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Effect of temperature and trace metal exposure on early life stages of European flat oysters and Pacific oysters

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ABSTRACT

Ocean warming and metal pollution pose a threat to coastal ecosystems worldwide. In the German Bight, efforts to restore biogenic reefs using the native European flat oyster (*Ostrea edulis*) face challenges due to environmental conditions and potential pollutants of the North Sea. Besides *O. edulis*, the non-native Pacific oyster (*Crassostrea gigas*) inhabits the North Sea. Larval stages of bivalves are known to be sensitive to pollution. In this study, we investigate the effect of the trace metals copper (Cu), zinc (Zn), cadmium (Cd) and lead (Pb) in combination with water temperatures of 18° and 24 °C on the embryo-larval development of *C. gigas* and acute mortality of *C. gigas* and *O. edulis* D-larvae. This multi-stressor approach revealed that Cu was the most toxic metal, regardless of temperature, species or life stage. While elevated temperatures mitigated the negative effects of metal exposure on embryo-larval development, larval mortality was species- and metal-dependent at the tested temperatures. *O. edulis* D-larvae demonstrated a greater absolute tolerance to metal exposure at both temperatures, but a species comparison showed that *O. edulis* D-larvae had lower relative tolerance to the combined stress of warming and metal exposure than *C. gigas*. Based on the resulting toxicity thresholds, an environmental risk assessment for Cu was conducted to identify potentially hazardous areas for *O. edulis* restoration to be included in future habitat suitability studies and site selection for restoration. The identified areas may also indicate problematic environmental conditions for larval stages of other invertebrate species or fish.

1. Introduction

Worldwide, coastal ecosystems such as coral reefs, seagrass meadows, mangroves, saltmarshes and oyster reefs are facing alarming degradation (Bayraktarov et al., 2016). Oyster reefs are the temperate equivalent to tropical coral reefs (McAfee and Connell, 2020), and provided vital ecosystem services and functions, including the provision of hard substrate habitat, shelter, food, water quality via filtration and climate regulation (Padilla, 2010; Colsoul et al., 2021; Thomas et al., 2022). Such biogenic reefs were a dominant habitat structure in temperate coastal and estuarine systems but have experienced drastic declines (Beck et al., 2011; McAfee and Connell, 2020). While the European flat oyster (*Ostrea edulis*) inhabited vast areas across European seas until 150 years ago (Vera et al., 2016; Hayer et al., 2021; Thurstan et al., 2024), it is under threat or even functionally extinct today (Merk et al., 2020; Zu Ermgassen et al., 2023; 2024). Overfishing is recognized

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as the primary driver of destruction of *O. edulis* oyster beds (Thurstan et al., 2013; Pogoda, 2019), but other anthropogenic stressors such as diseases, invasive species and pollution also contributed to native oyster populations decline (Moreira et al., 2018; Hughes et al., 2023).

In addition to the decline in native oyster beds, the shellfish industry was confronted with a significant drop in *O. edulis* production due to the outbreak of epizootic diseases caused by *Marteilia refringens* and *Bonamia ostreae* (Mérou et al., 2023). This led the aquaculture industry to seek a robust alternative species for production which was found in the Pacific oyster *Crassostrea gigas* (*Magallana gigas*; see Salvi and Mariottini, 2017; Bayne et al., 2017; Grizel and Héral, 1991; Vera et al., 2016). Today, 98 % of global oyster farming focuses on *C. gigas*, making it the most extensively cultivated oyster species worldwide (Azéma et al., 2017), and the second most harvested aquatic species in 2022 (FAO, 2024). Despite expectations that cooler summer temperatures in the Northwest Atlantic and North Sea would hinder natural reproduction, the non-native *C. gigas* has spread across European coastal waters in recent decades, likely supported by ocean warming (King et al., 2021). Concurrently, several conservation and restoration projects across Europe aim to reestablish the ecological key-role of *O. edulis* (Pogoda et al., 2019, 2020; Maathuis et al., 2020; Colsoul, 2022). Both species form biogenic reefs along European coastal and shelf seas (Nielsen et al., 2017; Zwerschke et al., 2018) and will be facing a multi-stressor environment including ocean warming (Venegas et al., 2023) and ocean pollution by trace metals (Rodríguez et al., 2021; Dinh et al., 2022), organic pollutants (Gan et al., 2021; Chowdhury and Rahman, 2023) or plastic particles (Paul et al., 2024; Mackay-Roberts et al., 2024).

Temperature is a key environmental factor, profoundly affecting marine invertebrates (Byrne, 2011). Rising temperature leads to shifts in species distribution (King et al., 2021), affects physiological and biochemical responses (Somero, 2012), shapes morphology (Visconti et al., 2017), and modulates timing of the reproduction phase (Przeslawski et al., 2008), as well as development in meroplankton (Garaventa et al., 2010; Martino et al., 2025), potentially altering marine ecosystems and biodiversity (Byrne, 2011). As a mean global sea surface temperature rise of 3.5 °C is predicted without countermeasures for 2099, with regional marine heatwaves potentially leading to water temperatures more than 6 °C warmer than in the past, marine organisms might face extreme temperature effects in the future.

Coastal areas are among the most densely populated regions globally. They experience the rapid expansion of human population and activities (Curran et al., 2002) and face severe pollutant entry into the marine environment (He and Silliman, 2019). Trace metals are an important group of contaminants in coastal ecosystems (Rainbow et al., 1990; Pan and Wang, 2012) and can persist in the environment with the potential for bioaccumulation. Although metals can be classified as essential (e.g. Cu, Zn) and non-essential metals (e.g. Cd, Pb), even essential metals can cause toxic effects when their concentrations exceed the critical homeostatic level of the organism (Ahearn et al., 2004; Rainbow, 2007; Przytarska et al., 2010). The negative impact of metal pollution on embryonic and larval development in marine invertebrates is a major concern for coastal areas as early life stages are more sensitive to metal exposure than adults (Connor, 1972; Verriopoulos and Moraïtou-Apostolopoulou, 1982; Gopalakrishnan et al., 2008). For oysters, the embryonic and larval stages represent the bottleneck for resilience to environmental changes (Moreira et al., 2018) as well as for population dynamics, including recruitment, dispersal, and connectivity among populations (Puritz and Toonen, 2011; Cowen and Sponaugle, 2009; Bashevkin et al., 2020; Rodríguez-Perez et al., 2020; Sidorenko et al., 2025). Whereas embryo-larval bioassays in *C. gigas* have provided valuable insights under conditions of single-temperature and single-metal exposure (Martin et al., 1981; His et al., 1999; Mai et al., 2012; Markich, 2021) less studies have investigated the combined effects of temperature and trace metals on oyster embryos (Gamain et al., 2017) and the availability of data for larvae stages is even more limited. To date, only one study has examined the effects of mercury on *C. gigas* larvae stages (Beiras and His, 1994) and only three studies have investigated the response of *O. edulis* larvae to mercury (Connor, 1972), copper (Alzieu et al., 1980), and zinc (Walne, 1970), using different endpoints and exposure durations. Notably, Zu Ermgassen et al. (2023) highlighted vast knowledge gaps regarding the impacts of many pollutants on *O. edulis*.

Due to the lack of comprehensive LC_{50} data for D-larvae of both species, we conducted basic toxicological research applying concentrations significantly higher than those typically found in the environment of the German Bight. These elevated concentrations were chosen by prior executed range-finding tests to establish clear dose–response relationships and determine median lethal concentrations (LC_{50}) as environmental levels are often too low to elicit measurable effects under laboratory conditions. For reference, median metal concentrations at 10 m water depth in the coastal German Bight in 2006 were 0.61 μg L^{-1} for Cu, 1.67 μg L^{-1} for Zn, 0.025 μg L^{-1} for cadmium (Cd), and 0.09 μg L^{-1} for lead (Pb) (BSH, 2013). However, concentrations can change several orders in magnitude resulting in extreme values as reported near the Elbe River outflow in summer 1998 for Cu reaching 97 μg L^{-1} and Zn reaching 1970 μg L^{-1} (MUDAB) (BfG, 2024).

The knowledge gap in threshold concentrations is particularly critical in the context of coastal pollution and global warming, where marine organisms are exposed to multiple environmental stressors simultaneously. Understanding how elevated temperatures modulate metal toxicity is important to accurately predict embryo-larval development and larval mortality, as these factors directly influence the recruitment success and survival of future oyster populations. Such insights are crucial for identifying suitable restoration sites and designing effective larval dispersal corridors that ensure reliable connectivity among populations and stable future oyster populations.

