Sediment toxicity testing in German estuaries under different temperature and oxygen conditions using biomarkers in the ragworm *Hediste diversicolor*

Cumulative Dissertation

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Abstract

Human activities release various chemical contaminants into the environment, many of which tend to accumulate in sediments, particularly in estuaries. These contaminants have the potential to cause adverse effects on benthic organisms and estuarine ecosystems, but the likelihood of such effects needs to be characterized. This process, known as ecological risk assessment of sediment contamination, relies on tools such as sediment toxicity testing, among others, and can be done prospectively for sediments facing future contamination or retrospectively for sediments that have already been contaminated. Recently, there has been growing concern about changes in the abiotic environment that may alter the adverse effects of sediment contamination, necessitating the consideration of environmental change in risk assessment. In this context, this dissertation presents three sediment toxicity tests conducted under different temperature and oxygen conditions to contribute to the prospective assessment of sediment contamination by copper and the retrospective assessment of sediment contamination in the Oder and Elbe estuaries. These tests used the ragworm *Hediste diversicolor*, an ecologically important annelid in European estuaries, and focused on biomarkers as early warning signs of toxicity.

Kurzfassung

Durch menschliche Tätigkeiten werden verschiedene chemische Schadstoffe in die Umwelt freigesetzt, von denen sich viele in den Sedimenten, insbesondere in Ästuaren, anreichern. Diese Schadstoffe haben das Potenzial, schädliche Auswirkungen auf benthische Organismen und Ästuarökosysteme zu haben, aber die Wahrscheinlichkeit solcher Auswirkungen muss bewertet werden. Dieser Prozess, der als ökologische Risikobewertung der Sedimentkontamination bekannt ist, stützt sich unter anderem auf Werkzeuge wie Sedimenttoxizitätstests und kann prospektiv für Sedimente, die einer zukünftigen Kontamination ausgesetzt sind, oder retrospektiv für bereits kontaminierte Sedimente durchgeführt werden. In jüngster Zeit gibt es zunehmende Bedenken, dass Veränderungen in der abiotischen Umwelt die schädlichen Auswirkungen von Sedimentkontaminationen verändern könnten, so dass die Berücksichtigung von Umweltveränderungen in der Risikoabschätzung notwendig wird. In diesem Zusammenhang werden in dieser Dissertation drei Sedimenttoxizitätstests vorgestellt, die unter verschiedenen Temperatur- und Sauerstoffbedingungen durchgeführt wurden, um zur prospektiven Bewertung der Sedimentkontamination durch Kupfer und zur retrospektiven Bewertung der Sedimentkontamination im Oder- und Elbeästuar beizutragen. Die Tests wurden mit dem Schillernden Seeringelwurm *Hediste diversicolor*, einem ökologisch wichtigen Ringelwurm in den europäischen Ästuaren, durchgeführt und konzentrierten sich auf Biomarker als Frühwarnindikatoren für Toxizität.

List of abbreviations

ADME	Absorption, distribution, metabolism, and excretion
ADP	Adenosine diphosphate
AEC	Adenylate energy charge
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATP7A	ATPase copper-transporting alpha
CAR	Carbohydrates
CAT	Catalase
CEA	Cellular energy allocation
CES	Carboxylesterase
CITS	Climate-induced toxicant sensitivity
CPR	Cytochrome P450 reductase
ERA	Ecological risk assessment
ETS	Electron transport system
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione transferase
LIP	Lipids
LOE	Line of evidence
LOEC	Lowest observed effect concentration
MDA	Malondialdehyde
MGO	Methylglyoxal
MT	Metallothionein
NADPH	Nicotinamide adenine dinucleotide phosphate
NOEC	No observed effect concentration
PAH	Polycyclic aromatic hydrocarbon
PC	Protein carbonyls
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
PRO	Proteins
PUFA	Polyunsaturated fatty acid
RCS	Reactive carbonyl species
ROS	Reactive oxygen species
SOD	Superoxide dismutase

SPM	Suspended particulate matter
SQG	Sediment quality guideline
SQT	Sediment quality triad
SSD	Species sensitivity distribution
TAC	Total antioxidant capacity
WOE	Weight of evidence

1 The big picture

1.1 Sediment contamination in estuaries

Humans tend to settle in river basins and coastal areas, which provide abundant natural resources and facilitate trade and transportation (Fang et al., 2018). However, human development in these areas has placed multiple and increasing pressures on aquatic ecosystems (Ceola et al., 2019; Freeman et al., 2019). One of the greatest pressures is the release of chemical contaminants into the environment (Borgwardt et al., 2019; Persson et al., 2022). These include various substances intentionally synthesized for industrial and other applications (e.g., agriculture, medicine, and food production), unintended by-products of manufacturing and combustion processes, and naturally occurring substances (e.g., trace metals) that are concentrated by human activities (EFSA, 2014; Wang et al., 2020). They enter surface waters through multiple emission pathways, including point sources (e.g., wastewater treatment plants) and diffuse sources (e.g., agricultural runoff) (Fuchs et al., 2010; Holt, 2000). Many contaminants are resistant to degradation (Cousins et al., 2019; Zacharia, 2019) and tend to adsorb to suspended particulate matter (SPM), including fine-grained mineral and organic particles that can be deposited as sediments (Burton, 2002; Salomons and Brils, 2004). The adsorption and deposition are particularly enhanced in estuaries due to unique conditions such as increased salinity (salting-out effect), turbulent mixing, and reduced water flow, leading to the accumulation of contaminants in sediments (Chapman and Wang, 2001; Turner, 2003).

Sediment contamination is a hazard (i.e., has the potential to cause adverse effects) to estuarine ecosystems (Chapman, 2007). Among estuarine biota, benthic organisms living in or on the bottom are most frequently exposed to contaminated sediments (Pinto et al., 2009). Uptake of contaminants can occur through ingestion of sediment particles and direct contact (e.g., via skin or gills) with pore water and overlying water (Holt, 2000; Wang and Fisher, 1999). Contaminants can bioaccumulate in benthic organisms and be transferred through the food web to higher trophic level species, such as birds and mammals (Salomons and Brils, 2004). In addition, human activities (e.g., dredging and disposal of dredged material) and natural events (e.g., storms and floods) can disturb estuarine sediments, remobilizing contaminants and making them available for uptake by a wider range of species (Crawford et al., 2022; Roberts, 2012). Once in the body, contaminants or their metabolites can interact with molecular targets or alter the microenvironment, causing cellular dysfunction and injury (Lehman-McKeeman, 2019). Without appropriate repair and adaptation, organisms can suffer a variety of toxic effects, including mortality or impaired growth, development, and reproduction (Di Giulio and Newman, 2019; Newman, 2015). These organismal effects can cascade to higher biological organization levels (i.e., populations and communities) (Clements, 2000; Fleeger et al., 2003), resulting in negative effects on entire estuarine ecosystems and a decline in their ecosystem services (Barbier et al., 2011). Given these potential scenarios, assessing the risk (i.e., the likelihood of adverse effects) associated with sediment contamination in estuaries is critical to inform management decisions (Apitz, 2011; Ausili et al., 2022).

1.2 Ecological risk assessment of sediment contamination

Risk assessment of sediment contamination often follows the ecological risk assessment (ERA) framework (Apitz, 2011; Tarazona et al., 2014). In general, ERA evaluates the risk of adverse effects on ecological receptors (e.g., populations, communities, or ecosystems) from exposure to environmental hazards (or stressors), including chemical contaminants (Chapman, 2016; Di Giulio and Newman, 2019). An ERA has three main steps: problem formulation, exposure and effects analysis, and risk characterization. Briefly, problem formulation aims to specify the stressors of concern and the ecological receptors to be protected. Exposure and effects analysis determines exposure concentrations and describes exposure-effect (dose-response) relationships. Based on these results, risk characterization estimates the risk and associated uncertainties (Chapman, 2016; Di Giulio and Newman, 2019).

ERA of sediment contamination can be prospective or retrospective (Diepens et al., 2014; Tarazona et al., 2014) (Figure 1). Prospective ERA addresses the risk from new or existing chemicals that may lead to future sediment contamination and is often applied in the context of chemical or product registration and authorization (Diepens et al., 2017; Diepens et al., 2014). In contrast, retrospective ERA focuses on the risk from known or suspected contaminated sediments and is often used to monitor and manage specific sites or areas (Bruce et al., 2020). This difference in goals leads to significant differences in their implementation, especially in terms of tools (Di Giulio and Newman, 2019; Tarazona et al., 2014).

Specifically, a typical prospective ERA addresses a single chemical at a time (Beyer et al., 2014; Diepens et al., 2017). In the exposure analysis, fate models are often used to obtain the predicted environmental concentration (PEC) of the chemical in sediments (Amiard and Amiard-Triquet, 2015; Koelmans et al., 2015). In the effects analysis, sediment toxicity tests and species sensitivity distribution (SSD) models are the main tools to derive the predicted no-effect concentration (PNEC), i.e., the concentration below which no adverse effects are expected (Diepens et al., 2017). The PEC/PNEC ratio (risk quotient) is then used to characterize the risk (Amiard and Amiard-Triquet, 2015).

Meanwhile, a typical retrospective ERA deals with field sediments, which usually contain a mixture of contaminants (Ankley and Mount, 1996). The exposure and effects analysis often involves the investigation of multiple lines of evidence (LOE), and the risk characterization integrates them through a weight of evidence (WOE) approach (Bruce et al., 2020; Chapman, 2016). A notable example is the sediment quality triad (SQT), which consists of three LOEs, namely sediment chemistry, sediment toxicity, and benthic ecology (Chapman, 1990; Chapman and McDonald, 2005). The first LOE uses chemical analyses to measure contaminant concentrations and compares them with benchmarks such as sediment quality guidelines (SQG) (Birch, 2018; Burton, 2002). The second LOE evaluates the potential combined effects of contaminants in sediments experimentally using sediment toxicity tests (Ankley and Mount, 1996; Burgess et al., 2013). The third LOE assesses the realized effects based on field

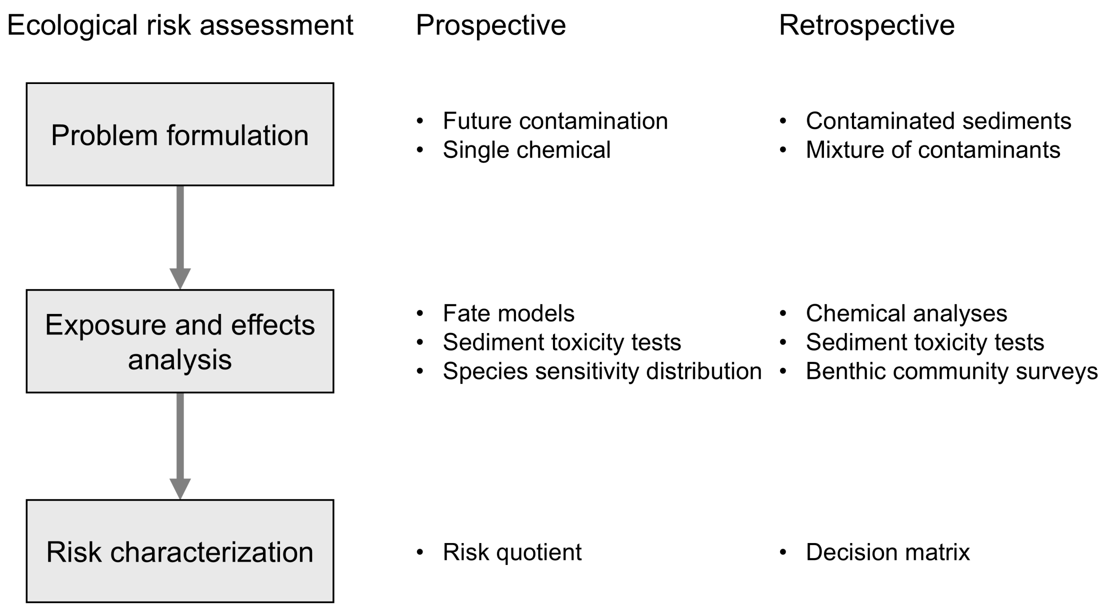


Figure 1: Comparison of prospective and retrospective ecological risk assessment of sediment contamination.

observations (surveys) of the benthic community (Pinto et al., 2009). A decision matrix, which is a simple form of WOE, is then used to make a conclusion about the risk (Chapman, 2007).

1.3 Incorporating environmental change into risk assessment

Changes in the abiotic environment can occur naturally, but can also be accelerated by human activities, including the release of chemical contaminants. A notable example is greenhouse gases (e.g., carbon dioxide, methane, nitrous oxide, and fluorinated gases), which trap heat in the atmosphere and raise the Earth's temperature (Lashof and Ahuja, 1990). This warming leads to changes in other climate variables such as humidity, wind, and precipitation (Mitchell et al., 2006). As a result, surface water parameters such as temperature, dissolved oxygen, and salinity are also affected (Stockmayer and Lehmann, 2023). Another example is macronutrients (e.g., nitrogen and phosphorus), which cause eutrophication of aquatic ecosystems, leading to algal blooms and subsequent oxygen depletion (i.e., hypoxia) (Conley et al., 2009; Diaz, 2001). Although these contaminants are often outside the scope of ERA, the widespread changes in environmental conditions that they cause can influence the exposure to and effects of other contaminants being assessed (Landis et al., 2013; Stahl et al., 2013) (Figure 2).

Specifically, changes in exposure can result from changes in the emission or fate (i.e., transport and transformation) of contaminants. For example, global warming can alter the distribution of arable land and the occurrence of plant pathogens, affecting the use and release of pesticides (Gouin et al., 2013; Roos et al., 2011). In addition, extreme rainfall events can

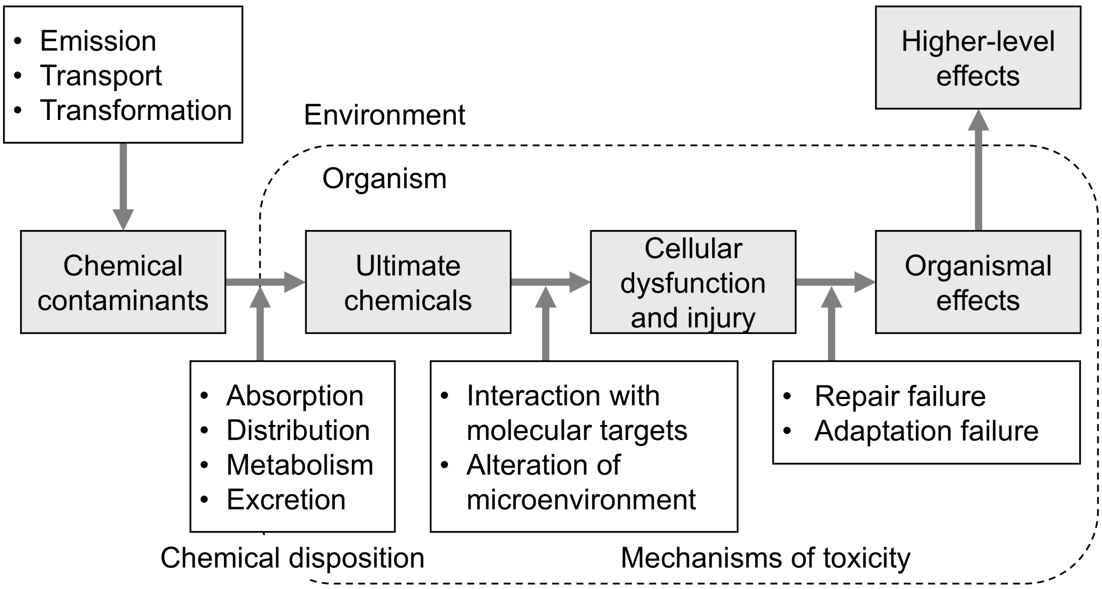


Figure 2: Processes involved in the exposure to and effects of chemical contaminants. Inspired by Lehman-McKeeman (2019) and Jager et al. (2011).

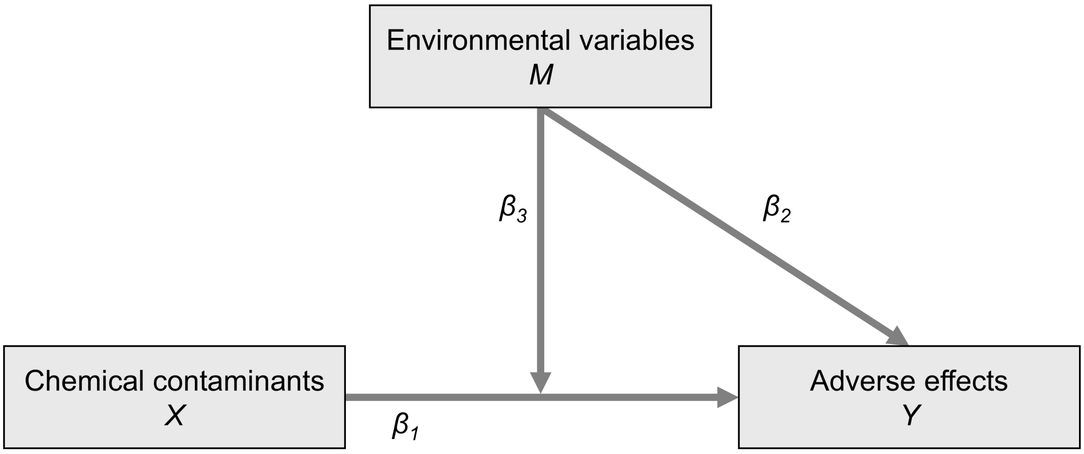
increase agricultural runoff and pesticide inputs to surface waters (Schiedek et al., 2007). The degradation of these pesticides can be enhanced by elevated temperatures, but inhibited under hypoxic conditions (Noyes et al., 2009; Vink and van der Zee, 1997).

As a bridge between exposure and effects, the bioaccumulation of contaminants can also be influenced through changes in chemical disposition (toxicokinetic processes), i.e., absorption/uptake, distribution, metabolism/biotransformation, and excretion/elimination (ADME) (Lehman-McKeeman, 2019; Newman, 2015). For instance, elevated temperatures often increase the uptake rate of trace metals in aquatic organisms, but may have less effect on the elimination rate, leading to increased net metal accumulation (Sokolova and Lannig, 2008). The biotransformation of organic contaminants also tends to increase at higher temperatures, which may increase or decrease toxicity depending on the nature of the resulting metabolites (Kennedy and Walsh, 1997; Meynet et al., 2020).

Effects on organisms can be modified through changes in mechanisms of toxicity (toxicodynamic processes), such as interactions with molecular targets and repair mechanisms (Lehman-McKeeman, 2019). For example, pyrethroid pesticides can bind to voltage-gated sodium channels in neurons and prolong their open state, leading to excessive sodium influx and nerve hyperexcitation (Costa, 2019). Higher temperatures can speed up the recovery of sodium channels, thereby reducing pyrethroid toxicity (Harwood et al., 2009).

Effects at the population and community levels can also be affected by environmental change (Moe et al., 2013). As an example mechanism, the genetic adaptation to higher temperatures may come at the cost of reduced tolerance to contaminants (i.e., an evolutionary trade-off). Therefore, global warming may increase the proportion of thermotolerant taxa in benthic communities, but these communities may be more sensitive to subsequent contaminant exposure (Sinclair et al., 2024).

