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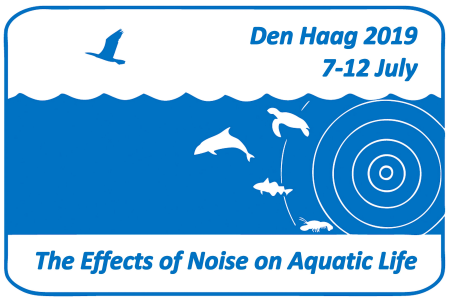

Effects of low-frequency noise and temperature on copepod and amphipod performance

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Offshore wind farms (OWF) are bound to increase as a mitigation strategy to reduce the emission of greenhouse gases, it is crucial to address all of their potential impacts on key ecosystem components in detail. Especially, the chronic effect of noise created during OWF turbine operations (duration 20-25 years) must be understood. As sensitive receptors cover the whole body of crustaceans to detect their surroundings, those low frequency noises may disrupt basic ecological (prey detection and predator avoidance) and physiological (metabolism) functions. Here we present an investigation designed to understand the joint effect of noise and increased temperature on copepod. The pelagic copepod *Acartia tonsa* is commonly used as a proxy for a range of fundamental processes that relate to marine planktonic crustaceans. Given that higher temperatures increase metabolic demands, the experiment was conducted at three different temperature levels (18, 21, 24°C) combined with silent and noise treatments. We assessed the combined effects on energetic balance, and oxidative stress indicators. The outputs of the project will provide important information on the potential impact of low-frequency noise on marine invertebrate key organisms with implications for secondary production and ecosystem functioning.

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1. INTRODUCTION

Climate change affects the resources and services provided by marine ecosystems, and the way we need to manage them sustainably. In the last decade, research on the side-effects of climate change such as warming, acidification and nutrient limitations using marine key species has increased. Noise can also constitute a potential stressor, but remains understudied in this global assessment. For example, the operational constant soundscape from offshore wind farms, that are bound to increase as a mitigation strategy against global warming, are still unaccounted for [1].

Phytoplankton, micro- and mesozooplankton taxa represent good biological models to understand the cell stress mechanisms that allow plasticity and resilience in changing environments. These organisms are: 1) available all year; 2) the main focus of many long survey records allowing the match of current responses with past conditions, and allow better predictions for the future; 2) small in size, which facilitates multi-stressors experimental design and replication; and 3) part of a complex community, which allow a larger overview of the system vulnerability and resilience. Copepods dominate the mesozooplankton communities in terms of biomass and abundance. They usually pass through five nauplii and six copepodite stages of development before reaching maturity. Setae receptors on their antenna act as velocity and vibration detectors for prey recognition and predator avoidance [2, 3]. Mechanosensitivities is higher for species inhabiting quiet environment [4]. Sensing is so precise that it enables the organism to determine the position and quality of food particles, and to alter the near-field flow to capture and manipulate the particles for ingestion or rejection [5, 6]. It was also shown that temperature changes impact the mechanosensitivity of small zooplankton, more than the thermally-induced effects on metabolism [7]. These identified mechanisms are particularly relevant as anthropogenic noise propagation also differs with temperature, salinity and pressure [8].

Studies of anthropogenic noise on zooplankton taxa are still scarce, and contradict each other. A field experiment based on sonar observations and net sampling emphasized that air-gun survey decreases zooplankton abundance, and caused a two- to threefold increase in dead adult and larval zooplankton on a range of distance of more than 1 Km [9]. However, a controlled field air-gun exposure of the copepod *Calanus finmarchicus* followed by an assessment of its survival, escape behavior and gene expression only observed significant effects in the close vicinity (<10 m) of the exposure [10]. The establishment of a noise tolerance threshold is crucial in plankton as the results can be integrated in ecosystem models to extrapolate the effects into higher trophic levels (macrozooplankton, fish, marine mammals, etc.).

The joint effect of noise and temperature increase was assessed on the copepod *Acartia tonsa*, a species commonly used as a proxy for a range of fundamental processes that relate to marine planktonic crustaceans. We hypothesize that the effect of low-frequency noise, such as the one generated by offshore wind farms' turbines, could potentially altered their capacity in gathering the energy required to fulfil all their biological functions (*e.g.* development, growth, reproduction, and survival by mean of escaping behavior). The noise source used in our experiment was not copying a wind farm turbine, but it produces a tonal sound that overlap with copepods' natural environmental signals for prey and predator's. Oxygen consumption, prey ingestion and several oxidative stress markers were analyzed.