The southern North Sea has historically been regarded as a strongly polluted marine area (Mart and Nürnberg, 1986). Despite improvements of environmental conditions through concerted efforts such as stricter regulations on industrial discharges, enhanced wastewater treatment, and better management of agricultural runoff, no North Sea region has yet achieved the good environmental status (GES) required by the EU Marine Strategy Framework Directive (MSFD) (UBA, 2022). Consequently, pollution, combined with global climate warming, remains a significant issue in the southern coastal North Sea, potentially challenging both natural recruitment and aquaculture breeding efforts of oyster populations. The aim of this work is to assess the combined effects of warming and exposure to the essential trace metals Cu and Zn as well as to the non-essential trace metals Cd and Pb, on embryo-larval development of *C. gigas* and larval mortality of *C. gigas* and *O. edulis*. Two temperature treatments were selected: one corresponding to current summer temperatures of approximately 18 °C in the German Bight (Amorim et al., 2023) and the other simulating future ocean warming and/or marine heatwave scenarios at 24 °C in the North Sea already documented in the German Bight in summer 2018 (Kaiser et al., 2023). The temperature of 24 °C is also commonly used and recommended for larval rearing in aquaculture hatcheries (Robert et al., 2017).

Collected results and data were used to estimate the hazard of Cu pollution in the German Bight using a quotient-based risk assessment approach (Rohr et al., 2016). This approach could be integrated as an additional factor in habitat suitability analysis for European flat oyster restoration (Pogoda et al., 2023), providing new insights into suitable and unsuitable areas for oyster restoration in the context of metal pollution.

2. Material & methods

Experiments were conducted at IFREMER Bouin station (46° 57′ 51″ N, 2° 2’ 40” W) located at the Bay of Biscay (NE Atlantic Ocean), in France.

2.1. Broodstock

Broodstock of *O. edulis* (n = 30, shell length 79.2 ± 5.5 mm, total wet weight (TWW) 62.4 ± 10.3 g) originated from IFREMER La Tremblade station (Charente-Maritime, France), whereas the broodstock of *C. gigas* (n = 30, shell length 58.1 ± 11.2 mm, TWW 60.0 ± 17.8 g) was obtained from IFREMER Argenton station (Brittany, France). Both oyster species were reared at IFREMER Bouin station and were approximately 24 months (*O. edulis*) and 18 months (*C. gigas*) old at the start of the experiment in February 2024. Broodstock of *O. edulis* (n = 250) and *C. gigas* (n = 200) was split in two equal parts per species. The separated broodstocks were kept at 8 °C from November 2023 until 8th of January or 2nd of February, respectively to reset their reproductive cycle and pause gametogenesis. Thereafter water temperature was increased 1 °C per day until 21 °C or 17 °C for each species separately to initiate a staggered oyster conditioning and ensure the availability of gametes (*C. gigas*) and larvae (*O. edulis*) during the experimental phase from March to April 2024. Each broodstock was kept in 100 L tanks with a water exchange rate of 40 L h^{-1}. Seawater was filtered through 1 μm bag filtration (PALL CWF- X100), UV sterilized (BIO UV TTPE 1160 HO) and an active carbon column (Filtracarb® EX64D) with a flow rate of 250 L h^{-1}. The broodstock was fed with 10 L h^{-1} of *Skeletonema marinoi* (Polder-Bouin-strain) at a concentration of 1.1 × 10^6 ± 2.0 x10^5 cells mL^{-1} with a weekly addition of approximately 25 L of *Tisochrysis lutea* (CCAP 927/14) at a concentration of 10 × 10^6 cells mL^{-1}. No mortality of the broodstock was observed during the experimental phase.

2.2. Chemicals and seawater

For the bioassays, seawater was prepared by using 1 μm prefiltered (PALL CWF- X100) and UV sterilized (BIO UV TTPE 1160 HO) Bouin hatchery water additionally filtered through a 1 μm cartridge filter (3M Betapure series NT-T 9″3/4) and a 0.2 μm Polyethersulfon (PES) membrane (Thermo Scientific Nalgene Rapid Flow 90 mm Unit −1000 mL). Bioassay water parameters including salinity (27.2 ± 0.8), pH (8.0 ± 0.1) and oxygen (94.6 % ± 2.8 %) were measured with a multiparameter meter (Multiline® 3630 IDS). Bioassay water was stored in Schott bottles, covered with tin foil, in darkness in an incubator at 18 °C ± 1 °C (Sanyo MLR 351) or in a temperature-controlled room at 24 °C ± 1 °C and used within 2–4 days. For background metal concentrations, 0.2 μm filtered bioassay water (250 mL) was filled into acid-washed plastic bottles and preserved with 8.3 mL of 30 % nitric acid (1 % v/v). Samples were stored at +4 °C in the dark for further metal analysis.

Before chemical analyses, plastic and Teflon equipments involved in the experiments were acid-cleaned before use. Acids were of ultratrace analytical grade (Suprapur, Merck®). For experiments, metal solutions of Cu (II), Zn (II) Cd (II) and Pb (II) were prepared by diluting Cu $(NO_3)_2{\cdot}3H_2O$, Zn $(NO_3)_2{\cdot}6H_2O$, Cd $(NO_3)_2{\cdot}4H_2O$, and Pb $(NO_3)_2$, monoelemental solutions (SPC PlasmaCal®, 1000 μg mL^{-1}) in distilled Milli-Q water with resistivity of 18.2 MΩ *cm (Millipore®) under clean conditions.

2.3. Metal analysis by ICP-MS

Filtered water samples were acidified to 1 ‰ v/v HNO_3 and stored at 4 °C prior to preparation based on the study by Biller and Bruland (2012). Briefly, in the clean room at IFREMER, samples were preconcentrated and separated from the saline matrix using columns filled with Nobias Chelate PA1 resin (Hitachi High Technologies®). Then, 100 mL of each sample, adjusted to pH 6.2 ± 0.2 with buffer solution (NH_4Ac 2.5 M), was applied to the columns at a rate of 1 mL min^{-1} using a peristaltic pump (ISMATEC). The columns were then rinsed with 5 mL of NH_4Ac 0.05 M, and the metals (Mn, Fe, Co, Ni, Cu, Zn, Cd, and Pb) were eluted with 1 mL of HNO_3 1 M. To control the quality of the preconcentration, blanks consisting of clean seawater (free of trace metals) and reference material (CASS-6, seawater, NRC, Canada) were run with each series.

Concentrations of Cd, Co, Cu, Fe, Mn, Ni, Pb and Zn were analysed using a TQ-ICP-MS (iCAP-TQ Thermo®) in KED mode with He as collision gas. Selected metal isotopes were the following: 55Mn, 57Fe, 59Co, 60Ni, 63Cu, 66Zn, 111Cd and 208 Pb. External calibration with multi-element standard solutions (SCP Science) was performed at the beginning of each analysis session. Indium (115In, 2 ppb) was used as the internal standard in each sample. The recovery of the CASS-6 reference material was 100 % ± 10 % for metals.

2.4. C. gigas embryo bioassay

Embryo-larval bioassays were based on the Ecological Effects Guidelines OCSPP 850.1055 (2016). For fertilization, 15 to 20 oysters of *C. gigas* were kept out of water overnight to promote spawning. The following day, oysters were individually placed in beakers and exposed to alternating water temperatures between 14 °C and 29 °C (thermal shock) every 15 min until spawning was initiated. Gamete selection was conducted based on the microscopic observation; only samples with abundant highly motile spermatozoa from 3 to 10 (median = 8) males and even, pear shaped oocytes from 2 to 6 (median = 3) females were used. Sperm was collected in a beaker after sieving through a 40 μm mesh to remove faeces and debris, while eggs were collected on a 20 μm mesh after faeces and debris removal on an 80 μm mesh. Eggs were placed in a 500 mL beaker, and the sperm concentration was adjusted to achieve an average of 5–10 spermatozoa attached to a single egg.

After 15 min eggs were rinsed again on a 20 μm mesh to remove excessive sperm. Fertilization success was validated under the microscope (Leica CME) and 5 replicates of 50 μl droplets were counted on a Sedgwick-Rafter Chamber at magnification of 100 to enumerate embryos. For each metal, a sterile 24 multi-well cell culture plate made of Polystyrene (Clearline® Biosigma) was used for the bioassay. Culture plates were loaded with 6 metal concentrations, including 5 nominal metal concentrations (Table 1) and the control (no metal addition) as quadruplicates per concentration. For each metal, titration of the mother solutions was performed to verify that the effective concentrations matched the intended nominal concentrations. Metal concentrations reported are nominal values, supplemented by measured background trace metal concentrations in the bioassay seawater (Table 2). No corrections were made for potential complexation or changes in metal speciation due to seawater chemistry (e.g., salinity,

Table 1
Nominal concentrations of the tested metals copper (Cu), zinc (Zn), lead (Pb) and cadmium (Cd), depending on species, stage and temperature during the acute toxicity tests (24–48 h) based on range-finding tests.