In the above examples, environmental variables can be considered moderators of exposure-effect relationships, potentially enhancing or reducing the adverse effects of contaminants (Figure 3). Because many environmental variables are strongly influenced by global climate change, this moderation is sometimes referred to as climate-induced toxicant sensitivity (CITS) (Hooper et al., 2013). In general, failure to account for environmental change can lead to under- or overestimation of the risk from chemical contaminants (Heugens et al., 2001; Holmstrup et al., 2010). Unfortunately, this issue is still often overlooked in ERA, including the ERA of sediment contamination (Bruce et al., 2020; Stahl et al., 2024).



$$E(Y \mid X, M) = \beta_0 + (\beta_1 + \beta_3 M)\, X + \beta_2 M = \beta_0 + \beta_1 X + \beta_2 M + \beta_3 (X \times M)$$

Figure 3: Conceptual diagram of a moderation model in which the adverse effects (Y) of chemical contaminants (X) are moderated by environmental variables (M). The moderation effect is captured by an interaction term $(\beta_3 XM)$ in a non-additive linear model. Only in the absence of this interaction effect (i.e., in an additive linear model) does the main effect of contaminants $(\beta_1 X)$ have a meaningful interpretation. The main effect of environmental variables $(\beta_2 M)$ is often not of interest.

2 An experimental approach

2.1 Sediment toxicity testing

Sediment toxicity testing is an important tool in the ERA of sediment contamination. In these tests, organisms are exposed to sediments under controlled conditions and their biological responses are measured (Simpson et al., 2016). Corresponding to prospective and retrospective ERA, prospective toxicity tests use formulated or natural sediments artificially spiked with the contaminants of concern, whereas retrospective toxicity tests are performed on natural sediments collected from the areas of interest (Leppanen et al., 2024). Despite this difference, both types of tests share similar considerations regarding key components such as test matrices, test organisms, test endpoints, and environmental conditions (ASTM, 2021) (Figure 4).

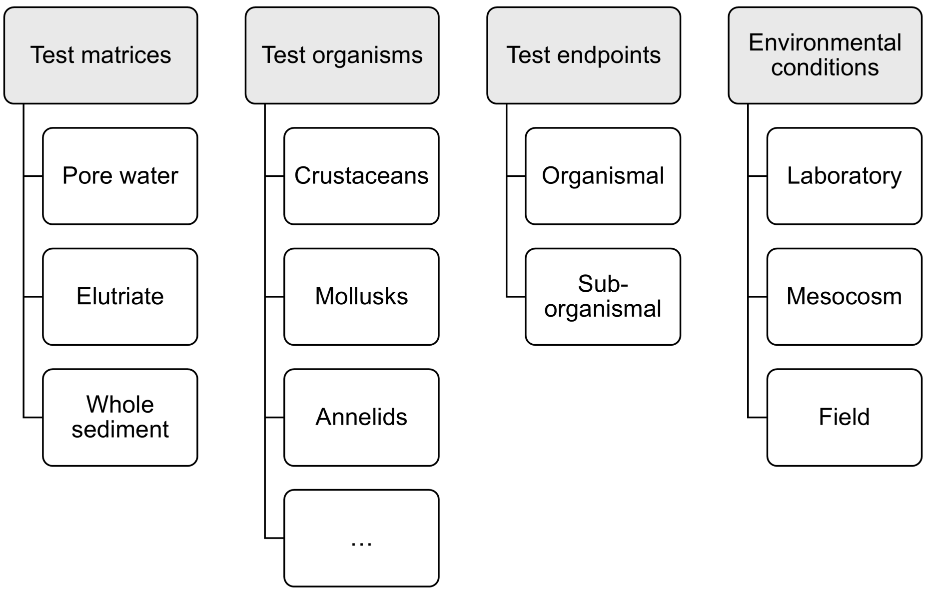


Figure 4: Multiple options for constructing sediment toxicity tests.

Specifically, sediment toxicity tests are often conducted on three matrices: pore water, elutriate, or whole sediment (Apitz, 2011; Harkey et al., 1994). Pore water occupies the interstitial spaces between sediment particles and can be extracted by various methods such as centrifugation or compression (Ankley and Schubauer-Berigan, 1994), whereas elutriate is obtained by mixing sediments with water and extracting the supernatant (overlying water) after the mixture has settled (Sartori et al., 2021). While pore water testing assumes that pore water is the primary route of exposure to sediment-associated contaminants (Chapman et al., 2002b), elutriate testing simulates the release of contaminants into the water column following sediment disturbance events (Sartori et al., 2021). In contrast to these liquid phase tests, whole sediment tests directly use bulk sediment samples to provide realistic exposure through multiple routes (i.e., sediment particles, pore water, and overlying water) (Diepens et al., 2014; Harkey et al., 1994). Regardless of the matrices used, toxicity tests must include control or reference sediments, ideally uncontaminated, to establish baseline values for test endpoints. This is straightforward in prospective testing, where the control sediment undergoes the same spiking procedure but without the addition of contaminants (Grønlund et al., 2024). However, identifying suitable reference sediments for retrospective testing is more challenging (Chapman et al., 2002a; Chapman and Wang, 2001). These sediments are often collected from nearby sites with lower levels of contamination or from the site where the test organisms were obtained (Simpson et al., 2016).

In toxicity testing, test organisms serve as surrogates for the ecological receptors to be protected (USEPA, 1994). To date, many test organisms of different habitats and taxonomic groups have been used (Simpson et al., 2016). Although early sediment toxicity tests used pelagic species exposed to pore water or elutriate, tests with benthic species using whole sediments have gained popularity (Diepens et al., 2014). Currently, whole sediment tests tend to favor crustaceans, especially amphipods, in part because of their presumed high sensitivity to contaminants (Chapman et al., 2013; Chapman and Wang, 2001). Tests using other invertebrates, such as mollusks and annelids, and those using microorganisms and sediment-rooted macrophytes are underrepresented (Diepens et al., 2014; Simpson et al., 2016).

Traditional toxicity tests (bioassays) typically measure endpoints at the organismal level, including fitness traits such as survival, growth, and reproduction (Simpson et al., 2016; USEPA, 1994). While these organismal endpoints are of high ecological relevance, toxic effects often take time to manifest at this level (Adams et al., 1989; Newman, 2015). Therefore, there is increasing interest in sub-organismal endpoints (biomarkers) as early warning signs of toxicity (Hagger et al., 2006; Hampel et al., 2016). A single toxicity test may use multiple biomarkers, and with advances in omics technologies, the number of measured endpoints can be very large (Gonzalez and Pierron, 2015; Pham and Sokolova, 2023).

Most sediment toxicity tests are conducted with a single species under laboratory conditions (Simpson et al., 2016), where environmental variables such as salinity, temperature, dissolved oxygen, and pH are usually kept relatively constant and within the optimal ranges of the test species (ASTM, 2021; Heugens et al., 2001). However, these tests do not account for the biotic and abiotic complexity of natural environments such as estuaries (Chapman and Wang, 2001; Elliott and Quintino, 2007). In contrast, multi-species mesocosm and field toxicity tests provide greater ecological realism, but are less common due to high costs (Alexander et al., 2016; Amiard-Triquet, 2015).

This dissertation focuses on whole sediment laboratory tests using biomarkers in an annelid, the ragworm *Hediste diversicolor*.

2.2 Ragworm *Hediste diversicolor* as a test organism

The ragworm *Hediste diversicolor* is a species in the family Nereididae (order Phyllodocida, class Polychaeta, phylum Annelida) (Wilson et al., 2023) (Figure 5). They are common in European estuaries, including those along the Atlantic coast and in the Baltic, Mediterranean, Black, and Caspian Seas (OBIS, 2023; Teixeira et al., 2022). They often inhabit shallow water sediments with densities up to several thousand individuals per square meter (Beyer and Sundt, 2006; Scaps, 2002). Their wide distribution and high abundance make them readily available for laboratory studies (Medeiros Aguiar et al., 2023).

Figure 5: Ragworms *Hediste diversicolor* from the Warnow Estuary, Germany.

Except for a brief pelagic larval period, ragworms spend most of their lives, which can last up to three years, as endobenthic organisms (Scaps, 2002). They build U- or Y-shaped burrows (with two openings at the sediment surface) and actively ventilate them by undulating body movements (Budd, 2008; Kristensen et al., 2012). Ragworms can partially crawl out of burrows to obtain food, including sediments (deposit feeding) and plants or animals (grazing, scavenging, or predation) (Zhu et al., 2016). They can also secrete and consume mucus nets that trap suspended particles in the water flowing through the burrows (filter feeding) (Riisgård, 1994). These bioturbation activities and feeding habits expose ragworms to contaminants through multiple routes, making them highly relevant for sediment toxicity testing (Simpson et al., 2016).

Ragworms are found in a variety of muddy and sandy sediments (Van Colen et al., 2014; Wiesebron et al., 2021) and can tolerate a wide range of salinity (Röhner et al., 1997), temperature (Fernandes et al., 2023), and oxygen (Budd, 2008). This tolerance facilitates their maintenance in the laboratory and allows toxicity testing of different sediment types under different environmental conditions of interest (Chapman and Wang, 2001; Simpson et al., 2016).

Ragworms are also an ecological receptor that needs to be protected in European estuaries. Their bioturbation activities influence microbial communities and biogeochemical processes in sediments (Laing et al., 2022). They are an important part of the estuarine food web, being the main prey of many crustaceans, fish, and birds (e.g., brown shrimp, common goby, and dunlin) (Rosa et al., 2008; Scaps, 2002). Ragworms also have potential in aquaculture, particularly for recycling fish waste and producing sustainable aquafeed (Wang et al., 2019). All these characteristics make ragworms an ideal test organism for sediment toxicity testing.

2.3 Biomarkers as test endpoints

Originating in medicine, biomarkers are objective, measurable indicators of biological states or disease processes (Strimbu and Tavel, 2010). They are often used as surrogates for clinical endpoints that directly reflect how patients feel, function, or survive (Califf, 2018). For example, stroke and heart attack are common clinical endpoints in cardiovascular trials, while blood pressure and low-density lipoprotein are popular biomarkers (Desai et al., 2006). The use of biomarkers has also expanded to other fields, including ecotoxicology (Van Gestel and Van Brummelen, 1996). Here, biomarkers refer to sub-organismal (e.g., molecular, biochemical, or cellular) endpoints that indicate the exposure to or effects of chemical contaminants (Lam and Gray, 2003). Similarly, they often serve as surrogates for ecologically relevant endpoints at the organismal level, such as fitness traits (Forbes et al., 2006).

Biomarkers in ecotoxicology can be classified in many ways. Traditionally, they are broadly divided into biomarkers of exposure (those related to chemical disposition) and biomarkers of effects (those related to mechanisms of toxicity) (Hook et al., 2014; Peakall and Shugart, 1993) (Figure 2). More recent views, however, favor a classification into biomarkers of defense, which reflect protective mechanisms, and biomarkers of damage, which capture resultant toxicities (Colas and Le Faucheur, 2024; de Lafontaine et al., 2000). It is also common to group biomarkers according to the more specific biological processes in which they are involved (Hampel et al., 2016; Mouneyrac and Amiard-Triquet, 2013). This dissertation focuses on three such groups: biomarkers of detoxification and antioxidant defense, biomarkers of oxidative and carbonyl stress, and biomarkers of energy metabolism.

Detoxification refers to processes that reduce the toxicity of contaminants. For organic chemicals, detoxification is often associated with metabolism (biotransformation) as part of chemical disposition (Lehman-McKeeman, 2019; Newman, 2015). Specifically, lipophilic chemicals that are readily absorbed into the cell can be converted into easily excreted hydrophilic metabolites by Phase I (hydrolysis, reduction, and oxidation) and Phase II (conjugation) enzymes (Parkinson et al., 2019). Examples of biotransformation enzymes commonly used as biomarkers are carboxylesterases (CES), which hydrolyze many organophosphate, carbamate, and pyrethroid pesticides (Wheelock et al., 2005), and glutathione transferases (GST), which conjugate the tripeptide glutathione (GSH) to various chemicals, such as reactive metabolites of polycyclic aromatic hydrocarbons (PAH) (Shimada, 2006). For trace metals, detoxification often relies more on excretion and distribution away from sensitive targets by metal transporters and metal-binding proteins (Ufelle and Barchowsky, 2019). For example, excess copper (Cu) in the cell can be sequestered by small, cysteine-rich metallothioneins (MT) and MT-like proteins, or actively exported by the transmembrane ATPase copper-transporting alpha (ATP7A) (Chen et al., 2022).

A common consequence of contaminant exposure is the increased generation of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) (Newman, 2015). This can occur through various mechanisms, including the redox cycling of organic chemicals by Phase I cytochrome P450 reductases (CPR) or the Fenton reaction catalyzed by trace metals (Lehman-McKeeman, 2019). To detoxify ROS, the cell produces enzymes such as superoxide dismutases (SOD), catalases (CAT), glutathione peroxidases (GPx), and glutathione reductases (GR), as well as antioxidants such as GSH and vitamins A, C, and E (Di Giulio and Newman, 2019). GPx, for example, catalyzes the redox reaction between H_2O_2 and GSH, producing water and glutathione disulfide (GSSG). The GSSG formed is then recycled back to GSH via the GR-catalyzed reaction with the reducing agent NADPH (Couto et al., 2016). The cumulative effect of all these enzymatic and non-enzymatic components in ROS neutralization is often referred to as total antioxidant capacity (TAC) (Regoli et al., 2002).

The imbalance between ROS production and antioxidant defense can lead to oxidative stress. Specifically, ROS can interact with polyunsaturated fatty acids (PUFA) in cell membranes, initiating lipid peroxidation, a chain reaction that generates many radical intermediates and degrades membrane lipids into hydrocarbons and reactive carbonyl (C=O) species (RCS) (Halliwell and Chirico, 1993). Two examples of RCS are malondialdehyde (MDA) and methylglyoxal (MGO), which are structural isomers ($CH_2(CHO)_2$ and CH_3COCHO, respectively). While MDA is a highly specific biomarker of lipid peroxidation (Del Rio et al., 2005), MGO can also be generated by many other pathways, including glycolysis and lipid, protein, and ketone metabolism (Lai et al., 2022; Semchyshyn, 2014). A metabolic shift to glycolysis, which often occurs under stress conditions, can result in increased MGO formation (Kaur et al., 2014). Both ROS and RCS can interact with protein side chains (e.g., proline, arginine, lysine, and threonine residues) and irreversibly introduce carbonyl groups, i.e., protein carbonyls (PC). The carbonylated proteins often lose their functions and form aggregates, contributing to cellular dysfunction and injury (Dalle-Donne et al., 2003; Rodríguez-García et al., 2020).

Exposure to contaminants often increases cellular energy demand due to the high energetic cost of defense and repair mechanisms (Calow, 1991; Mouneyrac and Amiard-Triquet, 2013). For example, in the case of protein carbonylation, the cell requires ATP to produce and activate the proteasome and Lon protease, which degrade damaged proteins and thereby mitigate aggregation (Ngo et al., 2013; Nyström, 2005). Cellular energy demand can be reflected in the activity of the mitochondrial electron transport system (ETS), which drives ATP synthesis through oxidative phosphorylation (Fanslow et al., 2001). In addition, increased energy demand can lead to depletion of cellular energy reserves, including carbohydrates, lipids, and proteins (CAR, LIP, and PRO) (Sokolova et al., 2012). The relationship between the energy available in these reserves and the rate of energy expenditure proxied by ETS activity is often summarized by a composite index known as cellular energy allocation (CEA) (De Coen and Janssen, 1997; Verslycke et al., 2004). Another important index of cellular energy status is adenylate energy charge (AEC), which represents the usable energy stored in the adenylate pool consisting of ATP, ADP, and AMP (Atkinson and Walton, 1967). Increased ATP consumption under contaminant exposure can shift the pool toward ADP and AMP, thereby lowering the AEC (Picado and Le Gal, 1999).

3 Three case studies

3.1 Copper as a re-emerging contaminant

Copper is one of the oldest known contaminants, with records of localized contamination dating back to the Late Neolithic (c. 5000 BC) (Grattan et al., 2016). However, copper contamination only became a widespread problem after the Industrial Revolution (18th-19th centuries), driven by massive copper production and coal burning (Cunha, 2020; Evans and Saunders, 2015). Since their peak in the 20th century, copper concentrations in European river sediments have generally declined as a result of environmental measures such as reduced dust emissions and improved wastewater treatment in metallurgical plants (Ciszewski, 2003; Middelkoop, 2002). In recent years, however, there have been reports of increasing copper concentrations in coastal sediments, particularly in areas near ports and marinas (Larsen and Fryer, 2016). This increase has been attributed to copper leaching from antifouling paints on boat hulls, which is currently the main emission source of copper in European coastal waters (Comber et al., 2023). Previously, antifouling paints relied on tributyltin as the primary biocide, but following its global ban due to high toxicity two decades ago, copper compounds such as cuprous oxide (Cu_2O) and cuprous thiocyanate (CuSCN) have become the dominant alternatives (Brooks and Waldock, 2009). Cu^+ ions released from these compounds oxidize rapidly in seawater to Cu^{2+} ions, which can then bind to SPM and settle in sediments. In Germany, copper-based antifouling paints are subject to authorization, which requires a prospective ERA of water and sediment contamination to be conducted (Daehne et al., 2017; UBA, 2020).

3.2 Oder and Elbe estuaries as contamination hotspots

The Oder (Odra) and the Elbe (Labe) are two major rivers in Central Europe. Both originate in the Czech Republic, with the Oder River flowing into the Baltic Sea near the German-Polish border and the Elbe River flowing into the North Sea in Germany. The Oder River has a catchment area of almost 120 000 km^2, 90% of which is in Poland (Glasby et al., 2004), while the Elbe River has a catchment area of nearly 150 000 km^2, about two-thirds of which is in Germany (Netzband et al., 2002). Both river basins have long experienced extensive urban, industrial, and agricultural activities (Förstner et al., 2004; Müller et al., 2002). The Oder River, for example, passes through the Wroclaw metropolitan area, the metal mining region of Glogow, and the winegrowing region of Zielona Góra (Ciszewski, 2003; Schernewski et al., 2008). Similarly, the Elbe River runs through major industrial cities such as Dresden and Hamburg, as well as fertile agricultural regions such as the Magdeburg Börde (Netzband et al., 2002; Wollschläger et al., 2016). As a result of human activities, the sediments of the Oder and Elbe estuaries have been contaminated with various trace metals and organic chemicals (Müller and Heininger, 1999;

Wetzel et al., 2013). In this context, a retrospective ERA of sediment contamination in these estuaries is needed.

3.3 Research objectives and organization

This dissertation aims to contribute to the ERA of sediment contamination in the above cases through sediment toxicity tests using biomarkers in the ragworm *Hediste diversicolor*. Specifically, the following three toxicity tests were performed:

- A prospective test of copper-spiked sediments (**Publication 1**, Pham et al., 2023).
- A retrospective test of sediments from the Oder Estuary (**Publication 2**, Pham et al., 2024).
- A retrospective test of sediments from both the Oder and Elbe estuaries (**Publication 3**, Pham et al., 2025).

All these tests used ragworms from a population in the Warnow Estuary in Germany (site W1, Figure 6). In the first test, surface sediments were collected from the coast of the Warnow Estuary (site W0) and spiked with copper at concentrations of 0 (control), 10, and 20 mg kg^{-1}. In the second test, surface sediments were sampled at eight sites (O1 to O8) in three regions of the Oder Estuary, i.e., the Pomeranian Bay (reference), the Peenestrom, and the Szczecin Lagoon. In the third test, surface sediments were sampled at site W1 (reference), sites O3 to O8 (except O5), and six sites (E1 to E6) in two regions of the Elbe Estuary, i.e., the Transitional Elbe and the Limnic Elbe. The selection of reference sediments in the two retrospective tests was guided by sediment chemical analyses (e.g., Figure 7).