2. METHODS

A. EXPERIMENTAL SETUP

I. COPEPOD CULTURES

The experiment was performed with a single cohort of copepods. Adult calanoid copepods *Acartia tonsa* were fed *ad libitum* with the algae *Rhodomonas salina* (grown with F/2 medium) and maintained in the dark in 200 L cylindrical tanks at 18°C to produce eggs. Every day, eggs were siphoned from the bottom of the tanks and stored at 4°C in 0.2 µm filtered seawater (FSW) until use. Prior to the experiment, the hatching rate of the cohort was determined to estimate the number of nauplii hatched per volume of eggs after 48 hr. To minimize age differences between individuals, hatched nauplii were discarded after 24 hr to collect only the ones hatched between 24 and 36 hr.

II. NOISE AND WARMING TREATMENTS

The experimental setup was mounted in a soundproof room (floor and wall). Three refrigerating and heating circulators (1x HUBER Compatible Control K6, Germany; 2x CBC 5 Control, IKA, Germany) were used to control the temperature. Identical glass bottles of 5 L were placed at 18°C (n = 4), 21°C, (n = 4) and 24°C (n = 4) for a total of 12. Added low-frequency noise treatments (n = 2 at each temperature) consisted of a vibration motor source of approximately 110 Hz contained in a waterproof egg standardized for small experimental units, similar to the device used in recent publications [11, 12]. Waterproof eggs without motor were used as control (n = 2 at each temperature). All eggs were fixed to a stone at 10 cm from the bottom with a thread to counterbalance the buoyancy. The experimental sound pressure and frequency was recorded with a calibrated hydrophone (Aquarian Scientific AS-1 hydrophone with PA4 preamplifier, sensitivity of -208 dB re: 1 V/uPa, AFAB Enterprises USA) connected to an audio recorder (Zoom UAC-2, Japan; sampling rate 44·1 KHz, 16 bit). The hydrophone was placed in the bottom of the bottle at 8 cm from its center. To assess the power spectral density of each replicate, R package 'tuneR' [13] and 'psd' [14] were used. Noise power spectral density comparison between control and added low-frequency noise treatments for each temperature is shown (**Figure. 1**).

In each bottle, 5,000 nauplii were incubated in 0.2 µm FSW until the first copepodite stage was reached. Nauplii were fed *ad libitum* with the algae *R. salina* (10,000 cells·ind^{-1}), which concentration was evaluated using a CASY particle counter (Scharfe Systems, Germany). Bottles were maintained in the dark. As temperature accelerates copepod development, the occurrence of the first copepodite stage was observed after three, six and nine days at 24°C, 21°C, and 18°C respectively. When the first copepodite stage was reached, the replicates were processed for oxygen consumption, ingestion rate, and preserved for oxidative stress indicators analysis.

B. OXYGEN CONSUMPTION AND INGESTION RATES

Oxygen consumption rates were measured at all temperatures with an oxygen ingress measurement system connected to an oxygen meter (Microx TX, PreSens GmbH, Germany). Calibration of the sensor was performed with aerated FSW as the 100% O_2 reference and with a saturated Na_2SO_3 solution as the 0% O_2 reference. For each experimental unit, FSW was added to three 12-mL glass vials equipped with sensor spots. Each vial has approximately ten copepodites each. The use of three control vials (FSW without copepodites) at each temperature allowed the correction for microbial oxygen consumption or production during incubation. Vials were placed in a dark box on a rocking platform shaker (IKA Rocker 2D digital, Germany) at 80 revolutions per minute (rpm) to avoid oxygen stratification within the vials. Oxygen data recording was realized every hour during 6 hr, but the first 60 min were discarded as this period represents the chamber

acclimation time. Copepodites were fixed in lugol (2%) and were counted posteriorly. Oxygen consumption was expressed in µg $O_2 \cdot h^{-1} \cdot ind^{-1}$.