Development stage	Temperature [°C]	Species	Metal/Nominal concentrations [μg L^{-1}]
Embryo	18, 24	*C. gigas*	Cu: 4, 8, 16, 32, 64 Zn: 40, 80, 160, 320, 640 Pb: 500, 800, 1280, 2048, 3280 Cd: 1000, 1320, 1740, 2300, 3000
D-larvae	18, 24	*C. gigas*	Cu: 100, 132, 174, 230, 304 Zn: 3800, 3990, 4190, 4400, 4620 Pb: 3600, 3780, 3970, 4170, 4380 Cd: 3600, 3780, 3970, 4170, 4380
D-larvae	18	*O. edulis*	Cu: 250, 365, 535, 780, 1140
D-larvae	24	*O. edulis*	Cu: 150, 200, 270, 370, 500
D-larvae	18, 24	*O. edulis*	Zn: 4000, 4500, 5010, 5770, 6500 Pb: 3500, 4060, 4710, 5460, 6340 Cd: 3500, 4060, 4710, 5460, 6340

Table 2
Bioassay water trace metal concentration [μg L^{-1}] of manganese (Mn), iron (Fe), cobalt (Co) nickel (Ni) copper (Cu), zinc (Zn), lead (Pb) and cadmium (Cd) during the experimental phase. Values in bold represent the mean ± standard deviation.

Mn	Fe	Co	Ni	Cu	Zn	Pb	Cd
0.68	0.39	0.16	0.58	1.12^{E}	26.96^{E}	0.36^{E}	0.02^{E}
0.57	0.60	0.12	0.51	$0.85^{E,L}$	$17.21^{E,L}$	$0.39^{E,L}$	$0.01^{E,L}$
0.45	0.60	0.12	0.44	0.71^{L}	12.40^{L}	0.51^{L}	0.01^{L}
0.32	1.39	0.05	0.52	0.71	4.79	0.08	0.01
0.92	1.12	0.17	0.60	0.95^{E}	15.16^{E}	0.41^{E}	0.02^{E}
0.55	0.46	0.21	0.55	0.90^{L}	11.55^{L}	0.41^{L}	0.02^{L}
0.58 ± 0.19 0.025^{P}	**0.76 ± 0.37** 0.15^{P}	**0.14 ± 0.05** n.d	**0.53 ± 0.05** 0.25^{P}	**0.87 ± 0.14** 0.10^{P}	**14.68 ± 6.71** 0.20^{P}	**0.36 ± 0.14** 0.02^{P}	**0.01 ± 0.0** 0.025^{P}

*Background metal concentrations in the bioassay water during embryo (E) and larvae (L) tests and upper values in pristine water of the Atlantic Ocean (P) according to OSPAR (2006).

pH). Therefore, actual bioavailable concentrations may differ from nominal values. However, pH and salinity were consistent across all treatments and reflected realistic environmental conditions, suggesting that the observed effects are likely representative of those in natural settings.

Prior to metal addition and embryo transfer (100 embryos mL^{-1}, OCSPP Ecological Effects Test Guidelines, 2016), wells were filled with the appropriate amount of bioassay seawater and trace metal solution to achieve a final volume of 2 mL per well after adding the embryos. Culture plates were kept at the target temperatures of 18 °C and 24 °C before embryo loading. Embryo-larval development tests were performed without feeding or light (Mai et al., 2012). After the embryo transfer, plates were incubated for 24 h at 24 °C ± 1 °C (Binder KT115) or 48 h at 18 °C ± 1 °C (Sanyo MLR 351). The longer incubation time at 18° was necessary to reach the D-larvae stage due to the slower developmental rate of embryos at colder temperatures. Following the incubation, samples were fixed with 40 μL of 8 % formalin solution. To determine the 24 h-EC_{50} (at 24 °C) and 48 h-EC_{50} (at 18 °C) for the respective developmental stage, 100 individuals per well were counted at 200x magnification using an inverted microscope (ZEISS Axiovert 25). Embryo bioassays were conducted three times at 24 °C using three different batches of oyster broodstocks, and twice at 18 °C using two different batches of oyster broodstocks. Abnormal shell development of the D-larval stage, based on the criteria of His et al. (1997) was used as toxicological endpoint (EC_{50}). If more than 30 % of the embryos failed to develop to a normal D-larvae in the control conditions, the bioassay was considered invalid (OCSPP Ecological Effects Test Guidelines, 2016). Before the final experiments, range finding tests for embryo-larval development were conducted based on the EC_{50} values of Martin et al. (1981). Since *O. edulis* is a brooding species and fertilization of eggs occurs within the female oyster's mantle cavity (Waller, 1981), conventional embryo-larval bioassays as typically applied to broadcast spawners such as *C. gigas* are not feasible. Therefore, we performed larval bioassays using D-larvae obtained after their release from the female (*O. edulis*) or 24 h post artificial fertilization (*C. gigas*), ensuring exposure at a defined and uniform developmental stage of both species. As the D-larvae stage of *C. gigas* represents the most sensitive larvae stage during the meroplanktic period under Hg exposure (Beiras and His, 1994) and the larval phase is the key stage in the oyster life cycle for dispersal and population connectivity, D-larvae were chosen for interspecies toxicity testing.

2.5. *C. gigas & O. edulis D-larvae bioassays*

The fertilized eggs of *C. gigas* were kept for 24 h at 24–25 °C in an aerated 30 L tank in 1 μm prefiltered (PALL CWF- X100) and UV sterilized (BIO UV TTPE 1160 HO) seawater without food to obtain D-larvae (batch size: $4.80 \times 10^6 \pm 2.43 \times 10^6$) with a shell length of 71.8 ± 3.2 μm (n = 100) for larval bioassays. Larvae of *O. edulis* (batch size: $0.71 \times 10^6 \pm 0.31 \times 10^6$) with a shell length of 185.3 ± 5.3 μm (n = 80) were collected by a 100 μm mesh placed below the broodstock tank outflow. Unlike *C. gigas,* the exact age of *O. edulis* D-larvae could not be determined due to unpredictable swarming events and ranged between 2 and 24 h. The culture plates were prepared in the same way as for the embryo-larval bioassays. Tests were performed with a larvae density of 50 D-larvae mL^{-1} without feeding or light (Mai et al., 2012). Larvae density was estimated by counting 5 replicates of 50 μL droplets with a Sedgwick-Rafter Chamber at 50x-100x magnification. Median lethal concentration (LC_{50}) of D-larvae was determined after 48 h of metal exposure (Beiras and His, 1994). Larvae were considered dead if no movement of cilia or internal organs was observed for 5 s (Garaventa et al., 2010), or if they exhibited a severe loss of shell integrity. For each well, 20 haphazardly chosen larvae of *O. edulis* and *C. gigas* were assessed at a magnification of 50x-100x (ZEISS Axiovert 25). Larval bioassays of *C. gigas* were repeated 5 times for Cu and Zn, while bioassays with Cd and Pb at 18 °C were conducted 4 times, with each test using different oysters from the broodstock. *O. edulis* larval bioassays were conducted 4 times using 3 different batches of oyster broodstock. Since no information was available regarding the response of D-larvae to metal exposure, the final nominal metal concentrations were determined based on prior range-finding tests conducted for each species (*O. edulis, C. gigas*), as shown in Table 1.

2.6. *Statistics*

Data analyses and plots were performed using R (version 4.2.2) with the RStudio interface (Version 2023.06.01). EC_{50}/LC_{50} estimates were conducted with the 'drc' package (Ritz et al., 2015). Model fitting was performed separately for the embryo-larval bioassays and the larval bioassays using developmental (EC_{50}) or mortality (LC_{50}) readouts. The best-fitting model for each species and trace metal was determined by comparing the model fit for each treatment using 'Log-logistic' and 'Weibull' family functions of pooled bioassay runs. Model selection was performed using the 'mselect' function, which ranks models by comparing the Log Likelihood, Akaike Information Criterion (AIC), Lack of Fit, and Residual Variance. Significance of the model parameters was assessed using the 'multcomp' package. Best model fit for each trace metal per species were used to estimate EC_{50}/LC_{50} values by the 'ED.drc' function, providing the median effective/lethal concentration with standard error (SE) and 95 % confidence intervals using the delta method. Intraspecific ratios of EC_{50}/LC_{50} estimates, derived from the same non-linear model per trace metal and species were compared using the function 'compParm' of the 'drc' package. For metal toxicity comparison, a global 'Weibull' function (W2.3u) was used for EC_{50} estimates, and the log-logistic function (LL.2) for LC_{50} estimates. Analysis was performed with the nominal concentrations (Table 1), adjusted with measured background metal concentrations during the experiments (Table 2). The x-axis values were plotted on a linear scale; log transformation was handled internally by the fitted models and not applied to the raw data or plots.

Differences in mortality between temperature treatments without metal addition of both species were assessed using the non-parametric Mann-Whitney-U test at a significance level of $p < 0.05$ after data were tested for normality (Shapiro-Wilk test) and homoscedasticity

(Levene's test). Values in the text are presented as mean ± standard deviation, unless specified otherwise.