In an effort to incorporate environmental change into the ERA, each sediment toxicity test was conducted under two environmental conditions (Figure 8). Specifically, the first and second tests were conducted under normal (12 or 10 °C) and elevated (20 °C) temperatures. The third test was conducted under normal and reduced dissolved oxygen conditions (continuous aeration and intermittent aeration). Each test lasted for three weeks (i.e., long-term exposure), after which the ragworms were retrieved for biomarker measurements (Figure 9).

In the statistical analyses of biomarker responses, the moderation effects of temperature or oxygen were assessed using the interaction terms in non-additive linear models, which quantify the interaction effects between sediment and temperature or oxygen (Figure 3). When no interaction effect was evident, additive linear models were used to assess the main effects of sediment. For brevity, the analyses in the retrospective tests presented here focus on estuarine regions rather than individual sampling sites.

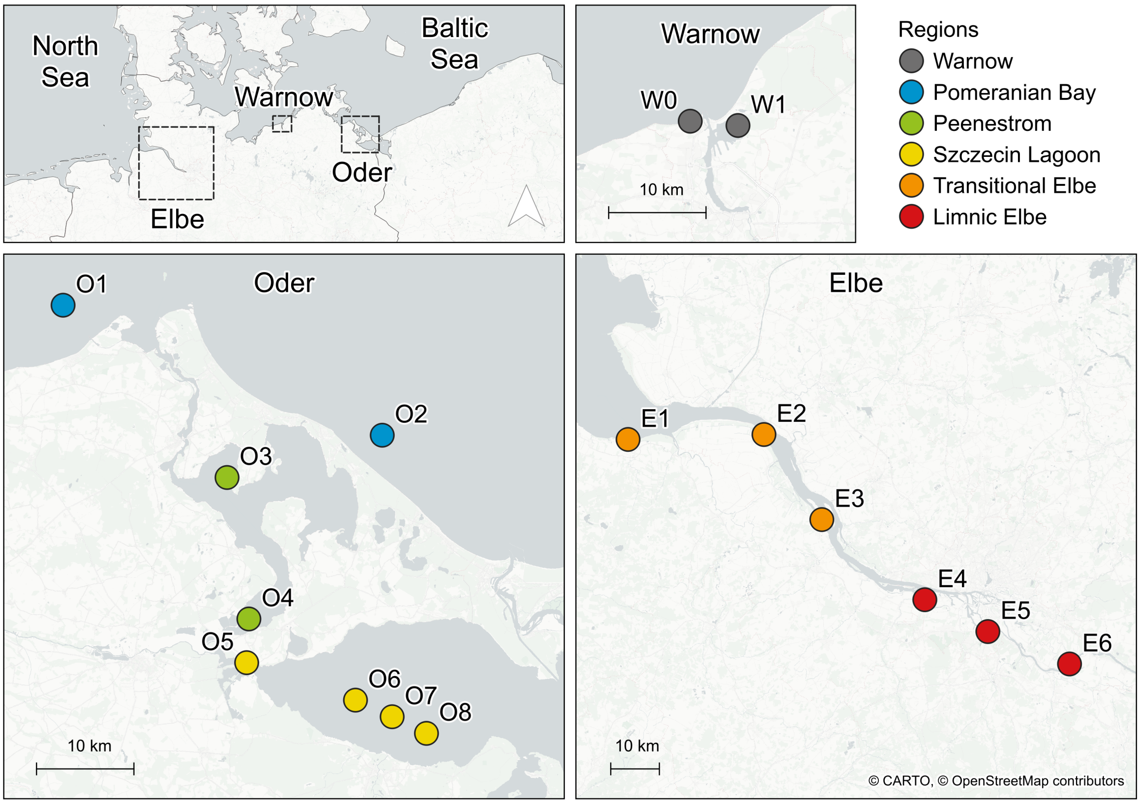


Figure 6: Sediment sampling sites in the Warnow, Oder, and Elbe estuaries. Adapted from Pham et al. (2025).

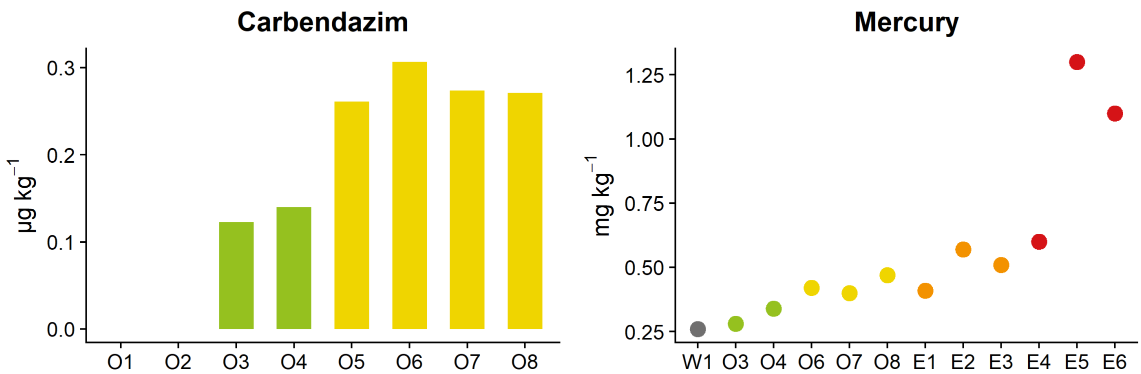


Figure 7: Concentrations of two representative contaminants (a pesticide and a trace metal) in the collected sediments. Adapted from Pham et al. (2024) and Pham et al. (2025).

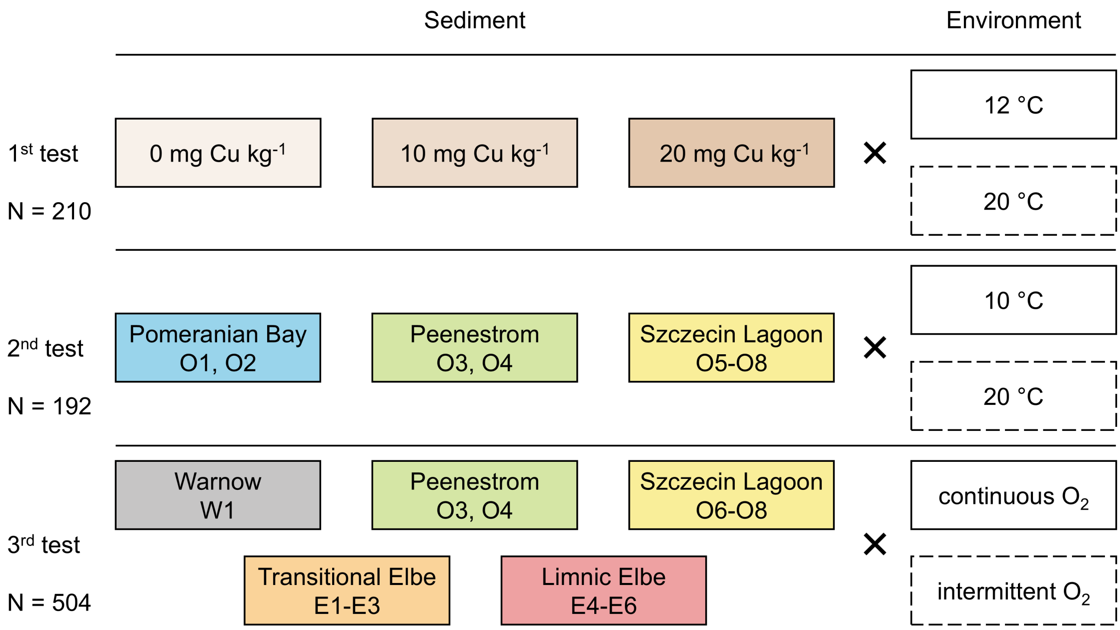


Figure 8: Experimental design for the sediment toxicity tests.

	Detoxification and antioxidant defense	Oxidative and carbonyl stress	Energy metabolism
1st test	MT, ATP7A	MDA, MGO, PC	CAR, LIP, PRO, ETS
2nd test	GST, GR, TAC	MDA, MGO	CAR, LIP, PRO, ETS
3rd test	CES, GST, GR, TAC	MDA, MGO, PC	CAR, LIP, PRO, ETS, AEC

Figure 9: Biomarkers used in the sediment toxicity tests. Those significantly affected by sediment exposure, either through interaction effects or main effects, are colored. Those showing interaction effects are further underlined.

4 Findings and reflections

4.1 Positive and negative results

Moderation effects of temperature or oxygen were rarely detected in the toxicity tests (Figures 9 to 16). One of the few instances where such effects were evident was in the expression of genes encoding MT and ATP7A in the test of copper-spiked sediments (Figure 10). The gene expression was not significantly altered by copper exposure at normal temperature, but was upregulated at elevated temperature, suggesting an increased need for copper sequestration and export, likely in response to higher copper uptake. Evidence for moderation effects was also found in the reserves of CAR and PRO in the third toxicity test (Figure 16). Specifically, exposure to sediments from the Oder and Elbe estuaries appeared to increase the content of CAR under normal oxygen, but decrease it under reduced oxygen. This could be explained by the higher availability of food (i.e., organic matter) in these contaminated sediments, which may compensate for the contaminant-induced CAR depletion (Mouneyrac et al., 2010; Pham et al., 2025). However, this compensation may not occur under limited oxygen supply, which can suppress feeding or metabolic activity (Sokolova, 2021). Sediment exposure also significantly increased the content of PRO under normal oxygen, but not under reduced oxygen. While a similar explanation as for CAR may apply, the increase of PRO content may also reflect the increased synthesis of detoxification enzymes or stress proteins (Ruffin et al., 1994).

Consistent effects of sediment exposure at different temperatures or oxygen conditions were observed more frequently in the toxicity tests (Figure 9). For example, all tests provided evidence for the main effects of sediment on MGO accumulation. In the two retrospective tests, MGO content increased significantly in response to sediments from the Szczecin Lagoon, the Transitional Elbe, and the Limnic Elbe (Figures 12 and 15). These results indicate substantial carbonyl stress induced by sediment exposure, probably due to increased glycolytic flux or impaired MGO detoxification via the glyoxalase system (Thornalley, 1990). Surprisingly, in the prospective test, copper-spiked sediments at 10 mg kg^{-1} resulted in a significant decrease in MGO content (Figure 10). Although the exact mechanism of this reduction remains unknown, it might be related to metal-induced overexpression of glyoxalases (Wang et al., 2024). Evidence for the main effects of sediment was also found in the activity of ETS in the first and third toxicity tests (Figures 11 and 16). Specifically, ETS activity increased significantly in response to copper-spiked sediments and sediments from the Limnic Elbe, suggesting an elevated cellular energy demand for defense or repair activities. Although less frequently, two other biomarkers, PRO and TAC, also showed evidence for the main effects of sediment. In the first test, copper-spiked sediments at 20 mg kg^{-1} significantly increased PRO content (Figure 11), likely due to additional synthesis of proteins involved in copper homeostasis. In the third test, sediments from the Szczecin Lagoon significantly reduced TAC (Figure 14), suggesting an increase in ROS production induced by contaminants. Although the Szczecin Lagoon sediments generally did not have the highest contaminant concentrations among the collected sediments, they stood out for their PAH levels (Pham et al., 2025). Therefore, the observed TAC result might be related to the presence of these chemicals (Ghribi et al., 2019).

All three toxicity tests frequently showed negative results regarding the effects of sediment exposure, i.e., no evidence was found for the interaction effects between sediment and temperature or oxygen, nor for the main effects of sediment (Figure 9). For example, the activities of the biotransformation enzymes CES and GST and the antioxidant enzyme GR were not significantly altered in the two retrospective tests (Figures 12 and 14). Similarly, no significant accumulation of MDA and PC was detected in any of the tests (Figures 10, 12, and 15). Regarding energy metabolism, none of the tests showed significant effects of sediment on LIP reserve (Figures 11, 13, and 16). In addition, no significant change in AEC was observed in the third toxicity test (Figure 16). These results suggest that contaminant concentrations in the sediments were generally not high enough to cause detectable increases in detoxification processes (i.e., hydrolysis, GSH conjugation, and GSH recycling), to induce substantial lipid peroxidation and protein carbonylation, or to result in extensive disturbances in cellular energy status.

4.2 Towards risk assessment

Unlike traditional toxicity tests, which typically use a single organismal endpoint (Diepens et al., 2014), biomarker-based toxicity tests often face the challenge of summarizing multiple biomarker responses. A popular summary approach is to normalize the responses to a common scale and integrate them into a final index for each test group. Many such integrated indices have been developed using diverse methods, but their validity remains controversial (Pham and Sokolova, 2023). A simpler approach is used here instead, which counts the instances in which evidence for adverse effects on biomarkers was found (Table 1). Changes in supposedly beneficial directions, such as increased PRO reserve or decreased MGO content, were not included (Figures 10, 11, and 16).

Table 1: Number of biomarkers showing adverse effects of sediment under different environmental conditions.

Toxicity test	Sediment	Normal temperature or normal oxygen	Elevated temperature or reduced oxygen
1st test	10 mg Cu kg^{-1}	1 (ETS)	2 (ETS, MT)
	20 mg Cu kg^{-1}	1 (ETS)	2 (ETS, ATP7A)
2nd test	Peenestrom	0	0
	Szczecin Lagoon	1 (MGO)	1 (MGO)
3rd test	Peenestrom	0	0
	Szczecin Lagoon	2 (TAC, MGO)	2 (TAC, MGO)
	Transitional Elbe	1 (MGO)	1 (MGO)
	Limnic Elbe	2 (MGO, ETS)	2 (MGO, ETS)

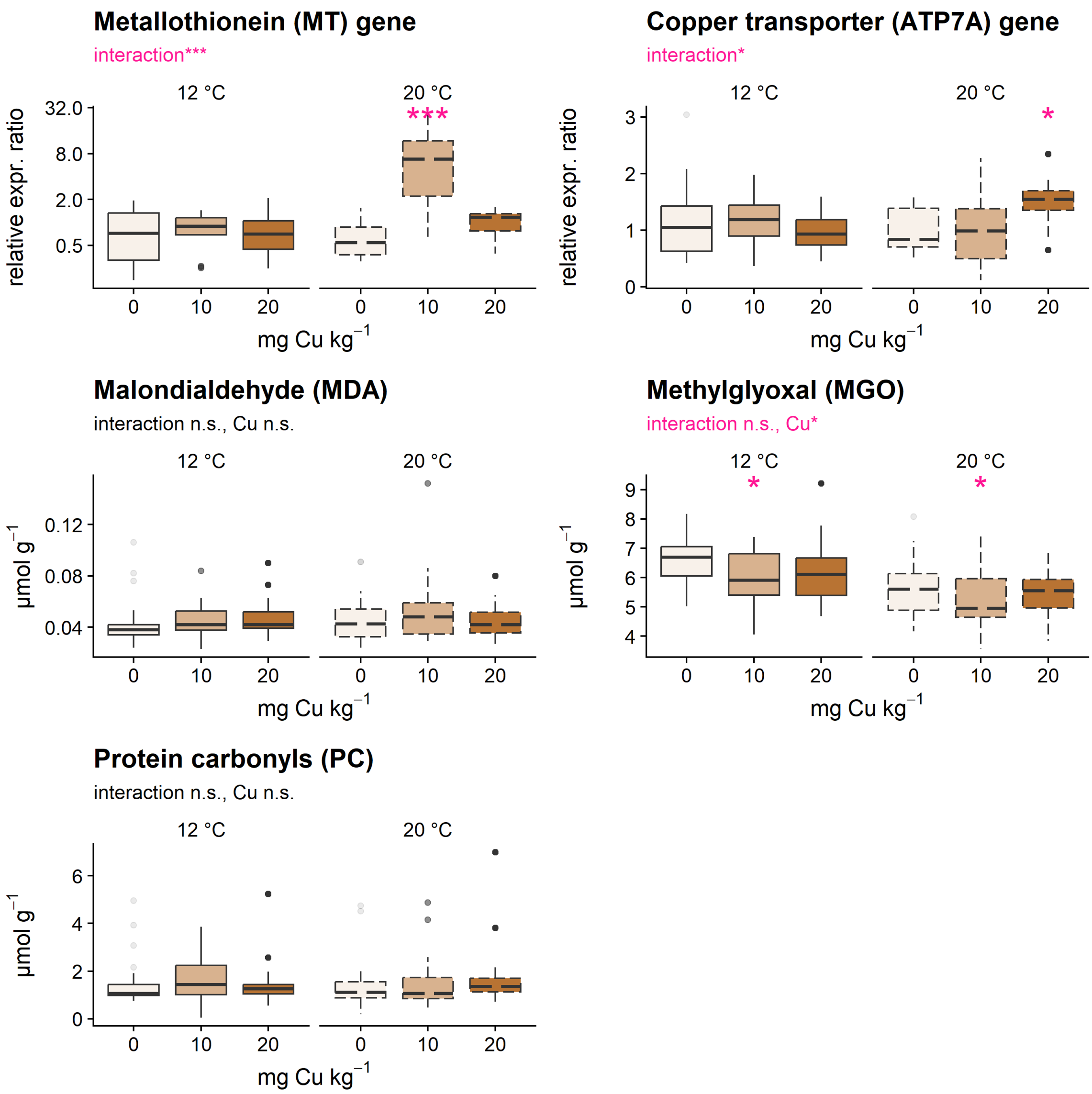


Figure 10: Effects of copper-spiked sediments on biomarkers of detoxification and antioxidant defense and on biomarkers of oxidative and carbonyl stress in the ragworm *Hediste diversicolor* under two temperature conditions. Results of omnibus tests and post-hoc tests against the control group are denoted as n.s. ($p > 0.05$) or by one to three asterisks ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$). Adapted from Pham et al. (2023).

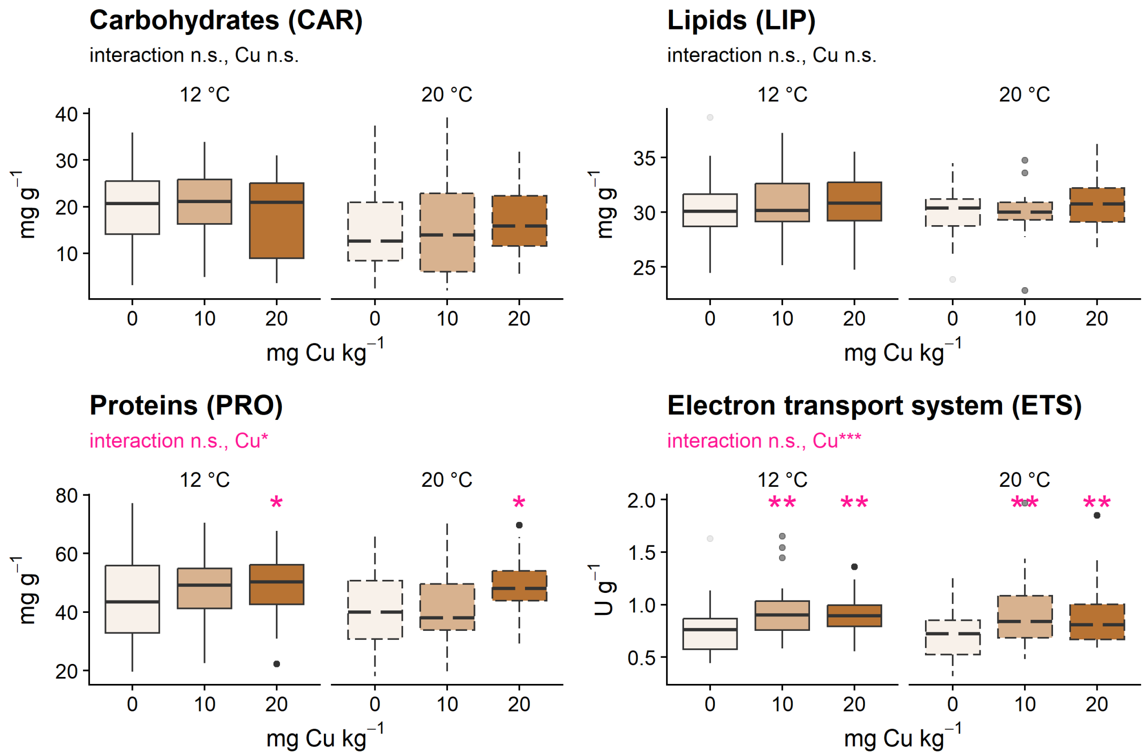


Figure 11: Effects of copper-spiked sediments on biomarkers of energy metabolism in the ragworm *Hediste diversicolor* under two temperature conditions. Results of omnibus tests and post-hoc tests against the control group are denoted as n.s. ($p > 0.05$) or by one to three asterisks ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$). Adapted from Pham et al. (2023).