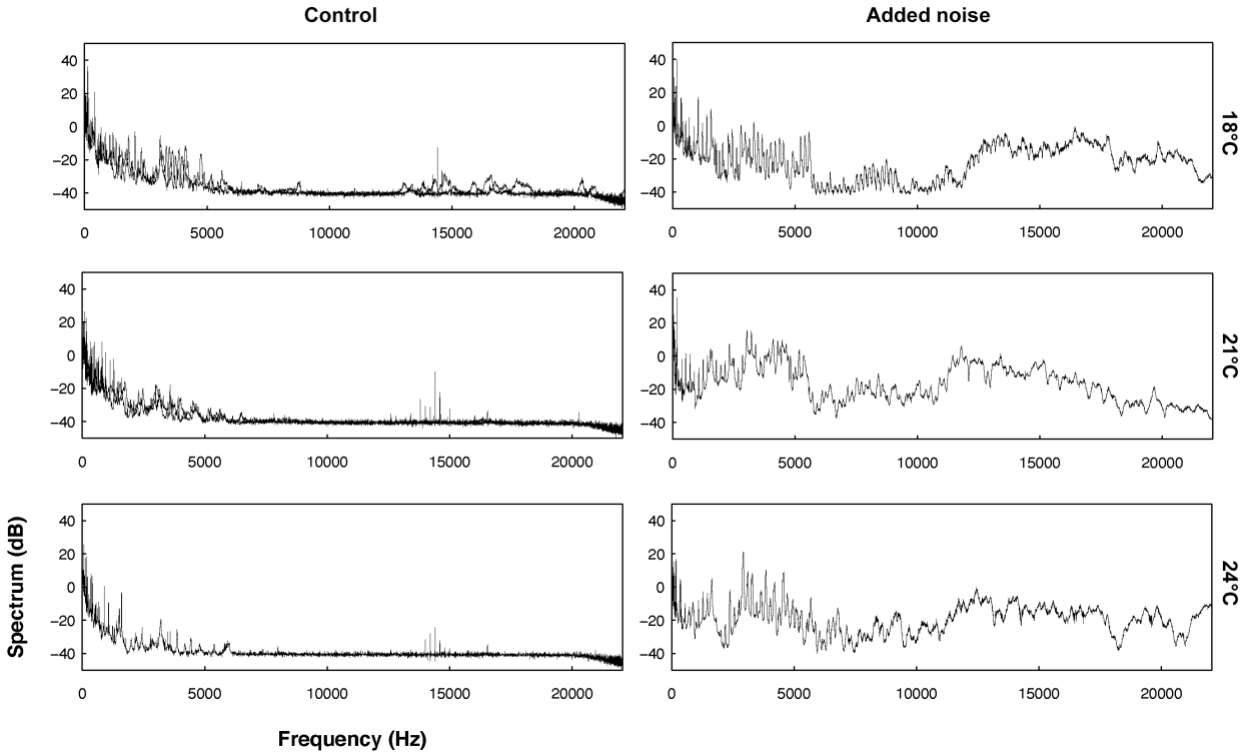

Figure 1. Noise power spectral density between control (left panels) and added low-frequency noise (right panels) treatments at 18, 21 and 24°C.

Algae cell concentration in the absence and presence of copepodites was used to estimate ingestion rates at 18°C and 24°C [15]. From each experimental replicate, three 15 mL tubes containing 15 copepodites each and filtered seawater (FSW) conditioned at the respective temperature was mixed with a volume of the algae *R. salina* corresponding to 20,000 $cells \cdot ind^{-1}$. For each temperature, the same volume of algae was used in three 15 ml tubes without copepodites to account for cell growth during the incubation. All tubes were attached onto a plankton wheel rotating in the dark with speed between 9 and 10 rpm for 24 hr. After this period, the copepods were retrieved using 60 µm mesh-sized sieve prior to fixation in lugol (2%) for counting. Cell concentrations at the beginning and end of the grazing incubation were measured with a flow cytometer (BD Accuri C6, Belgium). Ingestion rates were calculated as number of cells ingested per individual per day. Figures were realized using R.

C. OXIDATIVE STRESS INDICATORS

The remaining of the copepodites contained in each bottle at all temperatures was preserved for oxidative stress indicators biochemical analysis. Oxidative stress indicators give information about the mechanisms used by ectotherms to cope with respiratory imbalance (rapid shifts between over and under-oxygenation of tissues). As each cell of an organism produces a basal rate of approximately 0.1% of reactive oxygen species (ROS) from the oxygen it consumes under non-stressful aerobic conditions [16], the measurement of several antioxidant indicators of an organism under stress would indicate if the extra production of superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) has been neutralized. If antioxidant defense mechanisms do not counterbalance ROS, ROS react with transition metals (e.g. iron) that are not securely bound to a protein (by

Haber-Weiss or Fenton reactions) and form OH•, the most reactive and damaging ROS (proteins, lipids and DNA). In combination, cellular stress markers are used to assess how the physiological machinery responds to stressful conditions.