2.7. Hazard mapping

Based on measured environmental Cu concentration (MEC) in the German Bight, an environmental risk assessment was suggested by mapping (high) risk versus predicted no effect concentration (PNEC) areas (Hall and Anderson, 1999). MEC data were obtained from the Marine Environmental Data Base (MUDAB) (BfG, 2024). For spatial analysis, all Cu values of the water column from 1986 to 2021 were selected to ensure a dense data point net of the German Bight, as several stations were shut down in later years. As the spawning peak of both species occur in summer (Wilson and Simons, 1985; Lango-Reyoso et al., 2000), Cu concentrations from June to August were chosen. The PNEC was defined as EC_{10} estimates (Chapman et al., 1998) with an assessment factor of 5 to consider intraspecific embryotoxicity variation (Martin et al., 1981; Gamain et al., 2017) as well as uncertainties in D-larvae mortality. HQ values above 1 indicate an elevated risk of adverse effects for the exposed organism. Spatial HQ distribution of the German Bight was achieved by performing the Inverse Distance Weighting (IDW) interpolation method using QGIS (Version 3.24.2 – Tisler) with a power distance exponent of 4 (Barudžija et al., 2024).

3. Results

3.1. Trace metal background concentrations

Trace metal concentration measured at Bouin station were above measured background concentrations in pristine waters of the Atlantic (OSPAR, 2006). Nevertheless, abnormal larval development in the control and warming scenario without metal addition was always below the invalid test threshold of 30 % (OCSPP Ecological Effects Test Guidelines, 2016). Furthermore, mean larval mortality in the control and warming scenario without metal addition was low (<2 %).

3.2. Sublethal effects: behaviour and morphological abnormalities due to metal exposure

Under control conditions (no trace metal addition), *C. gigas* embryo development showed no statistically significant difference between 18° and the warming condition treatment at 24 °C ($W = 723$, $p = 0.66$). The frequency of abnormal development was 16.9 % ± 3.1 % at 18 °C and 17.3 % ± 5.2 % at 24 °C, respectively. Of the four tested trace metals, Cu displayed the highest embryonic toxicity at both temperatures as assessed by EC_{50} for larval abnormal development ($p < 0.001$), followed by Zn, Pb and Cd (Fig. 1, Table 3), leading to the identical toxicity ranking for both temperatures: Cu > Zn > Pb > Cd. Embryo abnormal development was more affected by metals at 18 °C than at 24 °C for all tested metals. The warmer temperature led to a toxicity decrease of 18.7 % ± 0.70 % in Cu and to a similar decrease in Pb (23.24 % ± 1.13 %) and Cd (23.14 % ± 0.70 %) treatments whereas embryo sensitivity to Zn exposure decreased only by 10.00 % ± 0.62 % at 24 °C relative to 18 °C (Table 3).

3.3. Temperature effects: species-specific metal toxicity

3.3.1. Mortality in control and warming scenario without trace metal addition

Comparison of D-larvae mortality in the controls at 18° and the warming scenario at 24 °C revealed no significant effect of temperature alone on *C. gigas* ($W = 2833$, $p = 0.73$) or *O. edulis* ($W = 2048$, $p = 1.00$). For *C. gigas* larvae, mortalities ranged from 0.49 % ± 1.71 % at 18 °C to 0.56 % ± 1.78 % at 24 °C, while for *O. edulis* larvae, mortality was 1.41 % ± 2.43 % at both temperatures.

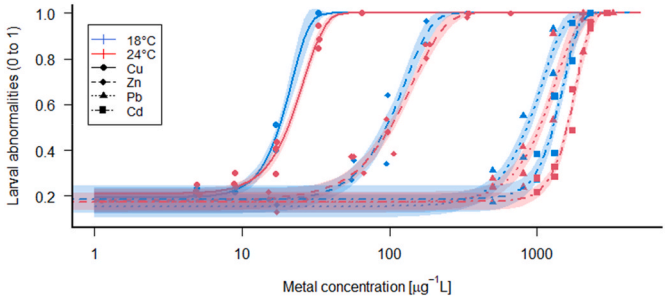

Fig. 1. Concentration-response curves with mean values and 95 % confidence intervals at 18 °C (blue colour) and 24 °C (red colour) for abnormal larval development after 24 h (24 °C) and 48 h (18 °C) for copper (Cu) represented with points and solid lines, zinc (Zn) with diamonds and dashed lines, lead (Pb) with triangles and dotted lines and cadmium (Cd) with boxes and dash-dotted lines. Bioassays responses for all tested metals are fitted to a three-parameter Weibull functions (W2.3u). Note: The x-axis is linear with tick marks manually placed at logarithmic intervals. Concentrations were log-transformed internally during model fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3.2. Sublethal effects: behaviour and morphological abnormalities due to metal exposure

Under metal exposure, early occurring clinical signs in the form of alterations in swimming behaviour were observed, starting at the lowest used concentration for all metals. While the larvae in the controls and warming scenario were mainly located in the water column of the well or, in the case of *O. edulis* larvae, agglomerated into rafts just below the surface, the metal-exposed larvae were largely located on the bottom of the well. Locomotion was either dysfunctional or inhibited, recognisable by circular movement patterns due to malformed, protruded and clumped velums (Fig. 2). Under Cu exposure, an additional distinct morphological sign was observed: a severe loss of ciliated velar cells with increasing Cu concentration in both species, as well as the condensation of the internal tissue into a spherical shape (Fig. 3; C, F).

The signs of Zn, Pb and Cd toxicity were similar, but differed from those observed under Cu exposure (Fig. 4). At median concentrations, Zn, Pb and Cd effects in *O. edulis* were visible as severe shell damages, with margins ranging from holes to severe shell fragmentation of individuals (A2, B2, C2), and isolated velums (A3, B3, C3). At the highest concentration, larvae showed a severe lack of shell integrity (A4, B4, C4) in comparison to treatment with no trace metal addition (A1, B1, C1). Shell damage was also observed in *C. gigas*, but features present in *O. edulis*, such as the detachment of the velum, were absent.

3.3.3. Metal toxicity and species-specific sensitivity between temperatures

Exposure of *C. gigas* and *O. edulis* D-larvae to trace metals resulted in increased mortality, with toxicity ranking was influenced by both metal type and temperature (Fig. 5, Table 4). Among the tested trace metals, Cu was the most toxic to both species at both temperatures, while Zn was consistently the least toxic metal. A temperature-dependent pattern was observed in the comparison between Zn and Pb. At 18 °C, toxicity differences between both metals were not statistically significant for either species (*C. gigas*: $t = 1.92$, $p = 0.055$; *O. edulis*: $t = 1.02$, $p = 0.307$). However, at 24 °C, Pb became significantly more toxic than Zn (*C. gigas*: $t = 4.66$, $p < 0.001$; *O. edulis*: $t = 9.22$, $p < 0.001$). Cd and Pb showed similar toxicities within species and across temperatures ($p > 0.05$). In terms of species-specific sensitivity, *O. edulis* D-larvae were generally more tolerant to metal exposure than *C. gigas* at both temperatures. However, *O. edulis* showed a greater relative sensitivity at 24 °C, as reflected by consistently higher LC_{50} ratios (Table 4). For Cu exposure, *C. gigas* tolerance decreased by 32.2 %, while *O. edulis* tolerance decreased by 45.5 % between 18 °C and 24 °C. For Zn, temperature effects were minimal (tolerance reduction: *C. gigas* – 1.3 %, *O. edulis* – 3.8 %). In contrast, species responses diverged for Cd and Pb: *C. gigas*

Table 3
EC_{50} estimates based on three-parameter Weibull functions (W2.3u) for copper (Cu), zinc (Zn) cadmium (Cd) and lead (Pb) for abnormal larval development ± standard error (SE) and 95 % confidence intervals (CI) of *C. gigas* after 48 h (18 °C) and 24 h of exposure (24 °C) and EC_{50} ratios of both temperature with reported t and P values derived from all test runs.