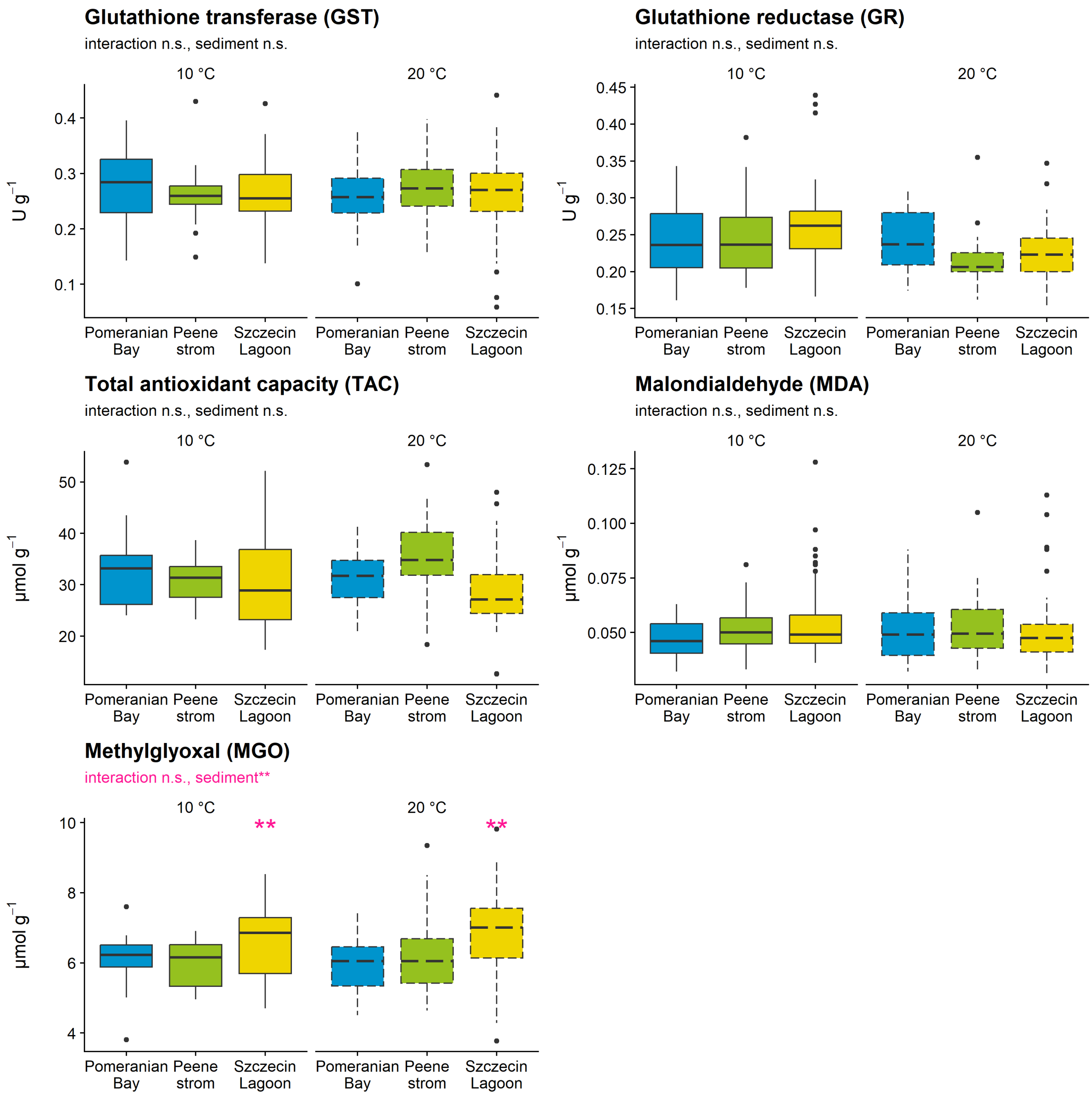


Figure 12: Effects of Oder estuary sediments on biomarkers of detoxification and antioxidant defense and on biomarkers of oxidative and carbonyl stress in the ragworm *Hediste diversicolor* under two temperature conditions. Results of omnibus tests and post-hoc tests against the reference group are denoted as n.s. ($p > 0.05$) or by one to three asterisks ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$). Adapted from Pham et al. (2024).

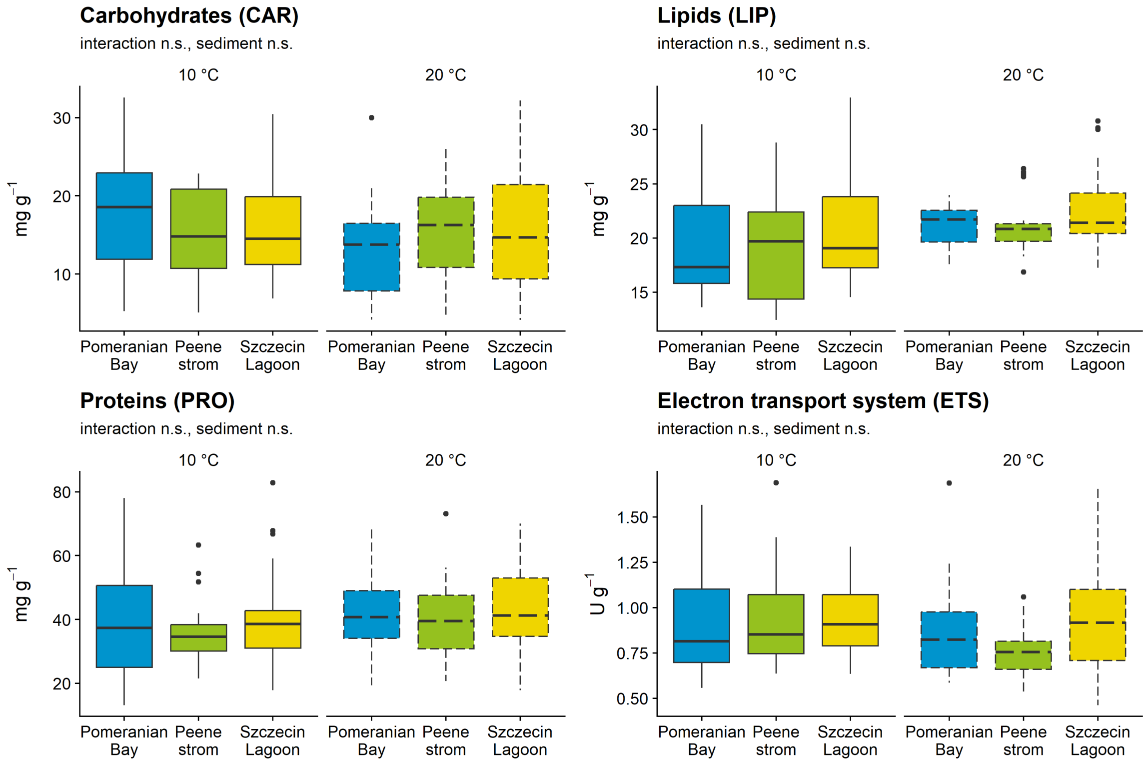


Figure 13: Effects of Oder estuary sediments on biomarkers of energy metabolism in the ragworm *Hediste diversicolor* under two temperature conditions. Results of omnibus tests and post-hoc tests against the reference group are denoted as n.s. ($p > 0.05$) or by one to three asterisks ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$). Adapted from Pham et al. (2024).

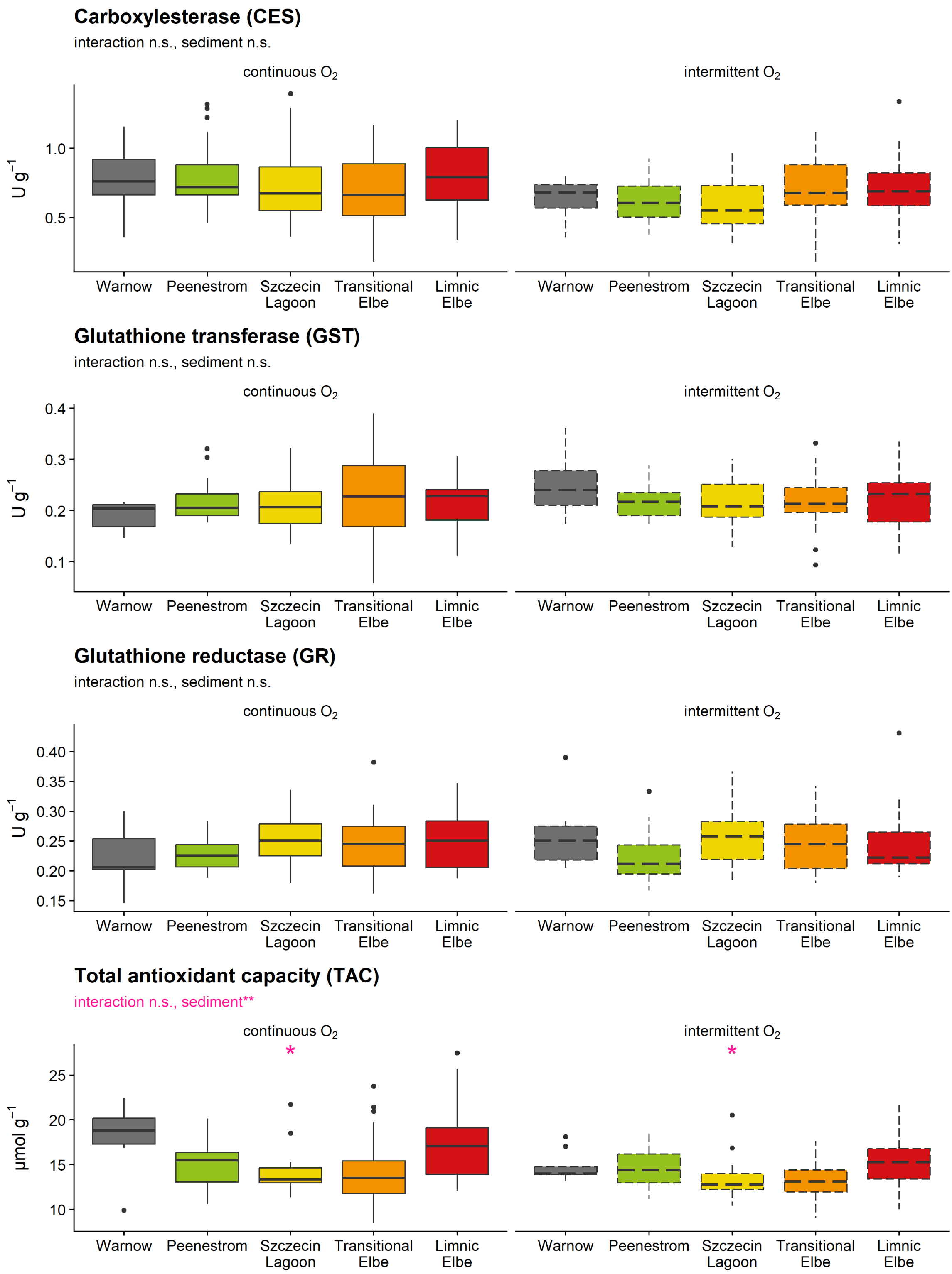


Figure 14: Effects of Oder and Elbe estuary sediments on biomarkers of detoxification and antioxidant defense in the ragworm *Hediste diversicolor* under two oxygen conditions. Results of omnibus tests and post-hoc tests against the reference group are denoted as n.s. ($p > 0.05$) or by one to three asterisks ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$). Adapted from Pham et al. (2025).

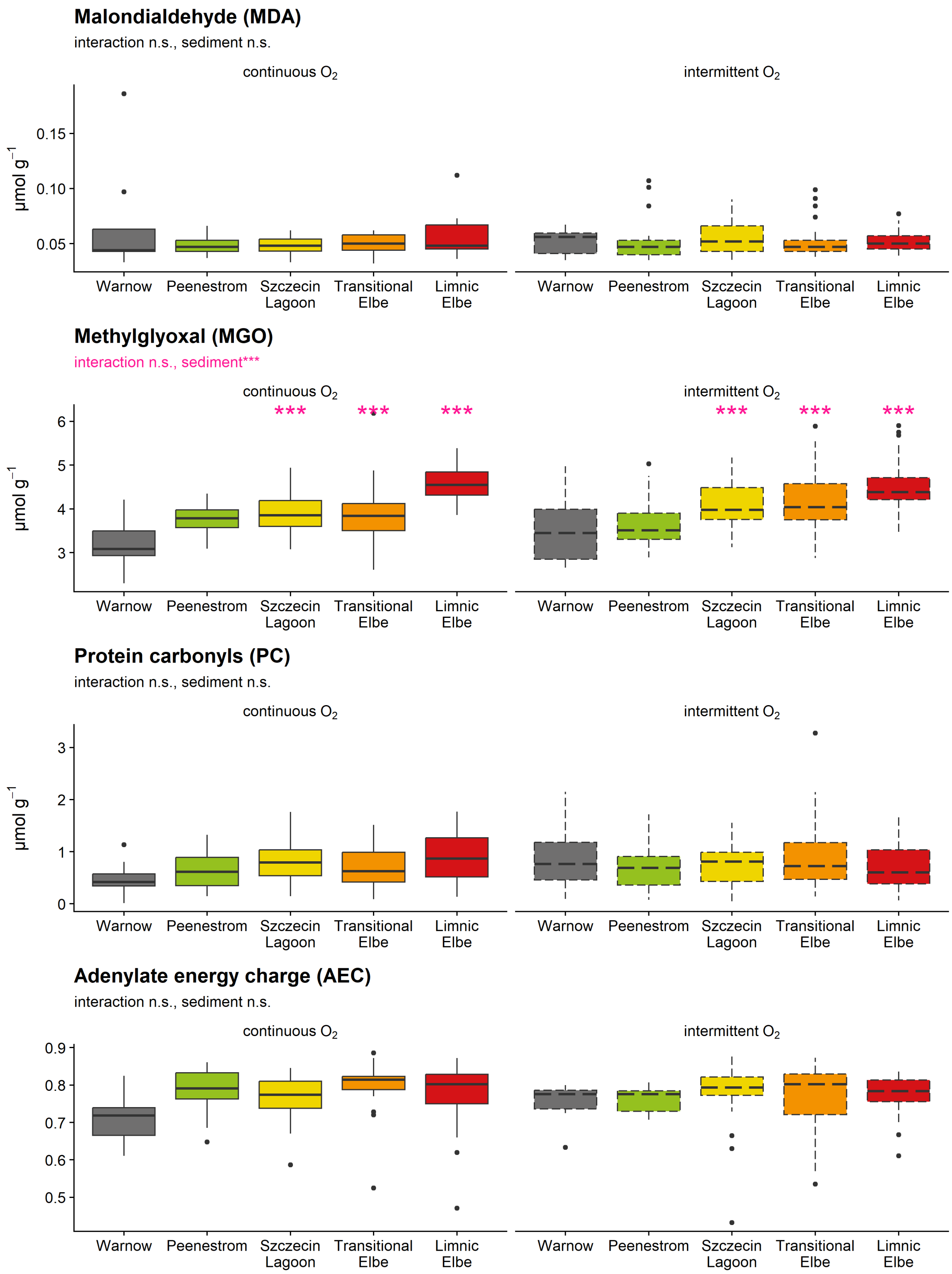


Figure 15: Effects of Oder and Elbe estuary sediments on biomarkers of oxidative and carbonyl stress and a biomarker of energy metabolism in the ragworm *Hediste diversicolor* under two oxygen conditions. Results of omnibus tests and post-hoc tests against the reference group are denoted as n.s. ($p > 0.05$) or by one to three asterisks ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$). Adapted from Pham et al. (2025).

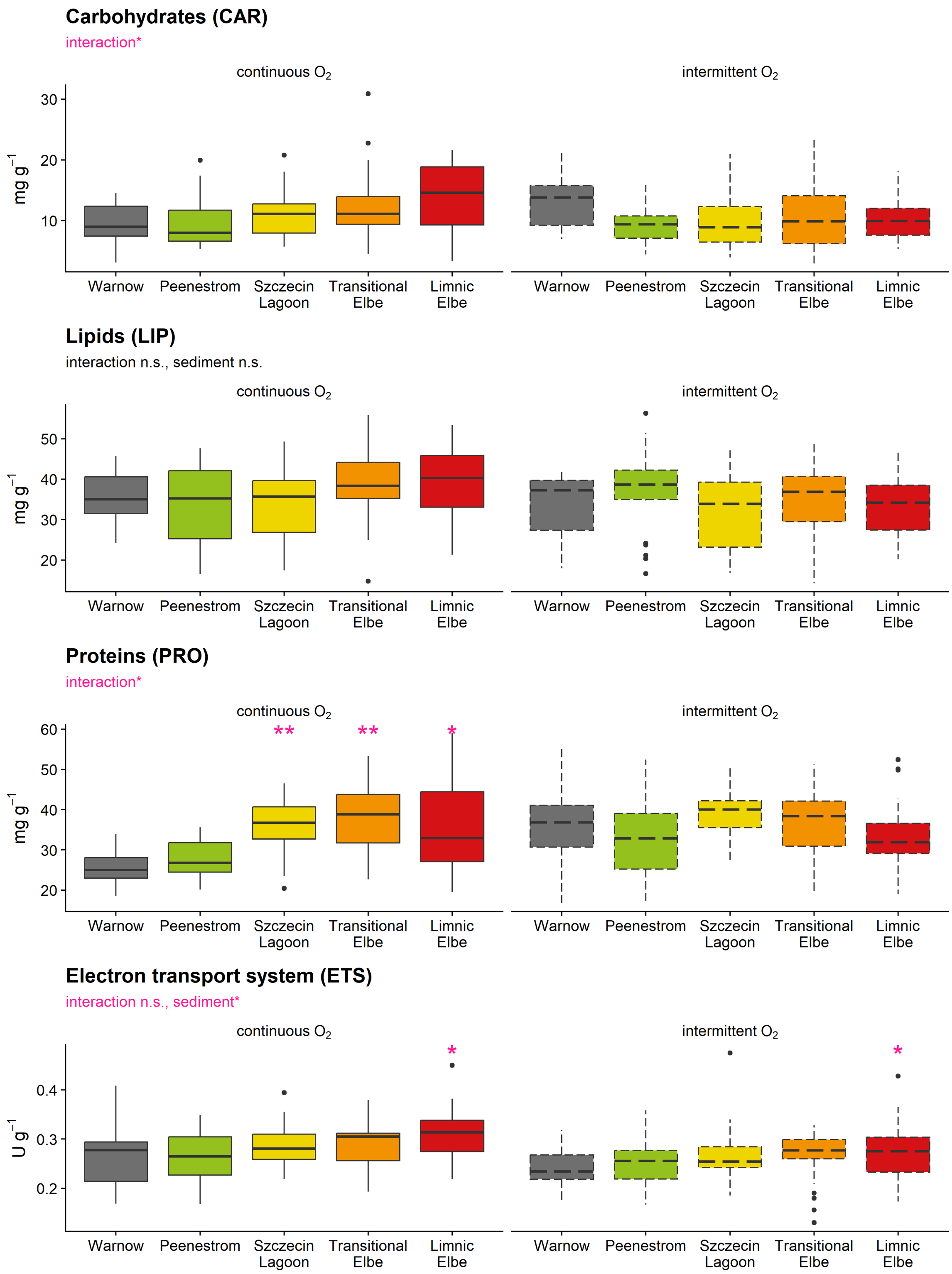


Figure 16: Effects of Oder and Elbe estuary sediments on biomarkers of energy metabolism in the ragworm *Hediste diversicolor* under two oxygen conditions. Results of omnibus tests and post-hoc tests against the reference group are denoted as n.s. ($p > 0.05$) or by one to three asterisks ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$). Adapted from Pham et al. (2025).