Copepodites from each experimental unit were sieved on a 60 µm mesh and were rapidly rinsed with distilled water to eliminate salts, transferred to two perforated 1.5 mL safe-lock tube using a micro scoop spoon, and snap-frozen in liquid nitrogen. The tubes were preserved at - 80 °C until biochemical analysis. One of the safe-lock tubes was used for enzymes and the other one was used for lipid peroxidation assessment. For the enzymes, samples were homogenized using a laboratory ball mill (MIXER MILL MM 400, Retsch, Haan, Germany) with 6-fold volume (w/v) of phosphate buffer solution (50 mmol L-1 potassium phosphate dibasic and monobasic mixture K_2HPO_4/KH_2PO_4, 50 mmol·L-1 EDTA, 1 mmol·L-1 phenylmethanesulfonyl fluoride, pH 7.5) and centrifuged at 23 897 g velocity for 3 min at 4 °C (HERMLE Z383K, Germany). Four antioxidant enzymes were analyzed in this supernatant in triplicate: the superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx). SOD converts superoxide radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and was measured using the xanthine-xanthine oxidase as a superoxide radical generating system and nitroblue tetrazolium as the detector [17]. CAT and GPx targets H_2O_2 and were measured at 240 and 340 ηm, respectively [18, 19]. GST transforms xenobiotics into other conjugates using reduced glutathione (GSH) as substrate and was estimated by detecting the formation of the thioether product from the reaction between GSH and 1-chloro, 2, 4-dinitrobenzene [20]. All antioxidant enzyme activities were measured at room temperature (20°C) using a micro-plate reader (THERMO Multiskan Spectrum, USA). Soluble protein content was measured in all supernatants using Bradford method [21] to obtain enzyme activities expressed in activity units (U) mg·proteins^{-1}. Quantification of malondialdehyde (MDA) formation (as lipid peroxidation proxy) was assessed in triplicate, and expressed as ηmol MDA g wet mass or WM^{-1} [22]. Figures were realized using R.

3. FIRST RESULTS AND DISCUSSION

Oxygen consumption rates of *Acartia tonsa* presented an increasing trend towards 24°C, without differentiation among noise and control treatments (**Figure. 2A**). In an opposite manner, ingestion rates at 24°C decreased significantly compared to 18°C (Kruskal-Wallis chi-squared = 5.3333, df = 1, p-value = 0.02092), independent of noise exposure (**Figure. 2B**). When temperature is the only stressor tested, previous experiments showed that zooplankton (*A. tonsa*, *Temora longicornis* and *Oxyrrhis marina*) preferentially ingest carbon-rich *Rhodomonas salina* to fulfil higher metabolic demands provoked by warmer temperatures [23, 24].

Oxidative stress indicators were affected by warming and noise. SOD, CAT and GPx activities were depleted under noise conditions at 21°C and 24°C (**Figure. 2C, D, F**). These three enzymes are linked as SOD converts $O_2^{\bullet-}$ to H_2O_2, which is neutralized by CAT and GPx. The depleted activity of these three enzymes could either mean that no $O_2^{\bullet-}$ and H_2O_2 were produced or that no elimination of these ROS occurred. The answer to this question will require further investigation analyzing the amount of $O_2^{\bullet-}$ and H_2O_2 produced in these treatments. Depleted antioxidant enzyme activities have also been observed in rodents exposed to noise [25, 26]. GST is a detoxifying antioxidant enzyme and was higher under noise at all temperatures compared to control (**Figure. 2E**). Its higher action may have help mitigating oxidative damages to lipids at 24°C (**Figure. 2G**).