Metal	Temp. [°C]	Duration [h]	EC_{50} ± SE [µg L^{-1}]	95 % CI [µg L^{-1}]	EC_{50} Ratio ± SE	t-value	p-value
Cu	18	48	19.7 ± 0.6	18.6–20.8	1.21 ± 0.05	4.08	<0.001
	24	24	23.4 ± 0.6	22.3–24.5			
Zn	18	48	109.0 ± 4.7	99.61–118.37	1.14 ± 0.06	2.34	<0.05
	24	24	119.9 ± 5.4	109.25–130.53			
Pb	18	48	943.3 ± 35.7	872.5–1014.0	1.23 ± 0.06	4.20	<0.001
	24	24	1162.5 ± 35.2	1092.7–1232.3			
Cd	18	48	1397.9 ± 34.2	1330.1–1465.6	1.22 ± 0.03	6.83	<0.001
	24	24	1721.3 ± 30.3	1661.3–1781.3			

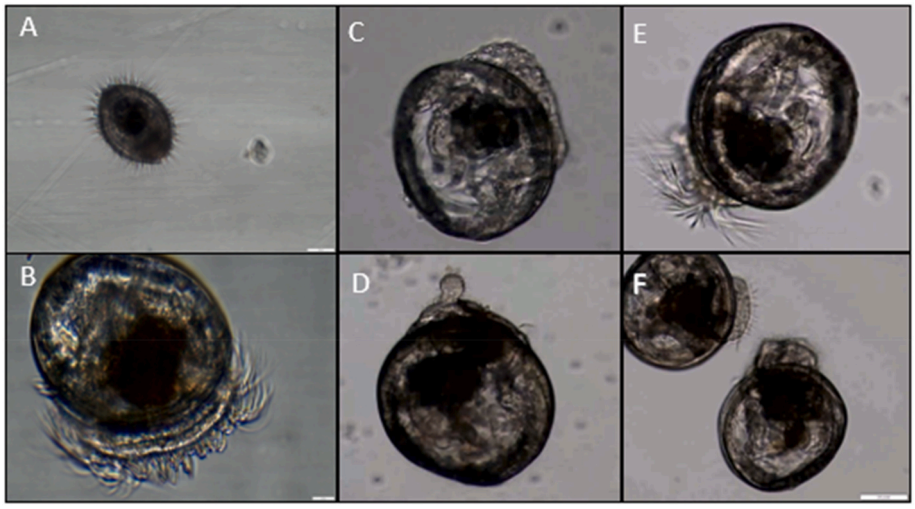

Fig. 2. Non clumped velum and well-organized cilia in *O. edulis* D-larvae (A, B) without trace metal addition and protruded, malformed velums at the lowest tested nominal concentration for copper (C), zinc (D), lead (E) and cadmium (F).

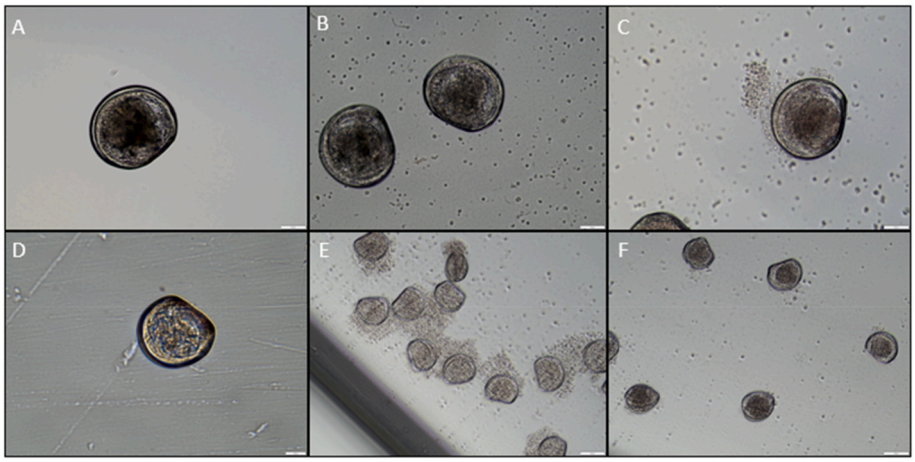

Fig. 3. Controls of *O. edulis* (A) and *C. gigas* (D) with normal tissue structure and intact cilia cells. Detachment of velum cells and condensation of the internal tissue into spherical shape in *O.* edulis (B, C) and *C. gigas* D-larvae (E, F) after 48 h at the lowest (B, E) and highest (C, F) Cu concentration at 24 °C.

exhibited slight tolerance increases (Cd: +2.3 %, Pb: +0.8 %) at 24 °C, while *O. edulis* showed reduced tolerance (Cd: 12.1 %, Pb: 9.7 %).

3.3.4. Hazard mapping: Spatial risk assessment of Cu exposure in the German Bight

Cu resulted in the most toxic response in both species with embryos of *C. gigas* representing the most sensitive stage at 18 °C and D-larvae larvae at 24 °C. Hazard quotient (HQ) calculation was applied and showed that the embryo-larval development of *C. gigas* could be affected in the vicinity of river plumes of Elbe, Weser and Ems as well as near harbours, ports and marinas, whereas the areas beyond the Wadden Sea islands, including the Marine Protected Areas (MPAs) Borkum Reef-ground and Sylt Outer Reef can be considered non-hazardous areas. In contrast, acute mortality on D-larvae of *C. gigas* and *O. edulis* is unlikely to be affected by environmental Cu concentrations as the HQ values are far below 1 (Fig. 6).

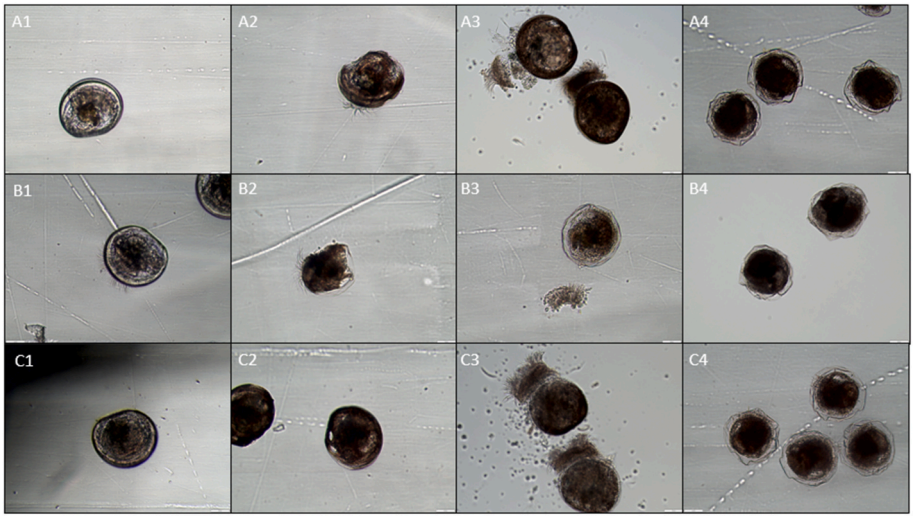

Fig. 4. Normal and intact shell shape of *O. edulis* D-larvae after 48 h at 24 °C (A1, B1, C1). Severe shell malformations after Zn (A2- A4), Pb (B2- B4) and Cd (C2- C4) exposure at median (A2, A3, B2, B3, C2, C3) and highest concentrations (A4, B4, C4) in *O. edulis* after 48 h at 24 °C.

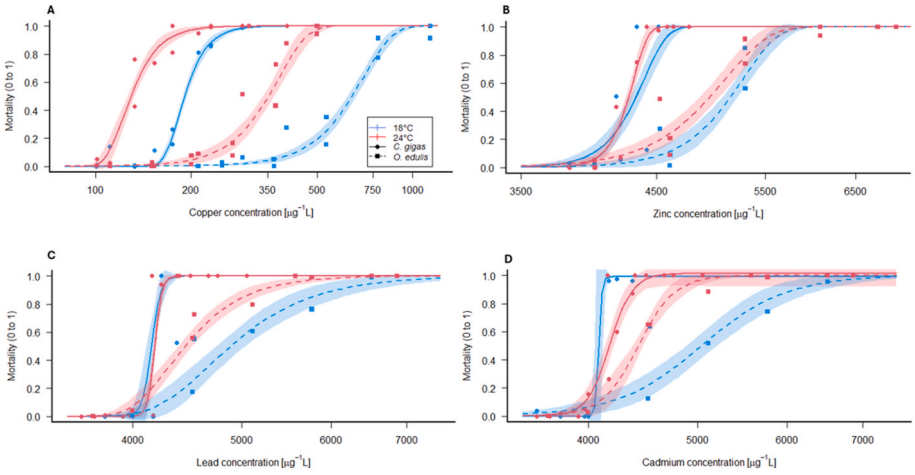

Fig. 5. Concentration-response curves with mean values and 95 % confidence interval at 18 °C (blue colour) and 24 °C (red colour) for larval mortality of *C. gigas* (points and solid lines) and *O. edulis* (boxes and dashed lines) D-veliger after 48 h exposure to **A** copper (model: *C. gigas*: W1.2; *O. edulis*: W2.2), **B** zinc (model: *C. gigas*, *O. edulis*: W2.2), **C** lead (model: *C. gigas*: LL.3; *O. edulis*: LL.2) and **D** cadmium (model: *C. gigas*: LL.2; *O. edulis*: W1.2). Note: The x-axis is displayed on a linear scale; log transformation of concentrations was applied internally during model fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Embryos