Overall, the prospective test showed that elevated temperature increased the adverse effects of copper-spiked sediments (Table 1). However, due to the limited number of test concentrations, 10 mg Cu kg^{-1} was considered the lowest observed effect concentration (LOEC) at both temperatures, i.e., the lowest test concentration that showed significant effects (Amiard and Amiard-Triquet, 2015). Since lower concentrations were not tested, the no observed effect concentration (NOEC), i.e., the highest test concentration that showed no significant effects, could only be approximated, for example, as LOEC/2 (Schudoma, 2001), or 5 mg Cu kg^{-1}. However, this NOEC is specific to the ragworm and the sediment used for spiking in this test. A complete derivation of the PNEC for copper in estuarine sediments would require NOEC data from additional species as well as normalization to sediment physicochemical properties such as fine fraction and organic matter content (Roman et al., 2007).

In the retrospective tests, environmental conditions did not change the adverse effects of sediments from the Oder and Elbe estuaries (Table 1). Both tests confirmed that Peenestrom sediments were not toxic to ragworms, while sediments from other regions, particularly the Szczecin Lagoon and the Limnic Elbe, were toxic. A more complete risk assessment of sediment contamination in the two estuaries would require additional sediment toxicity tests with other species as well as field surveys of benthic communities (Wetzel et al., 2013).

Notably, the three sediment toxicity tests demonstrated the high responsiveness of ETS and MGO, together with the lack of moderation effects of environmental variables on these biomarkers (Figure 9, Table 1). These results suggest that ETS and MGO may be sensitive and robust indicators of contaminant exposure, making them particularly valuable in cases of screening or conservative risk assessments (Hope, 2006; Viscusi et al., 1997).

5 Publications

5.1 Publication 1

Pham, D.N., Kopplin, J.A., Dellwig, O., Sokolov, E.P., Sokolova, I.M., 2023. Hot and heavy: Responses of ragworms (*Hediste diversicolor*) to copper-spiked sediments and elevated temperature. Environmental Pollution 332, 121964. https://doi.org/10.1016/j.envpol.2023.121964

Declaration of personal contributions

I hereby declare that my personal contributions, as defined in the Contributor Role Taxonomy (CRediT), to **Publication 1** are as follows:

- I took a leading role in **Methodology**, **Investigation**, and **Data curation**.
- I was solely responsible for **Software**, **Formal Analysis**, **Visualization**, and **Writing – original draft**.

Rostock, 2025-04-06

Duy Nghia PHAM

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Hot and heavy: Responses of ragworms (*Hediste diversicolor*) to copper-spiked sediments and elevated temperature☆

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ABSTRACT

Sediment contamination and seawater warming are two major stressors to macrobenthos in estuaries. However, little is known about their combined effects on infaunal organisms. Here we investigated the responses of an estuarine polychaete *Hediste diversicolor* to metal-contaminated sediment and increased temperature. Ragworms were exposed to sediments spiked with 10 and 20 mg kg^{-1} of copper at 12 and 20 °C for three weeks. No considerable changes were observed in the expression of genes related to copper homeostasis and in the accumulation of oxidative stress damage. Dicarbonyl stress was attenuated by warming exposure. Whole-body energy reserves in the form of carbohydrates, lipids and proteins were little affected, but the energy consumption rate increased with copper exposure and elevated temperature, indicating higher basal maintenance costs of ragworms. The combined effects of copper and warming exposures were mostly additive, with copper being a weak stressor and warming a more potent stressor. These results were replicable, as confirmed by two independent experiments of similar settings conducted at two different months of the year. This study suggests the higher sensitivity of energy-related biomarkers and the need to search for more conserved molecular markers of metal exposure in *H. diversicolor*.

1. Introduction

Estuarine ecosystems have changed rapidly in recent decades due to many anthropogenic and climatic pressures (Cloern et al., 2016; Mitchell et al., 2015). The introduction of hazardous substances is considered one of the greatest threats to estuarine communities (Borgwardt et al., 2019). Elevated water temperature due to climate change is also a major stressor, given its direct effects on the physiology of organisms and its common association with unfavorable events such as harmful algal blooms and hypoxia (Harley et al., 2006; Kimmerer and Weaver, 2013). Understanding how estuarine biota, living in inherently highly variable environment (Elliott and Quintino, 2007), responds to these additional stressors is therefore critical to maintaining estuarine ecosystem functions and services (Barbier et al., 2011; O'Brien et al., 2019).

Among estuarine species, macrobenthos is an ecologically important but highly vulnerable group (Pinto et al., 2009), often exposed to sediment-deposited contaminants, such as trace heavy metals (Brady et al., 2015; Wang and Fisher, 1999). Although some metals such as zinc (Zn) and copper (Cu) have essential functions (Festa and Thiele, 2011; Maret, 2013), their excessive amounts are toxic (Brix et al., 2022; Eisler, 1998; Rainbow, 2002). Metal toxicity is influenced by temperature, with increased temperature often enhancing metal uptake and accumulation, but concurrently facilitating detoxification and repair processes (Cairns et al., 1975). Higher temperature may also reduce the oxygen and energy supply required to offset the elevated maintenance costs due to metal exposure (Sokolova and Lannig, 2008). Because of these possible interactions, the combined effects of metal contamination and warming may be more or less extreme than the additive expectation of the individual effects, often referred to as synergism and antagonism, respectively (Crain et al., 2008; Folt et al., 1999; Piggott et al., 2015).

While several studies have examined the combined effects of metal

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contamination and warming on macrobenthic epifauna such as mussels and corals (Biscéré et al., 2017; Boukadida et al., 2016; da Silva Fonseca et al., 2019; Parry and Pipe, 2004; Tracy et al., 2020), similar research on infauna in contaminated sediments is lacking. In this study, we investigated the bioenergetic and stress responses of a benthic polychaete, ragworms *Hediste diversicolor* to Cu exposure and elevated temperature. Ragworms are widely used in ecotoxicological research because of their easy collection and maintenance, broad ecological niche, as well as burrowing behavior and diverse feeding modes that increase contaminant exposure (Scaps, 2002; Silva et al., 2020). Cu was chosen because of its often elevated levels in modern estuarine sediments, with reported contents ranging from tens to hundreds of mg kg^{-1} dry mass (Glasby et al., 2004; Müller and Heininger, 1999; Szefer et al., 2009; Wetzel et al., 2013). The increased use of Cu in antifouling paints to replace tributyltin over the last two decades also makes it of particular concern (Brooks and Waldock, 2009; Comber et al., 2022). Our preliminary analysis of surface sediments from the Warnow estuary, Germany showed a median Cu content of ~44 mg kg^{-1} (Fig. S1, LUNG, 2023; Rönspieß et al., 2020). Thus, we exposed ragworms to uncontaminated sediments and sediments spiked with 10 and 20 mg kg^{-1} of Cu at 12 and 20 °C. The temperature difference was chosen to mimic the warming scenario with surface temperatures in northern Europe projected to increase by ~3 °C by the mid-21st century (SSP5-8.5, 1995–2014 baseline, Carvalho et al., 2021; IPCC, 2021), and to account for daily temperature variations (~5 °C within a month). To assess the potential bioenergetic disruption from Cu and warming exposures, we measured whole-body levels of energy reserves (carbohydrates, lipids, and proteins) and mitochondrial aerobic capacity (electron transport system activity) and calculated the worm's cellular energy allocation (Verslycke et al., 2004). Potential cellular toxicity and molecular stress responses were evaluated using indices of dicarbonyl stress (methylglyoxal level), oxidative damage (malondialdehyde and protein carbonyl levels), and mRNA expression levels of genes encoding Cu transporters and metal-binding proteins (*ATP7A*, *CCS*, *MTS*, and *MTL*), metal stress-protective proteins (*GSTO1*, *HSP70MAJ*, and *HSP70MIN*), and a hemoglobin linker chain (*HBL2*) (Green Etxabe et al., 2021; McQuillan et al., 2014). To examine the research replicability (Romero, 2019), we conducted two experiments with similar setups in two different months of the year.

2. Materials and methods

2.1. Sediment collection and characterization

Surface sediment (upper 10 cm) was collected in December 2020 from a beach in Warnemünde, Germany (54.1787, 12.0666) with no point sources of pollution nearby (Fig. S1). Wet sediment was sieved through a 1-mm mesh to remove larger particles and organisms. The sediment was oven-dried at 60 °C for 96 h and stored at room temperature.

Physicochemical properties of wet sediment were measured using conventional procedures (Blake, 1965; Davies, 1974; Robertson et al., 1984; Verardo et al., 1990). The sediment had a bulk density of 1.49 g cm^{-3}, a water content of 17.9% w/w, a total organic matter content of 0.18% w/w, and a C:N mass ratio of 36.9. The sediment was composed of 2.5% mud, 0.2% very fine sand, 34.7% fine sand, 60.4% medium sand, and 2.2% coarse sand (Wentworth, 1922) with a median grain size of 409 μm.

2.2. Animal collection

Ragworms *Hediste* (*Nereis*) *diversicolor* were collected in January and March 2021 near Schnatermann, Germany (54.1728, 12.1414), a contaminated area due to intense port activity (Fig. S1, Abraham et al., 2017). Worms were sieved out of the sediment and transported in seawater-filled plastic drums to the laboratory. They were transferred to 20-L plastic trays pre-filled with the dried sediment (6 cm thick) and aerated artificial seawater (Pro-Reef, Tropic Marin, Germany) at 12 ± 0.5 °C and salinity 15 ± 0.25, which approximated the average annual conditions of their habitat. The worms were fed on alternate days with fish food (TetraMin Flakes, Tetra, Germany) and the overlying seawater was changed daily during a 10-day acclimation period to assist depuration of any pre-existing Cu burdens in the worms (Ozoh, 1994).

2.3. Sediment spiking

Copper (II) chloride ($CuCl_2{\cdot}2H_2O$, CAS 10125-13-0, Carl Roth, Germany) was used to prepare Cu-spiked sediments (Green Etxabe et al., 2021; Hutchins et al., 2009; Ward et al., 2015). Stock solutions of $CuCl_2$ were made in deionized water and diluted to the desired Cu masses in 125-mL water volumes in 800-mL glass beakers. The beakers were orbitally shaken for 90 min (Dual-Action Shaker KL 2, Edmund Bühler, Germany) and 500 g of the dried sediment was gradually added to obtain a 6-cm sediment thickness. Nominal Cu contents were 0 (control, no Cu salt addition), 10, and 20 mg kg^{-1} dry sediment. After 24 h settling without pH adjustment, the thin layer of overlying water was gently removed using syringes and replaced by 500 mL of artificial seawater (salinity 15). The beakers were kept in the dark at 12 ± 0.5 °C for one week until exposures.

2.4. Cu and warming exposures

Two two-factor experiments were conducted with the ragworms collected in January (Jan experiment) and March (Mar experiment). In each experiment, worms were randomly assigned to one of six exposure groups corresponding to the combinations of three Cu contents (0, 10, and 20 mg kg^{-1}) and two temperatures, i.e., 12 °C (control) and 20 °C (elevated). Five beakers were used for each group. The Jan experiment had three worms per beaker (wet mass 298 ± 160 mg) and the Mar experiment had four worms per beaker (wet mass 299 ± 137 mg). All beakers were kept in an environmental room at 12 ± 0.5 °C for three weeks, which is the common exposure duration for ragworms in toxicological experiments (Buffet et al., 2013; Fernandes et al., 2006; Mouneyrac et al., 2003; Zhou et al., 2003). Beakers receiving warming treatment were placed in a circulating water tub equipped with an aquarium heater (HT 75, Tetra). Temperature was set to gradually increase from 12 to 20 °C during the first week (to avoid excessive thermal stress) and remain stable at 20 ± 0.5 °C during the following two weeks. The overlying water was aerated to maintain high dissolved oxygen levels (>9 mg L^{-1} at 12 °C and >8 mg L^{-1} at 20 °C, pH > 8). The worms were fed daily with fish food (TetraMin Granules, Tetra) and overlying seawater was renewed once a week to avoid nitrate buildup. Dead worms and unconsumed food were promptly removed from the sediment surface to maintain the water quality.

After the three-week exposure, sediment and pore water were sampled in each beaker to measure Cu concentrations ($n = 5$). Surface sediments (~10 g, upper 1 cm) were freeze-dried for 24 h (Alpha 1–4 LSCplus, Martin Christ Gefriertrocknungsanlagen, Germany), homogenized using a porcelain mortar and pestle, and stored at room temperature for analysis of total Cu. Pore waters (~10 mL) were extracted from the bottom sediments using rhizon samplers (0.15-μm pore size, 19.21.23 F, Rhizosphere Research Products, Netherlands, Seeberg-Elverfeldt et al., 2005), acidified with high-purity HNO_3 (to 2% v/v), and stored at 4 °C for analysis of dissolved Cu. Worms were sieved out of the sediment, rinsed with seawater, shock-frozen in liquid nitrogen and stored at −80 °C to measure biomarker responses ($n = 15$ and 20 in the Jan and Mar experiments, respectively). After that, the remaining samples of worms from each beaker were pooled, freeze-dried for 72 h, and stored at room temperature for analysis of total Cu body burden ($n = 2$).

2.5. Cu analyses

Cu concentrations in sediments, pore waters, and worms were measured using inductively coupled plasma mass spectrometry (ICP-MS, iCAP Q, Thermo Fisher Scientific, Germany) following protocols described elsewhere (Dellwig et al., 2019; Lagerström et al., 2013). Details are given in the Supplementary Material.

2.6. Colorimetric assays

Whole worms were homogenized in ice-cold buffer (0.1 M Tris-HCl pH 8.5, 153 μM $MgSO_4$, 0.2% w/v Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride) using Potter-Elvehjem glass-Teflon homogenizers. The levels of carbohydrates, lipids, proteins, methylglyoxal (MGO), malondialdehyde (MDA) and protein carbonyls (PC) were measured with colorimetric end-point assays (Bradford, 1976; Buege and Aust, 1978; Folch et al., 1957; Levine et al., 1990; Masuko et al., 2005; Mitchel and Birnboim, 1977; Van Handel, 1985). Mitochondrial electron transport system (ETS) activity was measured kinetically at 25 °C (De Coen and Janssen, 1997). Details are given in the Supplementary Material.

ETS activity was corrected for the exposure temperatures (12 and 20 °C) using the Q_{10} temperature coefficient of 2.0 previously reported for *H. diversicolor* (Galasso et al., 2018). Contents of carbohydrates, lipids, and proteins were converted to energy equivalents using the specific enthalpy of combustion of 17.5, 39.5, and 24 J mg^{-1}, respectively (Gnaiger, 1983) and summed up to obtain the total available energy (Ea). Energy consumption rate (Ecr) was calculated from the ETS activity using the oxyenthalpic equivalent of 484 kJ mol^{-1} O_2 and cellular energy allocation (CEA) as an energy budget index was computed as the Ea/Ecr ratio (Verslycke et al., 2004).

2.7. Quantitative reverse transcription PCR (RT-qPCR)

Transcript levels of the target genes associated with metal-induced stress (Green Etxabe et al., 2021; McQuillan et al., 2014) and the reference genes *GAPDH* and *HIS3* were determined in the anterior part of ragworms, including the head and a few segments, following RT-qPCR protocols described elsewhere (Falfushynska et al., 2019) with gene-specific primers (Table 1). The anterior part was chosen because this body region was highly responsive to Cu exposure (Bouraoui et al., 2015). The expression levels of the target genes were normalized against those of the reference genes (which were stable across the exposure groups, Table S1) using geometric averaging (Matz et al., 2013; Pfaffl, 2001; Vandesompele et al., 2002). Due to limited resources, RT-qPCR was only performed on worms from the Mar experiment ($n = 15$). Details on RNA extraction, cDNA synthesis, and qPCR are given in the Supplementary Material.

2.8. Data analyses

The effects of Cu-spiked sediments, elevated temperature, and their interaction on experimental outcomes were evaluated by permutation tests for multi-factor analysis of variance (Anderson and Braak, 2003; Howell, 2015; Manly, 2007), which is the recommended approach for analyzing randomized experiments (Ernst, 2004; Ludbrook and Dudley, 1998). Specifically, linear models (LMs), binomial generalized linear mixed-effects models (GLMMs) with logit link function, and linear mixed-effects models (LMMs, Bates et al., 2015) were used for the measured concentrations of Cu, worm survival, and biomarkers, respectively, with nominal Cu content and temperature as interacting fixed effects. Experimental beaker was added as a random intercept in GLMMs and LMMs to account for the potential non-independence of worms within each beaker (Colegrave and Ruxton, 2018). The wet mass of worms was added as a covariate in LMMs to control for possible influences of size on biomarker responses (Durou et al., 2005; Stomperudhaugen et al., 2009). The *F*-statistics from these models and their recalculated values under 1999 permutations were used to compute the *p*-values. The test results were reported in the language of evidence (Muff et al., 2022), in which evidence against the null hypotheses was considered very strong, strong, moderate, or weak when $p \leq 0.001$, 0.01, 0.05, or 0.1, respectively. Multiple comparisons tests were not used in this study (Kozak and Powers, 2017).

We computed Glass's delta effect size (Glass et al., 1981; Lakens, 2013) for the effects of Cu and warming exposures on biomarker responses compared with the shared control condition (0 mg kg^{-1} of Cu at 12 °C). Glass's delta (Δ) is a measure of the standardized difference between means and was used to determine whether an interaction is synergistic or antagonistic (Piggott et al., 2015). To assess the similarity in the responses of biomarkers, we calculated Spearman correlation and performed agglomerative hierarchical clustering using the correlation-based distance ($\sqrt{1-|r_s|}$) and average linkage (Chen et al., 2023).

