHz can affect the sperm quality in marine mussel *Mytilus galloprovincialis.* The significative decrease observed in sperm motility after only 10 minutes of exposure represents a crucial factor need to be considered because it can threat reproductivity of the species. This study provides useful data on potential impact of high frequency EMFs on aquatic animals and cells, that currently are poorly investigated. Future research could benefit from specific investigation on the impact of 5G to better monitoring effects on animal organisms and to fill the gap currently known about the interactions with artificial sources of electromagnetic fields.

Author Contributions: Conceptualization, L.D.D. and M.V.B.; methodology, R.P., E.M.S, M.C., S.C.P. and G.S.; investigation, C.S. and S.I.; data curation, S.I., C.S., S.C.P. and G.S.;

writing-original draft preparation, L.D.D. and M.V.B.; writing-review and editing, R.P., E.M.S., A.S. and M.V.B.; supervision, L.D.D., R.P. and M.V.B.; funding acquisition, L.D.D. and M.V.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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