Cu had the most severe impact on embryo-larval development, followed by Zn, Pb and Cd. The toxicity ranking of the tested metals is mostly in accordance with *C. gigas* embryo-larval toxicity tests reported by Martin et al. (1981) (Cu > Zn > Cd > Pb) and Markich (2021) (Cu > Zn > Pb > Cd). Inter-study comparisons showed that EC_{50} estimates for Cu in this study were in the higher range than in comparison to other published embryo-larval bioassays. Thus, after 48 h Cu exposure of *C. gigas* embryos at 17 °C ± 1 °C, EC_{50} (Cu) estimates reported by Martin et al. (1981) were 5.3 ± 0.5 µg L^{-1}, whereas in the present study the 48 h-EC_{50} (Cu) was 19.7 ± 0.6 µg L^{-1} at 18 °C. At 24 °C after 24 h of Cu exposure, Mai et al. (2012) reported an EC_{50} (Cu) of 12.5 µg L^{-1} while we estimated an EC_{50} (Cu) of 23.4 ± 0.6 µg L^{-1}. In contrast, His et al. (1999a) observed a higher value than in our experiments with an EC_{50} (Cu) of 37.0 ± 3.4 µg L^{-1} after 18–24 h exposure time at 24 ± 1 °C. EC_{50} (Pb) of 758 ± 20.4 µg L^{-1} and EC_{50} (Cd) of 611 ± 195.9 µg L^{-1} observed by Martin et al. (1981) were lower than the values in the present study with an EC_{50} (Pb) of 943.3 ± 35.7 µg L^{-1} and EC_{50} (Cd) of 1397.9 ± 34.2 µg L^{-1}, respectively. In contrast, His et al. (1999a) observed only 31.2 % ± 8.5 % abnormal larval development at Pb concentrations of 1200 µg L^{-1} after 18–24 h at 24 °C whereas in this study the EC_{50} (Pb) was already reached at 1162.5 ± 35.2 µg L^{-1}. For Zn, 48 h EC_{50} (Zn) estimates at 17 °C ± 1 °C from the study of Martin et al. (1981) with 119 ± 12.0 µg L^{-1} were comparable to our estimates of 109.0 ± 4.8 µg L^{-1}. This inter-study variability may reflect differences in the tolerance of the studied oyster populations to metals, and/or differences in assay conditions. Nonetheless, interlaboratory and interpopulation differences in acute EC_{50} estimates, ranging from two-to five-fold, are commonly observed (His et al., 1999b; Raimondo et al., 2016).

Variability in EC_{50} estimates between toxicity studies can be attributed to differences in abiotic factors such as salinity (Coglianese, 1982; Gamain et al., 2016), pH (Han et al., 2014; Riba et al., 2016), temperature (Boukadida et al., 2016; Gamain et al., 2017), water chemical composition (Persoone et al., 2009), exposure time (Barata et al., 1999; Connell et al., 2016), and biotic factors including life stages (Beiras and

Table 4
LC_{50} estimates for *O. edulis*, based on two-parameter Weibull function (W2.2) for copper (Cu) and zinc (Zn), two parameter Log-logistic function (LL.2) for cadmium (Cd) and a two-parameter Weibull function (W1.2) for lead (Pb). LC_{50} estimates for *C. gigas*, based on Weibull function W1.2 for Cu and W2.2 for Zn, three-parameter Log-Logistic function (LL.3) for Cd and LL.2 for Pb. LC_{50} estimate ± standard error (SE) and 95 % confidence intervals (CI) of *C. gigas* and *O. edulis* D-larvae after 48 h at 18 °C and 24 °C with intraspecific LC_{50} ratios ± SE for both temperatures and corresponding t and P values derived from all test runs.

Species	Metal	Temp. [°C]	Duration [h]	LC_{50} ± SE [µg L^{-1}]	95 % CI [µg L^{-1}]	LC_{50} Ratio ± SE	t-value	p-value
C. gigas	Cu	18	48	191.0 ± 1.5	188.1–193.9	1.49 ± 0.02	21.78	<0.001
		24	48	129.4 ± 1.6	126.3–132.5			
O. edulis	Cu	18	48	645.3 ± 10.1	625.3–665.3	1.84 ± 0.05	17.20	<0.001
		24	48	351.7 ± 6.7	338.3–365.0			
C. gigas	Zn	18	48	4327.9 ± 24.7	4279.1–4376.8	1.02 ± 0.01	3.15	<0.01
		24	48	4271.4 ± 12.2	4247.2–4295.5			
O. edulis	Zn	18	48	5155.8 ± 34.5	5087.3–5224.4	1.03 ± 0.01	2.91	<0.01
		24	48	4962.0 ± 44.3	4874.2–5049.9			
C. gigas	Pb	18	48	4150.7 ± 13.2	4124.63–4176.83	0.99 ± 0.00	−2.01	<0.05
		24	48	4183.3 ± 9.5	4164.70–4201.97			
O. edulis	Pb	18	48	5018.0 ± 78.2	4862.85–5173.19	1.08 ± 0.02	4.98	<0.001
		24	48	4529.2 ± 44.6	4440.73–4617.67			
C. gigas	Cd	18	48	4084.8 ± 13.5	4058.0–4111.6	0.98 ± 0.02	−1.17	>0.05
		24	48	4180.4 ± 21.9	4137.1–4223.7			
O. edulis	Cd	18	48	5039.3 ± 61.8	4917.7–5161.0	1.14 ± 0.02	8.35	<0.001
		24	48	4430.9 ± 34.2	4363.5–4498.3			

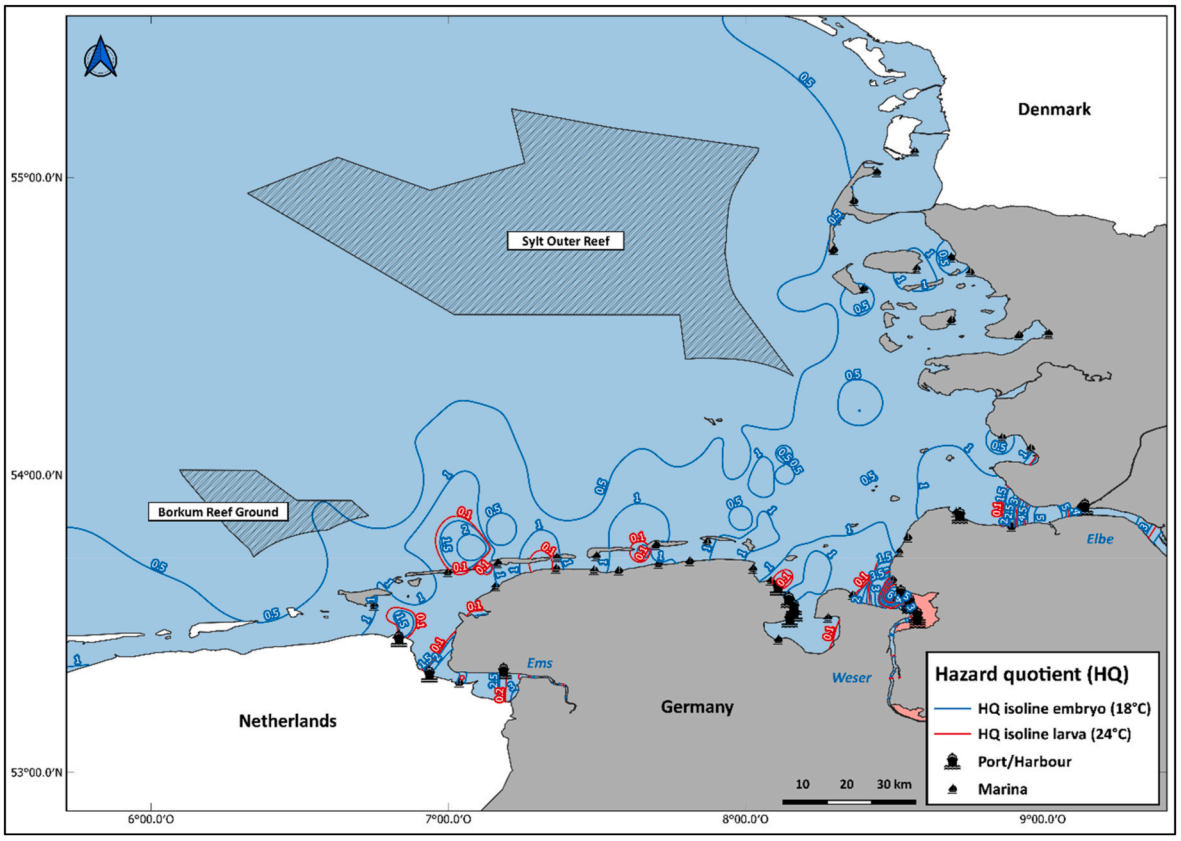

Fig. 6. Ecological risk assessment map of copper pollution in the German Bight for *C. gigas* embryo-larval development at 18 °C (blue isolines) and larval mortality at 24 °C (red isolines). HQ isolines≥1 indicate an adverse effect. Predicted no effect concentration (PNEC) was defined as EC_{10}/LC_{10} with and assessment factor (AF) of 5. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

His, 1994; His et al., 1999a) and the parental origin of study organisms (Weng and Wang, 2014; Gamain et al., 2017). In the context of the present study, the higher tolerance observed during embryo-larval development, compared to Martin et al. (1981) and Mai et al. (2012), may be attributed to genetic adaptation of the Bouin broodstock to elevated trace metal concentrations (Table 2). Consistent with this, Weng and Wang (2014) observed that embryos of *Crassostrea sikamea* from polluted areas exhibited significantly higher tolerance to Cu and Zn exposure than embryos from less polluted areas, by factors of 2.2 and 3.3, respectively.