All analyses were implemented in R v4.3.0 (R Core Team, 2023) using the *peramo* package v0.1.3 for permutation tests (Pham et al., 2022) and the *mbRes* package v0.1.7 for effect size calculation (Pham and Sokolova, 2023).

3. Results

3.1. Cu concentrations

Mean total Cu contents in the surface sediments in experimental beakers after the three-week exposure were 1.0, 3.8, and 7.5 mg kg^{-1} for the Jan experiment and 1.0, 1.6, and 3.8 mg kg^{-1} for the Mar experiment, corresponding to the nominal Cu contents of 0, 10, and 20 mg kg^{-1}, respectively. Respective mean dissolved Cu concentrations in the

Table 1
Primer sequences used for the amplification of the target and reference genes in *Hediste diversicolor*.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	NCBI accession number
ATP7A	CTACGAGAAGCCACGAGTCC	TCTCCAGGGACCACTTTCAG	–
CCS	AGCAGTTGGAGTCAGCAGGT	TGCCAGCTCTCCGTATTTCT	–
MTS	CATTGCACTGGGAATGTTTG	CATCACAGCATTGGATGGAC	–
MTL	GGAGCTTCTGTTTCCTGTGC	TCACAAATCCAGCACCATGT	–
GSTO1	CATCGCAGATTGAGGATTCA	TGTCCCTATGCCCAGAGAAC	–
HSP70MAJ	TTTCTGGCCTGAATGTCTTGCGTA	AGAGCGTTTCGTTCTCCTCCTACT	KX271712.1
HSP70MIN	ATTTGATGAAGCCTCTGTGCAATC	TTCTTCTCCGCCTTGTATTCAACT	KX271711.1
HBL2	GCTCGCTCATGGGATAATAACAAC	TCTCTGAAACTAACAGAGCACGAG	D58413.1
GAPDH	CATCCATGACCATCCTCAGCAA	GTTGTCATCAAACCCTCAACGATT	KX284894.1
HIS3	GTGAGATCCGTCGTTACCAGAAA	CAAGTCAGTCTTGAAGTCCTGGG	LC380659.1

Gene symbols: *ATP7A* – ATPase copper transporting alpha; *CCS* – copper chaperone for superoxide dismutase; *MTS* – Cd/Se metallothionein (small protein); *MTL* – atypical metallothionein-like protein (large protein); *GSTO1* – glutathione *S*-transferase omega 1; *HSP70MAJ* – 70 kDa heat shock protein major form; *HSP70MIN* – 70 kDa heat shock protein minor form; *HBL2* – linker chain L2 of the giant extracellular hemoglobin; *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase; *HIS3* – histone H3. Primer sequences for *ATP7A*, *CCS*, *MTS*, *MTL*, and *GSTO1* were retrieved from McQuillan et al. (2014).

pore waters of bottom sediments were 20.1, 183.1, 565.4 μg L^{-1} for the Jan experiment and 14.2, 195.9, and 337.1 μg L^{-1} for the Mar experiment. Respective mean total Cu burdens in worms were 24.6, 39.7, and 60.7 mg kg^{-1} for the Jan experiment and 9.5, 29.7, and 39.8 mg kg^{-1} for the Mar experiment. Cu levels in the sediments, pore waters, and worms were correlated (Fig. 1 and S2), determined by the nominal Cu contents but not affected by the exposure temperature ($p > 0.1$, Table S2).

3.2. Survival

At the end of the exposures, worm survival in six groups was 63.3% ($SD = 11.7\%$) in the Jan experiment and 84.2% ($SD = 5.8\%$) in the Mar experiment (Table S3). There was no evidence that Cu and warming exposures affected the survival ($p > 0.1$, Table S4).

3.3. Energy reserves

In the Jan experiment, there was no evidence that Cu exposure and temperature altered the carbohydrate content (Fig. 2a). Moderate evidence for the effect of temperature on carbohydrate content was found in the Mar experiment, in which worms exposed to 20 °C had lower carbohydrate levels than those at 12 °C. There was no evidence that Cu and warming exposures affected the lipid and protein contents (Figs. S3a and b).

In the Jan experiment, there was no evidence that Cu exposure and temperature affected the Ea (Fig. 2b). The Mar experiment showed moderate evidence for the effect of temperature, in which worms exposed to warming had lower Ea than those at control temperature.

3.4. Mitochondrial aerobic capacity and energy budget

Both experiments showed very strong evidence for the impact of temperature on mitochondrial ETS activity, in which worms exposed to 20 °C had elevated ETS activity compared with those at 12 °C (Fig. 2c). The effect of Cu on ETS activity was found with strong and weak evidence in the Jan and Mar experiments, respectively, in which worms exposed to Cu-spiked sediments had higher ETS activity than those in the control sediment.

Both experiments showed very strong evidence for the effect of temperature on CEA, in which worms exposed to 20 °C had misbalanced energy budget compared with those at 12 °C (Fig. 2d). Strong and weak evidence for the impact of Cu was found in the Jan and Mar experiments, respectively, in which worms exposed to Cu-spiked sediments had lower CEA than those in the control sediment.

3.5. Dicarbonyl and oxidative stress

Very strong and strong evidence was found for the effect of temperature on MGO level in the Jan and Mar experiments, respectively, in which worms at 20 °C had lower MGO levels than those at 12 °C (Fig. 2e). No evidence was found for the impact of Cu on MGO level. There was no evidence that Cu and temperature affected MDA and PC levels in both experiments (Figs. S3c and d).

3.6. Molecular markers of metal exposure and stress

In the Mar experiment, strong evidence was found for the effect of interaction between Cu and temperature on the expression level of *ATP7A* (Fig. 3a), in which warming led to an increase in the *ATP7A* transcript level in worms exposed to 20 mg kg^{-1} of Cu.

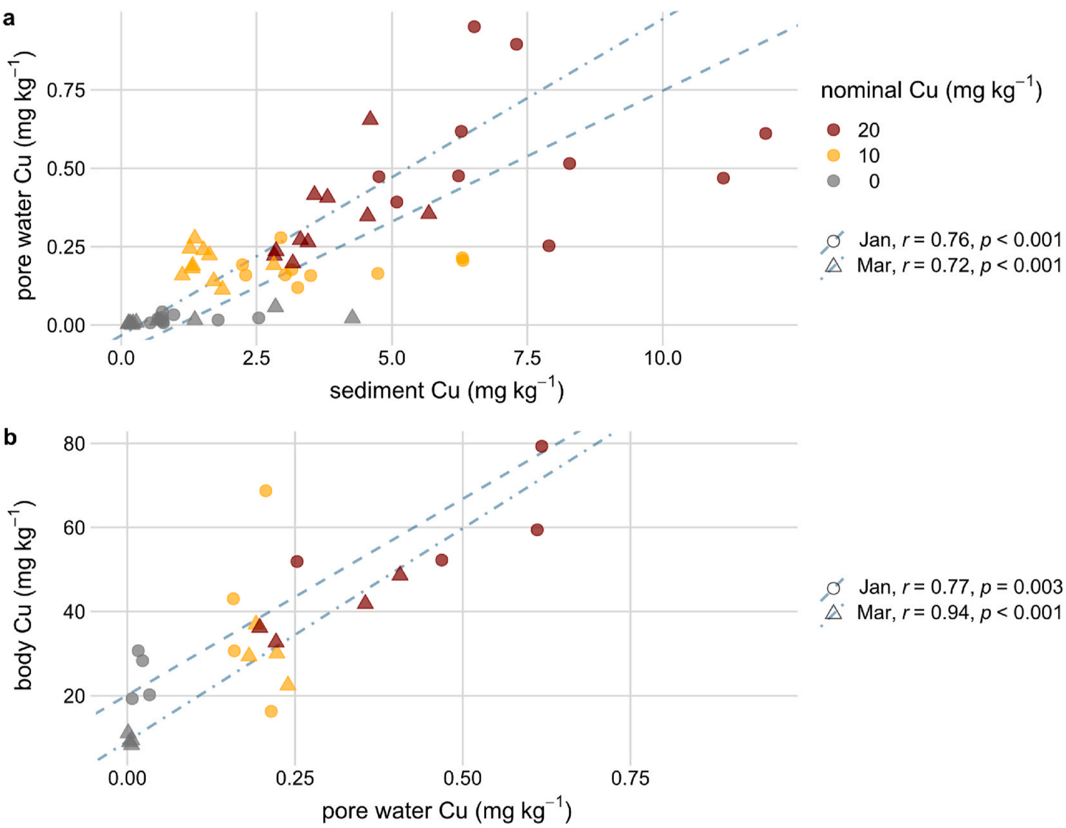

Fig. 1. Correlation between total Cu contents in surface sediments, dissolved Cu concentrations in pore waters of bottom sediments, and total Cu body burdens in worms at the end of the exposures in the Jan (circles) and Mar (triangles) experiments. Sediments were spiked with Cu at nominal contents of 0, 10, and 20 mg kg^{-1}. Standard deviation lines (Freedman et al., 2007), Pearson correlation and permutation p-values are given for each experiment.

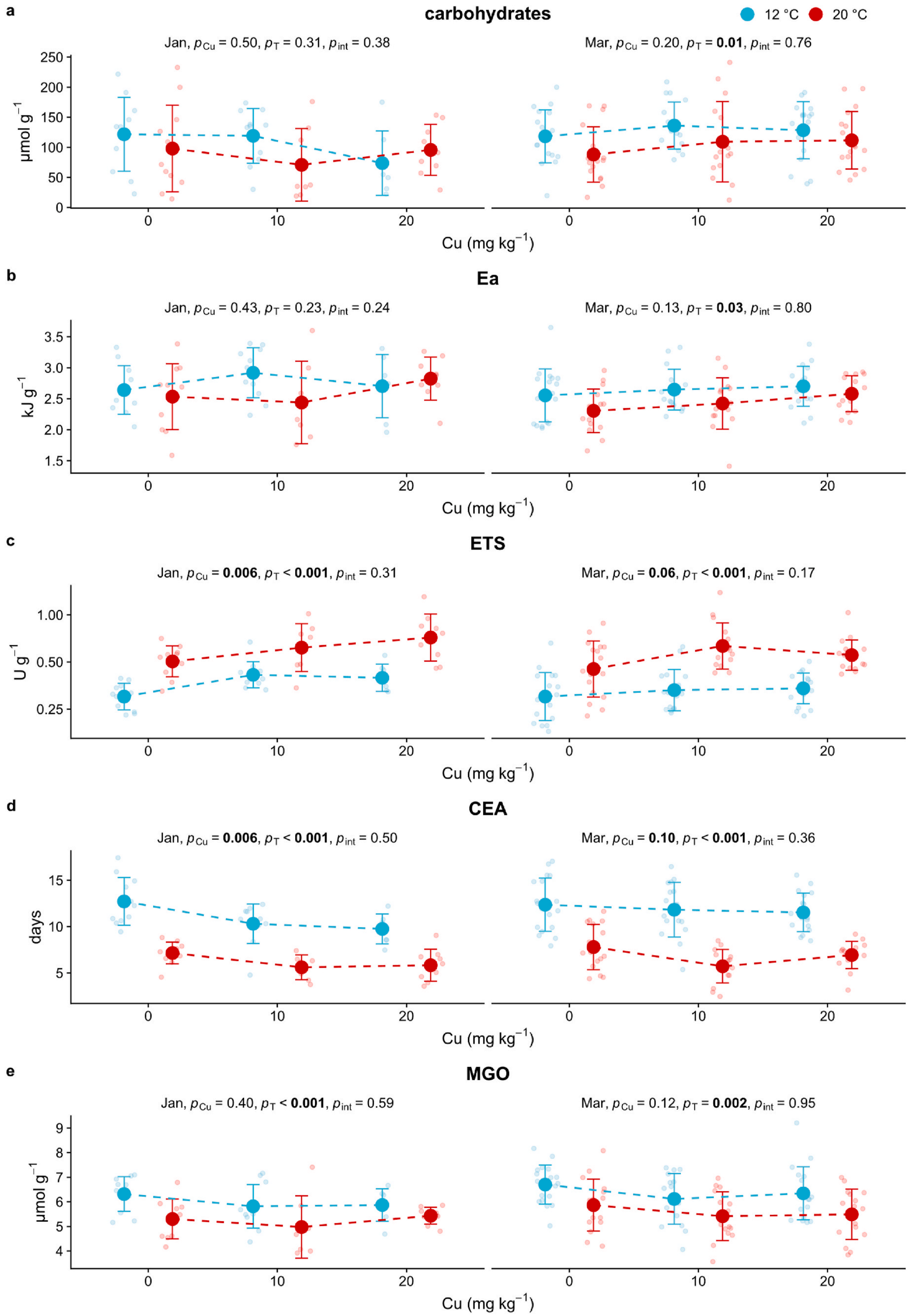

Fig. 2. Effects of Cu-spiked sediments and elevated temperature on carbohydrate content, total available energy (Ea), mitochondrial electron transport system (ETS) activity, cellular energy allocation (CEA), and methylglyoxal (MGO) level of *Hediste diversicolor* in the Jan and Mar experiments. Data are presented with individual observations and mean ± standard deviation. Permutation *p*-values are given for Cu, temperature (T), and their interaction (int). *F*-statistics are given in Table S5.

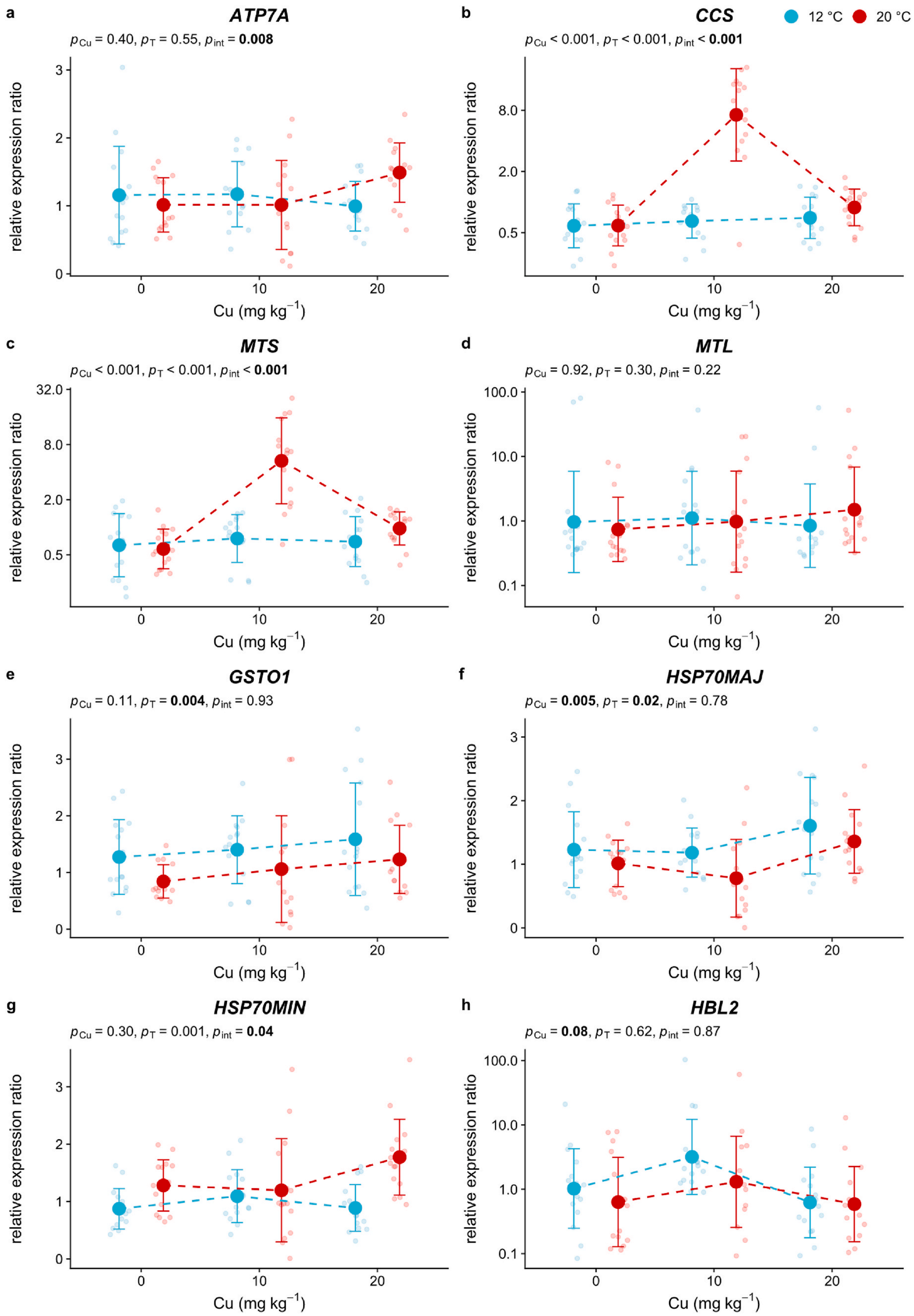

Fig. 3. Effects of Cu-spiked sediments and elevated temperature on mRNA expression levels of *ATP7A*, *CCS*, *MTS*, *MTL*, *GSTO1*, *HSP70MAJ*, *HSP70MIN*, and *HBL2* in the anterior part of *Hediste diversicolor* in the Mar experiment. Data are presented with individual observations and mean ± standard deviation. Permutation *p*-values are given for Cu, temperature (T), and their interaction (int). *F*-statistics are given in Table S5.

Very strong evidence was found for the interaction effect on the expression levels of *CCS* and *MTS* (Fig. 3b and c). The *CCS* and *MTS* transcript levels were strongly upregulated at the elevated temperature in worms exposed to 10 mg kg^{-1} of Cu. There was no evidence that Cu and warming exposures altered the expression level of *MTL* (Fig. 3d).

Strong evidence was found for the effect of temperature on the expression level of *GSTO1* (Fig. 3e), in which worms exposed to warming had downregulated *GSTO1* transcript levels compared with those at 12 °C.

There was strong and moderate evidence for the impacts of Cu and temperature, respectively, on the expression level of *HSP70MAJ* (Fig. 3f). Worms exposed to 10 and 20 mg kg^{-1} of Cu, respectively, showed downregulation and upregulation of *HSP70MAJ* expression compared with those in the control sediment, while worms exposed to warming showed the inhibition of *HSP70MAJ* expression compared with those at control temperature.

Moderate evidence was found for the interaction effect on the expression level of *HSP70MIN* (Fig. 3g), in which warming led to a greater increase in the *HSP70MIN* transcript level in worms exposed to 20 mg kg^{-1} of Cu than in those exposed to other Cu levels.

There was weak evidence for the effect of Cu on the expression level of *HBL2* (Fig. 3h), in which *HBL2* transcript levels were highest in worms exposed to 10 mg kg^{-1} of Cu and lowest in those exposed to 20 mg kg^{-1} of Cu.