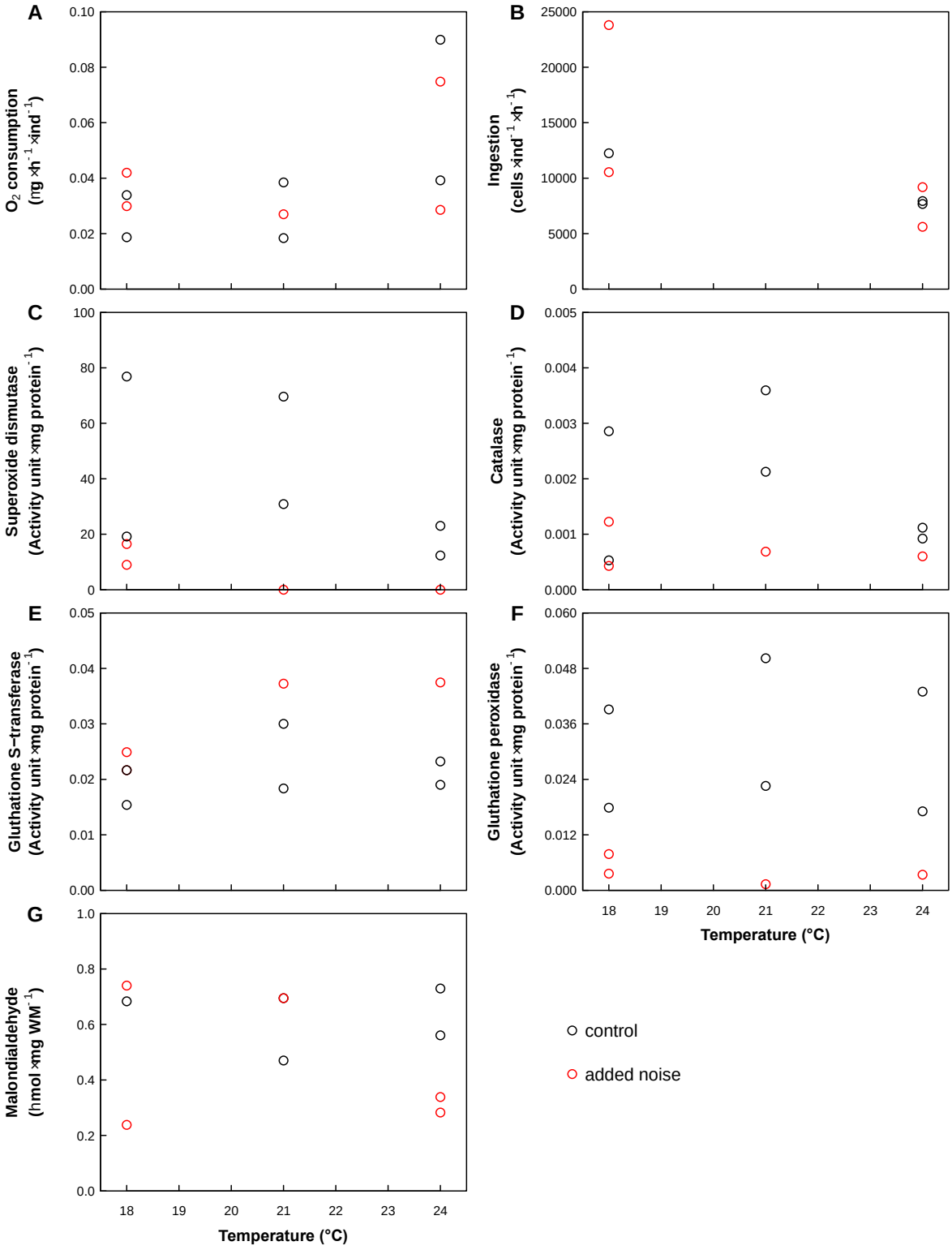

Figure 2. Effect of noise (red circles) and temperature on (A) oxygen consumption, (B) ingestion rates, and oxidative stress indicators of Acartia tonsa that reached copepodite stage: antioxidant enzymes - (C) superoxide dismutase, (D) catalase, (E) glutathione S-transferase, (F) glutathione peroxidase – and (G) malondialdehyde concentration as lipid peroxidation proxy.

4. CONCLUSION

The effect of low-frequency noise altered the antioxidant defense system of the copepod species *Acartia tonsa*, which means that potentially less energy is available to develop, growth, reproduce, or survive by mean of escaping behavior. More investigation is necessary to assess those energy sinkholes in this copepod species, looking at all ontogenetic stages, as well as other model species in terms of ecosystem functioning and nutrients recycling such as the amphipod *Echinogammarus marinus*. The full understanding and the settlement of noise threshold values at which the organisms are altered in a laboratory setting will allow to estimate gain or loss of the secondary production in the field.

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