Another explanation is the use of nominal concentrations in this multiwell plate approach. Factors such as adsorption to the well walls, uptake by embryos or larvae, and potential chemical instability may have reduced the actual metal concentrations during the exposure period (Gülden et al., 2015). Consequently, the derived EC_{50}/LC_{50} values may overestimate the true tolerance of embryos and larvae to

some extent. We acknowledge that this introduces a degree of uncertainty into the quantitative toxicity values reported. Nevertheless, the multiwell plate approach offers a viable alternative in terms of throughput, cost-efficiency, and methodological simplicity, making it a practical and economical tool for toxicity testing in comparison to conventional flasks (Ismail et al., 2002). Accordingly, a substantial portion of existing acute toxicity data is based on nominal concentrations (Raimondo et al., 2009).

In the absence of trace metal exposure, abnormal embryo-larval development of *C. gigas* was not affected by temperature, indicating that 18 °C and the warming scenario temperature (24 °C) are within the thermal tolerance window for embryogenesis of this eurythermal species (Di Poi et al., 2022). As expected in ectotherms, warming accelerated the embryo-larval development of *C. gigas* (Moreira et al., 2018), leading to a shorter exposure time, which is critical because the duration of exposure affects the build-up of internal contaminant concentrations (toxicokinetics) and the resulting biological effects (toxicodynamics) (Baas et al., 2010). Unlike 18 °C, 24 °C represents the optimal temperature for embryo-larval development in *C. gigas* (Gamain et al., 2017). Shorter exposure time until reaching the D-larvae stage as well as the optimal temperature (Tables 3 and 4), likely mitigated the toxic effects of trace metals by supporting efficient energy allocation toward detoxification, elimination, and cellular repair processes during embryo-larval development, mechanisms that may be compromised at peius temperatures (Lannig et al., 2006).

4.2. Larvae

In this study, *C. gigas* D-larvae demonstrated a much higher tolerance to metal exposure at 18 °C and at 24 °C than the embryo stage (Table 3, Table 4). These findings are supported by Beiras and His (1994) with a higher tolerance of *C. gigas* D-larvae to mercury exposure than embryos. This pattern was also observed in metal toxicity tests for *Crassostrea virginica* and *Mercenaria mercenaria* (Calabrese et al., 1977). Since later life stages of bivalves are typically more tolerant to metals than early developmental stages (His et al., 1999a), we assume that *O. edulis* larval stages are also less sensitive than the embryo stage. Nevertheless, the investigation of embryo-larval development under metal exposure conditions will be necessary to test this hypothesis. The reduced tolerance of embryos might be the result of a lower level of metallothioneins (MTs) in the embryo stage, compared to later larval stages (Pavičić et al., 1994). MTs are metal binding proteins, keeping the homeostasis of essential trace metals like Cu and Zn and acting as detoxification tool for non-essential metals such as Cd and Pb (Jenny et al., 2006). Interestingly, the susceptibility for Zn changed between embryo and larval stage of *C. gigas*, which could be the result of specific metabolic requirements for Zn depending on the life stage (Pavičić et al., 1994).

Of the four tested metals, Cu was the most toxic across both species and life stages. This high toxicity is likely due to Cu's redox properties (Young et al., 2023), which facilitate Fenton-like reactions converting hydrogen peroxide into highly reactive hydroxyl radicals (HO·) (Pham et al., 2013). These radicals can cause oxidative stress, causing cellular damage and ultimately apoptosis (Temple et al., 2005). Since Cu plays a role in controlled apoptosis of the velum during larval metamorphosis (Weng et al., 2019), an excess of Cu may have caused the unintended, severe loss of ciliated velar cells, as shown in Fig. 3. Besides the specific loss of velar cells at Cu exposure, all metals caused abnormal velum formation, even at the lowest tested metal concentrations (Fig. 2). Valve movement, including closure, is a widely observed avoidance behaviour in bivalves when exposed to pollution (Kramer et al., 1989) and was also observed for the D-larvae in this study. The velum protrusion might therefore be caused by a rapid shell closure which serves to protect the larvae from external contaminants, resulting in the velum becoming trapped (Beiras and His, 1994). This morphological abnormality compromises normal swimming activity for both species and likely impedes food uptake. The process of food uptake relies on the ciliary transport system, where large velum cilia move collected food particles to the ciliated tract located on the base of the velum, and from there, to the mouth (Yonge, 1926). Accordingly, impairment of velum functionality as observed at the lowest tested concentration in the acute toxicity tests in this study could lead to much lower LC_{50} estimates in chronic tests, due to starvation within a few days in *C. gigas* (His and Seaman, 1992) and *O. edulis* (Millar and Scott, 1967) as well as unintentional sedimentation due to a lack of active swimming behaviour (Finelli and Wethey, 2003, Fuchs et al., 2013).

Besides interspecific differences in metal tolerance in bivalves (Beaumont et al., 1987; Martin et al., 1981; Pavičić et al., 1994; Markich, 2021), the lower absolute sensitivity of *O. edulis* is likely related to its 2.6 times larger size (shell length) at the first larval stage. This may result from *O. edulis* reproduction biology and brooding strategy. *O. edulis* is an incubatory species, whereas *C. gigas* is a broadcast spawner (Stechele et al., 2022). Accordingly larviparous oyster species such as *O. edulis* only cast the sperm in the surrounding water whereas the eggs are fertilized in the mantle cavity of the female oyster by the incurrent stream and protected during the early development for 6–18 days post fertilization until release as D-larvae. On the opposite, the oviparous species *C. gigas* releases the gametes into the surrounding water, where fertilization, embryo and larval development takes place without any internal incubation (Waller, 1981).

The allometric relationships between organism size and toxicant sensitivity has been observed for limnic and marine zooplankton as well as for aquatic insects in the past. A smaller body size was typically associated with a lower tolerance to metal exposure (Koivisto and Ketola, 1995; Vesela and Vijverberg, 2007; Kang et al., 2019; Cadmus et al., 2020). This allometric relationship was also observed in the three main planktonic larval stages of *C. gigas* exposed to mercury, with D-larvae stages (68 μm) being most sensitive, followed by less sensitive umbonate stage (211 μm) and the most tolerant pediveliger stage (310 μm) (Beiras and His, 1994). The higher toxicity of smaller organisms might result from bigger surface-to-volume ratios and a greater weight-specific metabolic rate of smaller organisms increasing the uptake rate of toxins (Krantzberg, 1989; Kiffney and Clements, 1996; Echeveste et al., 2010).

Typically, warmer temperatures lead to a higher solubility of metal salts and solute movement across cell membranes (MacInnes and Calabrese, 1979) as well as an accelerated metabolism and therefore increased uptake, resulting in higher internal metal concentrations and toxicity (Sokolova and Lannig, 2008; Nin and Rodgher, 2021). In contrast to *C. gigas*, the higher LC_{50} ratios (Table 4) of *O. edulis* indicate that exposure to warmer temperatures consistently resulted in significantly reduced relative tolerance to metal exposure. This is in accordance with the general trend of elevated metal toxicity with increasing temperatures (Heugens, 2003; Chapman et al., 2006; Holmstrup et al., 2010). The effect of warming to metal toxicity was most pronounced for Cu, followed by Cd, Pb and Zn (Fig. 5, Table 4) in *O. edulis* D-larvae. *C. gigas* D-larvae response was less clear and differed per trace metal and temperature (Fig.5, Table 4). For Cu and Zn, a lower tolerance was observed at 24 °C, although the temperature-dependent increase in toxicity was less pronounced than in *O. edulis*. In contrast to *O. edulis*, *C. gigas* D-larvae sensitivity to Cd was not temperature-dependent. Interestingly, a higher tolerance of *C. gigas* D-larvae was observed in the Pb treatments at 24 °C relative to 18 °C. The higher relative tolerance of *C. gigas* in comparison to *O. edulis* larvae might be explained by interspecific differences in the width of the thermal tolerance window (Sokolova and Lannig, 2008). While Pacific oyster larvae are known to have a wide thermal window (Ben Kheder et al., 2010) with mortality rates below 10 % at 32 °C (Rico-Villa et al., 2009), larvae of *O. edulis* have an upper temperature limit below this threshold as shown by mortality rates of 84 % at 32.5 °C (Davis and Calabrese, 1969). Amid cumulative stress from various anthropogenic impacts and environmental changes, trace metals act as additional stressors. They pose ecological risks by narrowing the temperature tolerance window,

shifting the upper thermal limit for the studied oyster larvae (Pörtner and Farrell, 2008). This shift is particularly concerning for *O. edulis*, as the temperature-related shifts in LC_{50} ratios (Table 4, Fig. 5) were more pronounced compared to *C. gigas*. With future climate scenarios predicting increased marine heatwaves (Frölicher et al., 2018), conservation and restoration efforts must address the combined risks of heightened pollution and warming on the sensitive larval stages of invertebrates (Dinh et al., 2022). Observed effects range from reduced growth rates and disrupted development to larval death and low recruitment, altogether with the potential for severe impacts also for higher trophic levels and associated species communities. Passive restoration of ecosystems encompasses the identification and reduction of stressors relevant for the target species and/or habitats. Through this mechanism, the reduction of marine pollution including elevated metal concentrations must be addressed to achieve the good environmental status of European Seas and a favourable conservation status in MPAs (Pogoda et al., 2023).