3.7. Patterns of biomarker responses

There was a lack of evidence for the effect of the Cu × temperature

a **Jan**

	carbs	lipids	proteins	Ea	ETS	CEA	MGO	MDA	PC
Cu0-T12	0	0	0	0	0	0	0	0	0
Cu0-T20	-0.39	-0.06	-0.12	-0.27	3.57	-2.16	-1.44	-0.04	0.27
Cu10-T12	-0.04	0.48	0.85	0.71	1.96	-0.93	-0.71	-0.02	1.56
Cu10-T20	-0.83	0.03	0.02	-0.52	5.86	-2.76	-1.91	0.72	0.48
Cu20-T12	-0.78	0.24	0.64	0.16	1.65	-1.15	-0.63	0	1.6
Cu20-T20	-0.42	0.24	0.86	0.47	7.66	-2.67	-1.26	-0.15	2.3

b **Mar**

	carbs	lipids	proteins	Ea	ETS	CEA	MGO	MDA	PC
Cu0-T12	0	0	0	0	0	0	0	0	0
Cu0-T20	-0.68	-0.04	-0.33	-0.58	1.39	-1.59	-1.06	0.77	-0.09
Cu10-T12	0.4	0.03	0.11	0.22	0.22	-0.19	-0.74	0.43	-0.01
Cu10-T20	-0.21	-0.05	-0.28	-0.31	2.92	-2.31	-1.62	0.7	-0.02
Cu20-T12	0.23	0.28	0.25	0.34	0.23	-0.29	-0.45	0.9	-0.22
Cu20-T20	-0.15	0.13	0.16	0.06	2.06	-1.89	-1.53	0.69	-0.09

	ATP7A	*CCS*	*MTS*	*MTL*	*GSTO1*	*HSP70MAJ*	*HSP70MIN*	*HBL2*
Cu0-T12	0	0	0	0	0	0	0	0
Cu0-T20	-0.2	-0.02	-0.31	-0.34	-0.65	-0.36	1.15	-0.14
Cu10-T12	0.02	0.12	0.06	-0.2	0.2	-0.08	0.62	1.59
Cu10-T20	-0.2	29.34	13.13	-0.24	-0.32	-0.75	0.91	0.59
Cu20-T12	-0.23	0.38	0.01	-0.2	0.48	0.63	0.04	-0.25
Cu20-T20	0.46	0.94	0.37	-0.17	-0.06	0.22	2.55	-0.21

Glass's Δ: 2, 1, 0, -1, -2

c

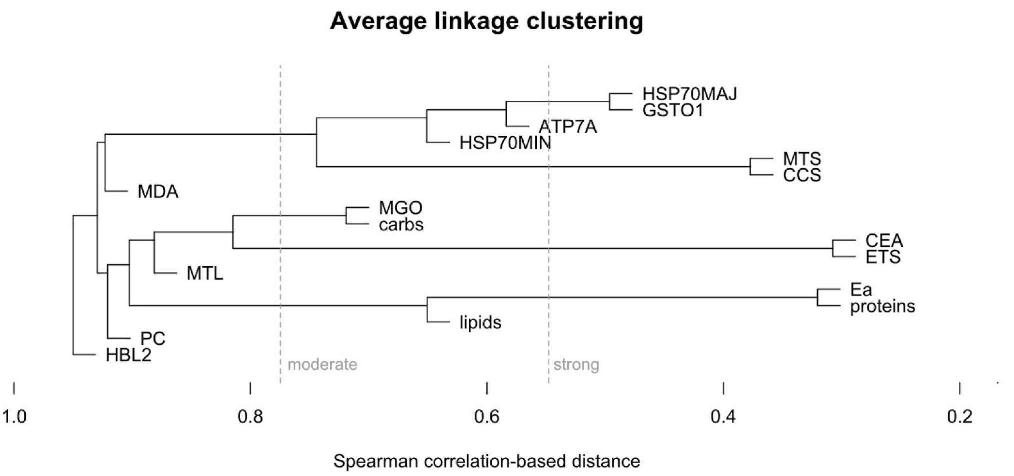

Fig. 4. Summary of biomarker responses of *Hediste diversicolor* to Cu-spiked sediments and elevated temperature with Glass's delta effect size (a, b) and correlation-based clustering (c). Glass's delta (Δ) was calculated against the responses in the shared control condition (0 mg kg^{-1} of Cu at 12 °C) and is 0 for this group. Smaller distances in the cluster analysis imply stronger correlations among biomarker responses, and dashed lines indicate thresholds for correlation strength (Akoglu, 2018). Exposure groups: Cu0, Cu10, and Cu20 for 0, 10, and 20 mg kg^{-1} of Cu and T10 and T20 for 12 and 20 °C, respectively.

interaction on bioenergetic and cellular responses in both experiments ($p > 0.1$, Fig. 2 and S3). Interaction effects, however, were found with stronger evidence in some molecular markers including *ATP7A*, *CCS*, *MTS* and *HSP70MIN* (Fig. 3). In these cases, the effects of the combined Cu (either 10 or 20 mg kg^{-1}) and warming exposures were more extreme than the total effects of the separate exposures, suggesting synergistic interactions (Fig. 4b). Both experiments also showed that ETS, CEA, and MGO were more affected than other studied biomarkers as indicated by notably large effect sizes (Fig. 4a and b).

Some biomarkers showed strong correlations ($|r_s| \geq 0.7$) in their responses (Fig. 4c). ETS activity and CEA were negatively correlated ($r_s = -0.91$), while Ea was positively correlated with the protein level ($r_s = 0.90$). Positive correlations existed between the expression levels of *CCS* and *MTS* ($r_s = 0.86$) and *GSTO1* and *HSP70MAJ* ($r_s = 0.75$). At the threshold distance corresponding to moderate correlations ($|r_s| \geq 0.4$), four clusters were identified, comprising the expression of *ATP7A*, *CCS*, *MTS*, *GSTO1*, *HSP70MAJ*, and *HSP70MIN* (1st cluster), carbohydrate and MGO levels (2nd cluster), ETS activity and CEA (3rd cluster), and Ea, proteins and lipids (4th cluster).

4. Discussion

4.1. Exposure conditions and Cu bioavailability

The sandy sediment in our study is not preferred by ragworms, which usually inhabit more muddy substrates (median grain size of ~50–225 µm, Van Colen et al., 2014). However, its limited fine grains and organic matter ensured low levels of contaminants that could mask the effects of spiked Cu (Simpson et al., 2004). Although the Cu content was not measured in the sediment before spiking, it should be as low as in the control sediment at the end of the exposures ($M = 1.0$ mg kg^{-1}). Because sandy sediments typically lack the main metal-binding solid phases (sulfides, iron hydroxides, and organic carbon, Seibert et al., 2019; Strom et al., 2011), we presume that a large fraction of the added Cu would have remained in the dissolved forms. Cu hydrolysis often causes the pH reduction in sediments after spiking, increasing the redox potential and favoring metals in dissolved phases (Hutchins et al., 2007). The lack of pH adjustment and the relatively short incubation time (Simpson et al., 2011) in our study could therefore explain the high Cu concentrations in pore waters (>100 µg L^{-1}). Cu levels in sediments and pore waters after spiking and during exposures could be higher than measured levels at the end, given the weekly renewal of overlying waters that likely removed copper from the beakers. Notably, the environmental Cu concentrations were generally lower in the Mar experiment than in the Jan experiment. This could be explained by the higher worm density in the Mar experiment, which enhanced bioturbation activity (François et al., 2002) and leakage of Cu into the overlying water. Furthermore, although elevated temperature often facilitates metal accumulation in particulate phases (Warren and Zimmerman, 1994), we found no effect of temperature on environmental Cu concentrations in either experiment, probably due to the mentioned shortage of sediment binding sites.

Given the low amount of sediment-bound Cu, we expect that Cu exposures in ragworms occurred primarily via the dissolved route (pore water, burrow water, and overlying water) rather than sediment ingestion (Simpson et al., 2011; Wang and Fisher, 1999). Because the artificial seawater used in our study did not have organic ligands that can form stable complexes with copper (II) (Paul et al., 2021; Waugh et al., 2022; Whitby et al., 2017), our experimental settings promoted the abundance of free Cu^{2+}, the most bioavailable copper species, in these water compartments. For *H. diversicolor*, the reported 96-h median lethal concentrations (LC_{50}) of Cu^{2+} in seawater without sediments vary from 125 µg L^{-1} (20 °C, salinity 20, Moreira et al., 2005) to 480 µg L^{-1} (7 °C, salinity 17.5, Jones et al., 1976) and >720 µg L^{-1} (12–22 °C, salinity 15.25, Ozoh, 1992). Thus, our observation that worm survival after three weeks was not affected by several hundred µg L^{-1} of Cu in pore waters seems surprising. A possible reason for the high tolerance of worms is that they have genetically adapted (Dinh et al., 2022) to the high contamination level in their habitat (Fig. S1, Abraham et al., 2017). Ozoh (1992) also found that in the presence of sandy sediment, the 96-h LC_{50} of Cu^{2+} increased ~3–5 times to several thousand µg L^{-1}. The reduced toxic effects of Cu in sandy sediment could be related to the secreted mucus that worms used for lining their burrow galleries, which can act as a protective layer that adsorbs dissolved Cu and reduces its bioavailability (Fernandes et al., 2009; Geffard et al., 2005; Mouneyrac et al., 2003).

Consistent with previous studies (Amiard et al., 1987; Geffard et al., 2005; Zhou et al., 2003), we observed positive correlations between Cu concentrations in worms and their environment (especially dissolved Cu in pore waters). Worms in the Jan experiment generally had higher baseline Cu body burdens than those in the Mar experiment, likely due to temporal variations of contamination levels in the field (Aydin-Onen et al., 2015). Cu bioaccumulation was also evident, as Cu body burdens were ~10-fold and 100-fold higher than those in sediments and pore waters, respectively. We found no effect of temperature on Cu body burdens in both experiments, probably because this species has relatively constant metal bioaccumulation parameters across different populations and these parameters are insensitive to temperature (Kalman et al., 2010; Rainbow et al., 2009). Cu toxicity, however, depends on the metabolically available Cu rather than the total accumulated amount (Berthet et al., 2003; Rainbow, 2002).

4.2. Cu homeostasis under temperature effects

Due to the reducing environment in the cell, imported copper is predominantly Cu^{+}, which is toxic and must be strictly regulated (Nevitt et al., 2012; Turski and Thiele, 2009). Cu homeostasis was examined in our study via the mRNA expression levels of related genes (McQuillan et al., 2014) including *ATP7A*, *CCS*, *MTS*, *MTL*, and *GSTO1*. The Cd/Se metallothionein (MTS) and atypical metallothionein-like protein (MTL) help buffer excess Cu in the cytoplasm (Amiard et al., 2006). The active Cu transporter ATP7A delivers intracellular Cu to the Golgi apparatus for cuproprotein synthesis and exports excess Cu from the cell (Zeid et al., 2019). The chaperone CCS inserts Cu into superoxide dismutase, which is important in the defense against reactive oxygen species (Banci et al., 2009). The Phase II omega class glutathione *S*-transferase (GSTO1) conjugates the common Cu ligand glutathione and detoxifies electrophilic compounds formed during oxidative stress (Hayes and Strange, 2000). We found little alterations in the transcript levels of these genes in response to Cu exposure at normal temperature. Our observation is consistent with the previous studies in which ragworms exposed to sediments from certain Cu-contaminated sites showed no difference in gene expression compared with the reference sites (Breton and Prentiss, 2019; McQuillan et al., 2014). The metallothionein protein level and GST enzyme activity in ragworms were also not affected by metal exposure in several field and laboratory studies (Bouraoui et al., 2009; Poirier et al., 2006; Solé et al., 2009). These findings taken together suggest that Cu homeostasis-associated genes might not be sensitive markers of Cu stress (Green Etxabe et al., 2021).

At the elevated temperature, the pattern of gene expression became more complicated with some synergistic effects. The combined exposure of 20 mg kg^{-1} of Cu and warming triggered a weak upregulation of *ATP7A*, suggesting an enhanced Cu efflux. Additionally, we detected a spike in the expression levels of *CCS* and *MTS* in the combined exposure of 10 mg kg^{-1} of Cu and warming. These unexpected non-monotonic responses to Cu might imply that at the 10-mg kg^{-1} exposure the accumulated intracellular Cu necessitated CCS and MTS synthesis (e.g., to prevent oxidative stress), whereas at the 20-mg kg^{-1} exposure, the cells' higher Cu efflux reduced that need. Notably, warming led to the downregulation of *GSTO1* regardless of Cu exposure. Although the exact mechanisms remain unknown, the reduction of GST enzyme activity in response to elevated temperature has been documented in other species

(Dorts et al., 2012; Madeira et al., 2013).

We measured the expression of *HSP70MAJ*, *HSP70MIN*, and *HBL2* encoding the major and minor forms of stress-70 proteins (Sanders, 1990) and linker chain L2 of the giant extracellular hemoglobin (Suzuki et al., 1994) following the suggestion that they might be conserved biomarkers of metal stress in polychaetes (Green Etxabe et al., 2021). However, we only observed inconsistent or weak responses of *HSP70MAJ* and *HSP70MIN* to Cu exposure. Warming had opposite effects on the expression of two stress-70 forms (i.e., inhibited the major form and induced the minor form) implying their different roles during heat stress. *HBL2* also did not present an ideal marker of metal exposure given its induction at 10 mg kg^{-1} of Cu but downregulation at 20 mg kg^{-1} and its very variable expression.

4.3. Cellular effects of Cu and warming exposures

While the molecular markers failed to differentiate the effects of Cu, we found a clear elevation of mitochondrial ETS activity, a proxy of cellular energy demand correlated to the standard metabolic rate (Fanslow et al., 2001; Sokolova, 2021) in response to Cu and warming exposures. The increased ETS activity due to warming is unsurprising given the dependence of biochemical processes on temperature in ectothermic animals (Willmer et al., 2005). However, the enhanced ETS activity in worms exposed to Cu might suggest the higher cellular maintenance costs to regulate Cu homeostasis and alleviate its toxic effects. Increased ETS activity in ragworms has also been recorded following exposure to various contaminants including mercury, carbon nanomaterials, nanoplastics, pharmaceuticals, as well as acidified seawater (Bhuiyan et al., 2021; De Marchi et al., 2017, 2018; Freitas et al., 2016, 2017; Pires et al., 2016, 2022; Silva et al., 2022). We did not find a considerable accumulation of MDA and PC, which are markers of lipid and protein oxidation, respectively, indicating that Cu-induced oxidative stress did not exceed the antioxidant capacity of ragworms. The lack of oxidative stress damage in ragworms despite exposure to contaminants was also observed in many studies, implying the high tolerance of this species to oxidative stress (Buffet et al., 2011; Buffet et al., 2013; Durou et al., 2007; Nunes and Costa, 2019; Pires et al., 2022; Urban-Malinga et al., 2021, 2022).

Given the higher energy demand during Cu and warming exposures, one would expect the declines in energy reserves and total available energy (Ea) as found in some other studies on ragworms (De Marchi et al., 2018; Durou et al., 2005; Durou et al., 2007; Freitas et al., 2016, 2017; Pook et al., 2009). However, we found only a weak reduction of carbohydrate content and Ea in the Mar experiment in responses to elevated temperature, probably due to the utilization of readily available glucose to fuel cellular respiration (Pires et al., 2022). In general, the worms were able to sustain their energy storage under Cu and warming exposures. This finding is consistent with other research which suggests that worms living in contaminated environments can still have high levels of energy reserves when the food is abundant (Mouneyrac et al., 2006, 2010; Pires et al., 2022). In our case, worms were fed daily during the exposure and the aeration of overlying water and the shallow depth of sediments allowed high dissolved oxygen levels for aerobic respiration. These exposure conditions, therefore, facilitated the ATP supply, which can be used for cellular maintenance or stored in energy reserves (Sokolova et al., 2012; Sokolova, 2021).

Due to the little variations in Ea, the energy budget index CEA was mostly dependent on ETS activity as shown by their strong negative correlation. CEA can be roughly interpreted as the time that worms can survive after the energy influx is stopped and the worms begin to deplete their energy reserves at a constant rate. CEA therefore could be an ecologically relevant biomarker (De Coen and Janssen, 1997; Pook et al., 2009; Verslycke et al., 2004) indicating the susceptibility of worms to environmental stressors under field conditions. Both experiments showed that Cu exposure and warming reduced CEA, prompting potentially adverse impacts of Cu and warming at higher biological organization levels.

MGO was another biomarker strongly affected by warming. This reactive glycolytic by-product can form adducts with proteins, lipids, and nucleic acids, causing pathophysiological issues (Allaman et al., 2015). Increased glucose metabolism is expected to increase the formation of MGO (Rabbani et al., 2020), which could explain the correlation between MGO and carbohydrate content in our study. Surprisingly, we observed the MGO decrease in response to warming, probably due to the enhanced activity of the glyoxalase system that detoxifies MGO (Thornalley, 1990).

4.4. Combined effects and research replicability

Interactive (synergistic) effects of Cu and warming were only detected in some molecular responses. At the cellular level, the combined effects of Cu and warming were additive with temperature as a stronger driver. Little effects were found on energy reserves and oxidative stress damage, whereas impacts on the energy consumption rate, energy balance, and MGO were large. Notably, although both experiments were conducted at different times and with different pools of ragworms, they resulted in similar biomarker response patterns.

Previous studies on the combined effects of metal exposure and warming on macrobenthos showed both additive and non-additive effects (Biscéré et al., 2017; da Silva Fonseca et al., 2019; Martino et al., 2021; Tracy et al., 2020; Wang et al., 2016). Systematic reviews on the combined effects of multiple stressors on aquatic organisms demonstrated diverse patterns in the responses, influenced by various factors such as stressor identity, level of biological organizations, and evolutionary history of animals (Crain et al., 2008; Dinh et al., 2022; Morris et al., 2022). Mack et al. (2022) suggested that the detection of interactive effects is influenced by research design rather than reflecting actual ecological processes.

The lack of interaction between Cu exposure and elevated temperature on cellular responses in our study therefore could be explained by several reasons. For example, temperature did not affect the uptake and elimination of Cu in *H. diversicolor* (Kalman et al., 2010; Rainbow et al., 2009). The generally weak effect of Cu and the absence of other stressors such as food scarcity and hypoxia may also leave less room for the interactive effects of Cu and temperature on worm energy balance.

4.5. Conclusions and future directions

We found that Cu exposure and elevated temperature had little impact on the expression of genes related to Cu homeostasis. Our study highlights the need to look for more conserved molecular markers of metal exposure in estuarine polychaetes. By contrast, our data showed additive and replicable effects of Cu exposure and warming on the energy balance of ragworms. We suggest that energy metabolism can be a focus to explore the effects of multiple stressors, because energy-related biomarkers have higher sensitivity and higher predictive power for ecological consequences. This research also indicates that warming could be a potent stressor for infaunal species. A more realistic study design, such as the use of field-collected sediment with multiple contaminants in laboratory exposure, can also be applied to examine the combined effects of sediment contamination and warming.

Credit author statement

Duy Nghia Pham: Methodology, Software, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Julie Angelina Kopplin**: Methodology, Investigation, Data Curation, Writing - Review & Editing. **Olaf Dellwig**: Methodology, Validation, Investigation, Data Curation, Writing - Review & Editing. **Eugene P. Sokolov**: Methodology, Validation, Writing - Review & Editing, Supervision. **Inna M. Sokolova**: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Research data are publicly available on Zenodo at https://doi.org/10.5281/zenodo.7978848.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121964.