4.3. Hazard mapping

Due to the lower toxicity of the other metals tested compared to Cu, along with their either low environmental concentrations (Pb, Cd) or concentrations well below embryo-larval toxicity thresholds (Zn) (BSH, 2013), HQ was only calculated for Cu and can be seen as worst-case scenario for metal pollution within the group of tested trace metals in the German Bight. The HQ map was generated based on the risk quotients for the less tolerant species (*C. gigas*) based on normal embryo-larval development at 18 °C and larval mortality in the warming scenario.

Overall, the risk of negative effects was only present for embryo-larval development in *C. gigas* but not for D-larvae of both species, as the HQ isoline values were well below critical Cu levels (Fig. 6). Adverse effects on *C. gigas* embryo-larval development in certain areas of the German Bight may serve as a useful proxy for potentially hazardous sites for *O. edulis* restoration, despite the inability to conduct embryo-larval bioassays on *O. edulis* due to its reproductive strategy as a mantle brooder (Waller, 1981).

Although the extent to which the results of *C. gigas* embryo copper tolerance are transferable to *O. edulis* remains largely hypothetical, it is grounded in common ecological reasoning. Based on previous observations of higher metal sensitivity of embryos compared to larvae in marine bivalves such as *C. virginica*, *M. mercenaria* and *C. gigas* (Calabrese et al., 1977; Beiras and His, 1994), we anticipate that the embryos of *O. edulis* are less tolerant to metal pollution than its larval stages. Furthermore, we hypothesize that *O. edulis* embryos might be more tolerant than *C. gigas* embryos, as the diameter of *O. edulis* eggs is 1.7–2.7 times larger (Kim et al., 2010), potentially buffering the lower relative sensitivity to metal exposure and elevated temperatures as observed in larval bioassays of this study. The larger egg size reduces the surface area-to-volume ratio, which is higher in smaller eggs and allows for greater metal uptake, potentially leading to increased toxicity. For example, a comparison of three rotifer species (*Brachionus* spp.) showed higher Cd toxicity in neonates hatched from smaller eggs (Kang et al., 2019). Nevertheless, the establishment of embryo-larval development tests for the Ostreidae oyster family is advised to test this hypothesis.

River outflows of Elbe, Weser and Ems were identified as vectors for Cu input shown by increasing upstream HQ isoline values, as result of strong industrial and urban development along these rivers (Von der Au et al., 2022; Dendievel et al., 2022). Additionally, ports such as Eemshaven, Wilhelmshaven and Bremerhaven as well as marinas were identified as point sources for elevated Cu concentrations, leading to a HQ $\geq$ 1 for embryo-larval development along the East-Frisian Islands (Fig. 6). The surroundings of Bremerhaven port revealed by far the highest Cu concentration. According to Pogoda et al. (2011), the highest mortality of adult *O. edulis* during field experiments in the German Bight was observed at the sampling site Wurster Arm in the Weser estuary, near the port of Bremerhaven. The authors attributed the low survival rates to poor water quality, caused by the presence of contaminants.

Elevated Cu concentrations in the vicinity of harbours and marinas have been reported in the past and are linked to the leakage of antifouling coatings from ship hulls and static harbour structures containing Cu (Moffett et al., 1997; Jones and Bolam, 2007; Turner et al., 2008; Turner, 2010). In contrast, the Wadden Sea of the North Frisian Islands showed a lower Cu contamination with only a single HQ isoline $\geq$1 in the vicinity of Föhr (Fig. 6). HQ isolines decreased with increasing distance from the coast, and offshore areas of the German Bight, including the MPAs Borkum Reefground and Sylt Outer Reef can be seen as favourable habitats for *O. edulis* restoration in terms of Cu impact, on the premise that *O. edulis* embryos are no less tolerant to copper exposure than *C. gigas*. However, nearshore areas in the sphere of river plumes and harbours, especially along the East Frisian islands, might need further investigations when considered as restoration or hatchery areas.

It is important to note that the HQ map offers only a general indication of potentially hazardous areas. In this study and due to the experimental design, only single-metal effects were assessed. In natural environments, organisms are exposed to a cocktail of trace metals (Wu et al., 2016), creating a multi-stressor environment with antagonistic, additive, or synergistic effects (Preston et al., 2000). Cumulative negative effects for oyster embryos and larvae are to be expected as well for other meroplanktic larval stages. Furthermore, Cu bioavailability is heavily influenced by its chemical speciation, which is altered by binding to organic and inorganic ligands and particulates (Hall and Anderson, 1999) and depends on water chemistry factors like pH and salinity (Fairbrother et al., 2007). Additionally, the origin of oysters and maternal effects may play a significant role in the embryos' and larvae's tolerance to metal exposure (Weng and Wang, 2014).

4.4. Study limitations

This study provides important first insights into the effects of trace metals and temperature on early life stages of *O. edulis* and *C. gigas*, but several limitations have to be considered. The use of elevated, nominal metal concentrations was necessary to determine toxicity thresholds but does not reflect environmental conditions, where speciation, complexation, and bioavailability vary. Consequently, actual exposure levels in the field may differ from those used here. The absence of embryo-larval tests for *O. edulis* required reliance on *C. gigas* as a proxy, which introduces uncertainty given differences in reproductive strategies and potential maternal protection. Only acute exposure was tested; chronic and sublethal effects, as well as interactions between multiple pollutants, were not addressed but will be ecologically relevant. Lastly, the use of a single broodstock limits generalizability, as local adaptation may influence metal sensitivity.

5. Conclusion

This study showed that of the four tested metals Cu was the most toxic one for embryos and larvae of both studied oyster species regardless of the temperature. The interspecific comparison demonstrated that *O. edulis* larvae had a higher tolerance to metal exposures than *C. gigas* larvae. However, *O. edulis* was consistently more vulnerable to warming than *C. gigas during metal exposure*. This suggests that D-larvae of native *O. edulis* are more likely to be adversely affected by the combination of ocean warming and metal exposure compared to the non-native *C. gigas*. The hazard map for the German North Sea revealed that the embryo larval development might be affected in estuaries and in the vicinity of ports and marinas. Considering the designated increase of offshore windfarm construction and the industrialized use of these areas, in the future, embryo larval development might be affected also beyond the above-mentioned point sources. Notably, larvae of *C. gigas* showed greater resilience to all metals compared to its embryos. This suggests that the embryonic stage of oysters might represent a critical bottleneck

for reproduction in metal-polluted areas and highlights the urgent need to develop reliable embryo-larval bioassays for the brooding species *O. edulis* to assess the impact of metal pollution and warming on reproduction and recruitment. These bioassays must account not only for potential ontogenetic variation in sensitivity to metals but also for the possible protective role of the maternal environment on developing embryos. Combined with the metal sensitivity data for D-larvae obtained in the present study, assessing embryo sensitivities will help identify suitable areas for ongoing restoration efforts of *O. edulis* in the German Bight and beyond. Furthermore, conducting chronic toxicity tests on larval stages using realistic concentrations is recommended to generate reliable data for environmental risk assessments. Such experiments should also consider the combined and potentially cumulative effects of trace metal mixtures (Manzo et al., 2010; Xu et al., 2011), as well as interactions between metals and other pollutants, such as polycyclic aromatic hydrocarbons (PAHs) (Gauthier et al., 2014; Meynard et al., 2021), pesticides (Bocquené et al., 1995; Filimonova et al., 2018), and microplastics (Lee et al., 2021; Cao et al., 2021), which can result in additive or even synergistic interactions.

CRediT authorship contribution statement

Dominique C. Noetzel: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Bérenger Colsoul:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Farida Akcha:** Writing – review & editing, Investigation, Conceptualization. **Nicolas Briant:** Writing – review & editing, Investigation, Conceptualization. **Jérémy Le Roy:** Writing – review & editing, Investigation, Conceptualization. **Virginie François:** Writing – review & editing, Conceptualization. **Christophe Stavrakakis:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Bernadette Pogoda:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Inna M. Sokolova:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Inna Sokolova was recently involved in preparing a report for the International Council on Mining and Metals (ICMM) Metals Environmental Risk Assessment Guidance (MERAG), entitled *"Environmental Risk Assessment of Metals: Guidance Review and Recommendations to Account for a Changing Climate,"* funded by the International Zinc Association (IZA). However, no financial support from ICMM or IZA was provided for the work described in the current manuscript, and their funding had no influence on the study's design, execution, or findings.

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Data availability

Data will be made available on request.

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