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Hot and heavy: Responses of ragworms (*Hediste diversicolor*) to copper-spiked sediments and elevated temperature

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Supplementary Material

1. Introduction

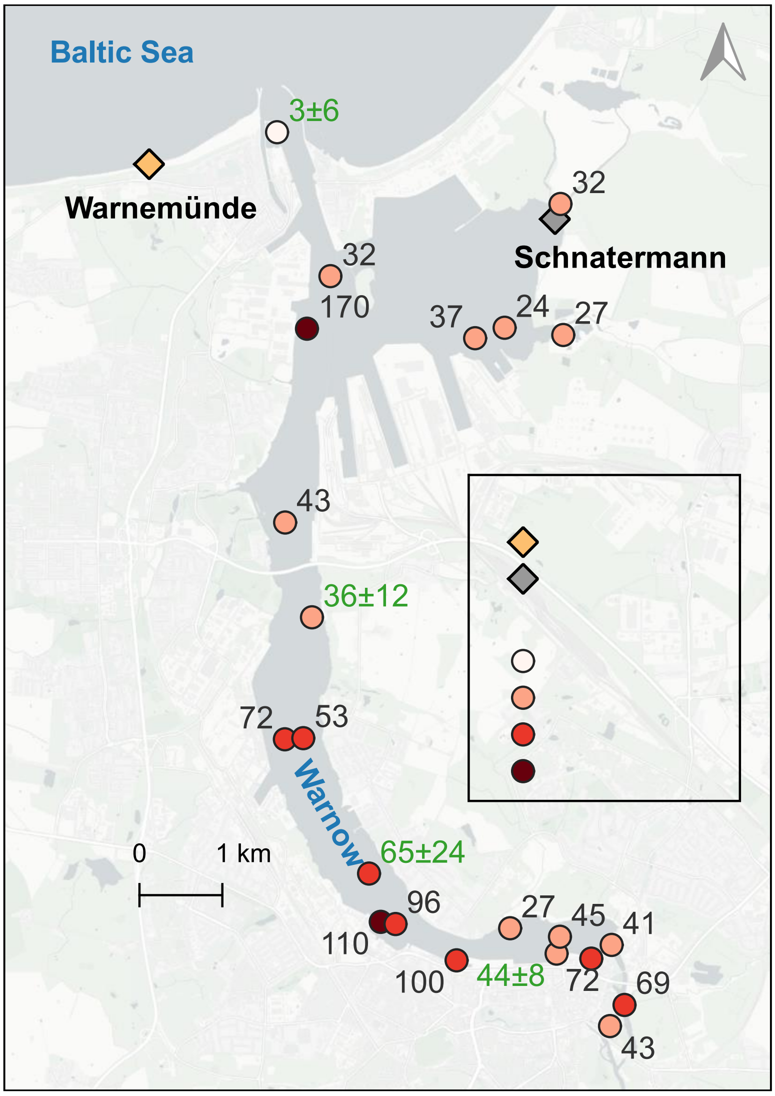

Figure S1. Collection sites of sediment and animals for the experiments and monitoring sites for Cu content in surface sediments (<2 mm) of the Warnow estuary, Germany. Data from our own analyses of samples collected by Rönspieß et al. (2020) in 2017 (*M±SD*, in green) and from the state database (LUNG, 2023) during 2002-2013. Basemap from https://carto.com/.

2. Materials and methods

2.5. Cu analyses

Cu was extracted from 200 mg of the homogenized sediment samples by adding 10 mL of 0.5 M HCl and shaking for 1 h at room temperature. The 0.45 µm-filtered solutions were diluted 50-fold with 2% v/v HNO_3. Pore water samples were diluted 25-fold with 2% HNO_3. Dried worm homogenates (55-300 mg) were digested with 3 mL of HNO_3 and 1 mL of $HClO_4$ in closed Teflon vessels at 180°C for 12 h. After evaporating the acids on a heated block at ~180°C and fuming three times with 6 M HCl, 20 mL of 2% HNO_3 was added to the near-dry residues. Rhodium was added to all samples as an internal standard. ICP-MS was performed with external calibration, using helium as a collision gas to minimize molecular interferences (Dellwig et al., 2019; Lagerström et al., 2013). For measurements of sediment samples, the trueness and precision based on the international reference material SGR-1b (USGS) were 1.6 – 2.1% and 1.4 – 1.5%, respectively. For measurements of pore water samples, the trueness and precision based on the Atlantic seawater (Ocean Scientific International, UK) spiked with 4.8 µg L^{-1} of Cu were 1.9 – 2.8% and 0.4 – 1.1%, respectively. For measurements of worm samples, the trueness and precision based on the international reference material SGR-1b were 2.7% and 0.2%, respectively.

2.6. Colorimetric assays

Briefly, carbohydrate content in the cytoplasmic fraction of homogenates expressed as glucose equivalents, was determined by the reaction with phenol-sulfuric acid forming a 492 nm absorbing product (Masuko et al., 2005). Lipid content in the chloroform-methanol extraction of homogenates (Folch et al., 1957) was assessed by the sulfo-phospho-vanillin reaction producing a 490 nm absorbing compound (Van Handel, 1985). Protein content in the cytoplasmic fraction expressed as bovine serum albumin equivalents, was quantified by the Bradford assay yielding a 595 nm absorbing protein-dye complex (Bradford, 1976). The level of methylglyoxal (MGO) in the cytoplasmic fraction was evaluated by the reaction with Girard's reagent T in borax solution that produces a disubstituted compound measurable at 325 nm using MGO standards (Mitchel and Birnboim, 1977). Malondialdehyde (MDA) level in the homogenates was measured by the reaction with thiobarbituric acid forming a product measurable at 530 nm with the extinction coefficient of 156 mM^{-1} cm^{-1} (Buege and Aust, 1978). Protein carbonyl (PC) level was determined by the reaction with 2,4-dinitrophenylhydrazine (DNPH) that forms protein-dinitrophenylhydrazone measurable at 370 nm using the extinction coefficient of 22 mM^{-1} cm^{-1} (Levine et al., 1990). The activity of mitochondrial electron transport system (ETS) expressed as oxygen consumption equivalents, was assessed by the NAD(P)H-dependent reduction of iodonitrotetrazolium chloride (INT) to formazan monitorable at 490 nm using the extinction coefficient of 15.9 mM^{-1} cm^{-1} (De Coen and Janssen, 1997). All assays were performed using a microplate reader (SpectraMax iD3, Molecular Devices, Germany).

2.7. Quantitative reverse transcription PCR (RT-qPCR)

The anterior part was lysed in TRI Reagent (Zymo Research, USA) in a FastPrep-24 homogenizer (MP Biomedicals, Germany) for RNA extraction. DNA was removed (TURBO DNA-free Kit, Thermo Fisher Scientific) and cDNA was synthesized from 1 µg of total RNA (RevertAid RT Reverse Transcription Kit, Thermo Fisher Scientific) in a thermal cycler (peqSTAR 96 Universal Gradient, PEQLAB Biotechnologie, Germany). Transcript levels were determined by quantitative PCR (Biozym Blue S'Green qPCR Kit Separate ROX, Biozym Scientific, Germany) in a StepOnePlus System (Thermo Fisher Scientific). Post-amplification melting curve analyses were conducted to confirm the specificity of the PCR product. Gene-specific amplification efficiencies (E) were computed using a cDNA standard dilution series (Pfaffl, 2001).

Table S1. Permutation tests for the effects of Cu, temperature (T), and their interaction (int) on the expression (quantification cycle) of reference genes in *Hediste diversicolor* at the end of the exposure in the Mar experiment[1].

reference gene	Factor	df_{num}	F	p
GAPDH	Cu	2	0.69	0.70
	T	1	1.08	0.38
	int	2	0.64	0.70
HIS3	Cu	2	0.24	0.80
	T	1	2.04	0.18
	int	2	0.96	0.40

[1] df_{den} are not available for linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

3. Results

3.1. Cu concentrations

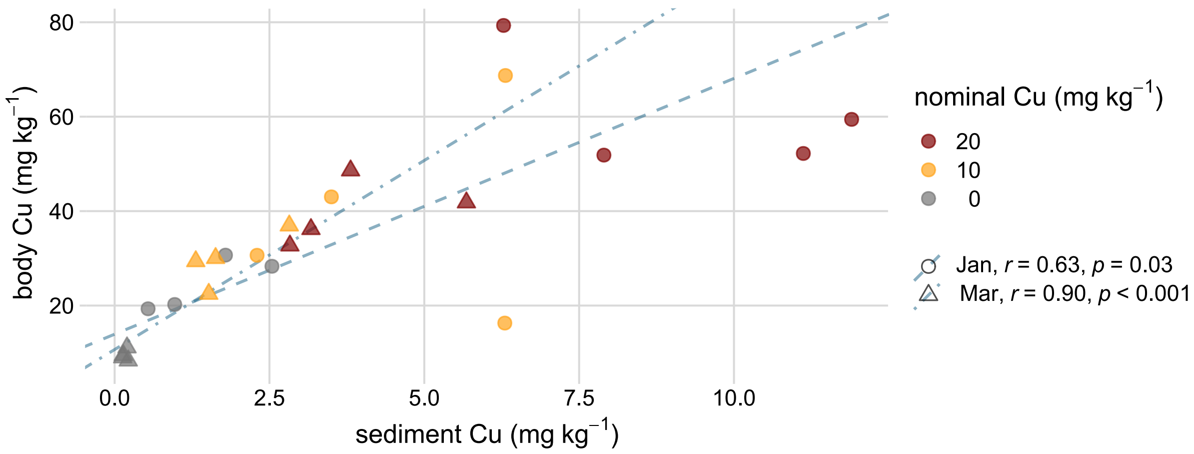

Figure S2. Correlation between total Cu contents in surface sediments and total Cu body burdens in worms at the end of the exposures in the Jan (circles) and Mar (triangles) experiments. Sediments were spiked with Cu at nominal contents of 0, 10, and 20 mg kg^{-1}. Standard deviation lines (Freedman et al., 2007), Pearson correlation and permutation *p*-values are given for each experiment.

Table S2. Permutation tests for the effects of nominal Cu content, exposure temperature (T), and their interaction (int) on total Cu contents in surface sediments, dissolved Cu concentrations in pore waters of bottom sediments, and total Cu body burdens in worms at the end of the exposures in the Jan and Mar experiments[2].

Medium	Experiment	Factor	*F*	*p*
Sediment	Jan	Cu	37.10	**< 0.001**
		T	0.88	0.37

[2] df_{num} are the same as in Table S1, df_{den} = 24 for sediment and pore water and 6 for body.

Medium	Experiment	Factor	F	p
		int	0.51	0.62
Sediment	Mar	Cu	19.30	**< 0.001**
		T	0.13	0.74
		int	0.61	0.56
Pore water	Jan	Cu	43.16	**< 0.001**
		T	0.03	0.89
		int	0.02	0.98
Pore water	Mar	Cu	39.74	**< 0.001**
		T	0.54	0.50
		int	2.15	0.14
Body	Jan	Cu	8.50	**0.02**
		T	1.14	0.33
		int	3.16	0.11
Body	Mar	Cu	31.80	**0.002**
		T	1.48	0.28
		int	0.50	0.57

3.2. Survival

Table S3. Survival of *Hediste diversicolor* at the end of the exposures in the Jan and Mar experiments.

Experiment	Number of lives	12 °C	20 °C
Jan, n = 15	0 mg kg^{-1}	11	11
	10 mg kg^{-1}	11	8
	20 mg kg^{-1}	7	9
Mar, n = 20	0 mg kg^{-1}	18	17
	10 mg kg^{-1}	16	15
	20 mg kg^{-1}	18	17

Table S4. Permutation tests for the effects of Cu, temperature (T), and their interaction (int) on the survival of *Hediste diversicolor* at the end of the exposures in the Jan and Mar experiments[3].

[3] df_{num} are the same as in Table S1, df_{den} are not available for linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

Experiment	Factor	*F*	*p*
Jan	Cu	1.22	0.29
	T	0.04	0.91
	int	0.84	0.51
Mar	Cu	0.93	0.29
	T	0.57	0.32
	int	0.01	0.97

3.3-6. Energy reserves-Molecular markers of metal exposure and stress

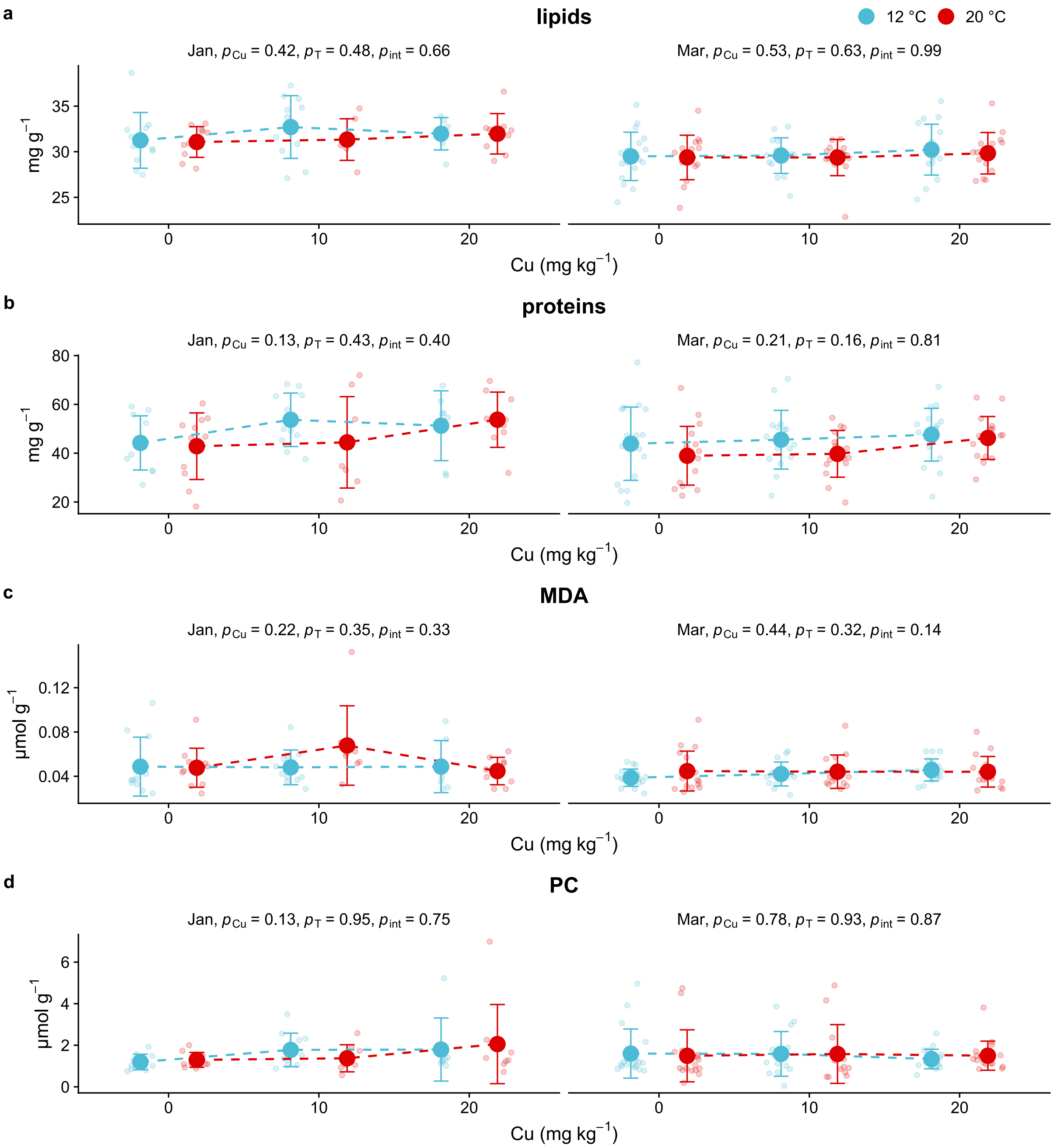

Figure S3. Effects of Cu-spiked sediments and elevated temperature on lipid and protein contents and malondialdehyde (MDA) and protein carbonyl (PC) levels of *Hediste diversicolor* in the Jan and Mar experiments. Data are presented with individual observations and mean ± standard deviation. Permutation p-values are given for Cu, temperature (T), and their interaction (int). F-statistics are provided in Table S5.

Table S5. F-statistics in permutation tests for the effects of Cu, temperature (T), and their interaction (int) on the biomarker responses of *Hediste diversicolor* at the end of the exposures in the Jan and Mar experiments[4].

Biomarker	Experiment	Cu	T	int
carb	Jan	0.84	1.18	1.03
	Mar	1.58	6.37	0.26
lipid	Jan	0.88	0.52	0.44
	Mar	0.65	0.23	0.01
protein	Jan	2.36	0.65	0.98
	Mar	1.68	2.32	0.21
Ea	Jan	0.81	1.50	1.56
	Mar	2.26	5.84	0.24
ETS	Jan	7.33	39.08	1.26
	Mar	3.33	53.05	1.96
CEA	Jan	8.41	97.07	0.74
	Mar	2.67	112.72	1.07
MGO	Jan	0.89	12.88	0.51
	Mar	2.42	15.78	0.06
MDA	Jan	1.14	0.77	0.95
	Mar	0.80	0.99	2.19
PC	Jan	2.08	0.01	0.38
	Mar	0.23	0.01	0.12
ATP7A	Mar	0.84	0.34	4.00
CCS	Mar	29.97	31.92	30.21
MTS	Mar	14.99	14.89	14.72
MTL	Mar	0.07	0.94	1.13
GSTO1	Mar	1.79	6.15	0.06
HSP70MAJ	Mar	6.61	6.16	0.24
HSP70MIN	Mar	1.30	12.29	3.32
HBL2	Mar	2.10	0.46	0.37

[4] df_{num} are the same as in Table S1, df_{den} are not available for linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

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5.2 Publication 2

Pham, **D.N.**, Ruhl, A., Fisch, K., El Toum, S., Heise, S., Sokolova, I.M., 2024. Effects of contamination and warming on ragworms *Hediste diversicolor*: A laboratory experiment with Oder estuary sediments. Estuarine, Coastal and Shelf Science 299, 108702. https://doi.org/10.1016/j.ecss.2024.108702

Declaration of personal contributions

I hereby declare that my personal contributions, as defined in the Contributor Role Taxonomy (CRediT), to **Publication 2** are as follows:

- I took a leading role in **Methodology**, **Investigation**, and **Data curation**.
- I was solely responsible for **Software**, **Formal Analysis**, **Visualization**, and **Writing – original draft**.

Rostock, 2025-04-06

Duy Nghia PHAM

Publication 2

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Effects of contamination and warming on ragworms *Hediste diversicolor*: A laboratory experiment with Oder estuary sediments

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Polychaete

A B S T R A C T

Knowledge about the effects of sediment contamination on estuarine benthos has come mainly from observational studies and spiking experiments. There is a lack of experimental studies with field-contaminated sediments, especially those assessing the combined effects of contamination and other environmental stressors. Here we investigated the biomarker responses of ragworms *Hediste diversicolor* to contaminated sediments and warming. Ragworms were exposed to sediments with different contamination levels from the Oder estuary (German-Polish border) at 10 and 20 °C for three weeks. The most contaminated sediments induced dicarbonyl stress, but did not result in enhanced detoxification, significant oxidative stress, or impaired energy balance. Warming altered antioxidant defense in ragworms and increased their energy reserves. The effects of contamination and warming were generally mild, and the interactive effects were absent. This study suggests the relatively high resilience of *H. diversicolor* to multiple stressors.

1. Introduction

While providing us with important ecosystem services and economic values, estuaries worldwide are under intense pressure from human activities (Barbier et al., 2011; Booi et al., 2022; Borgwardt et al., 2019; Freeman et al., 2019). Urbanization and coastal development have led to increased contamination, with many hazardous substances accumulating in estuarine sediments (Chapman et al., 2013; Sun et al., 2012). Concurrently, anthropogenic climate change has caused rapid warming of surface waters, especially in shallow, temperate estuaries (Kurylyk and Smith, 2023; Oczkowski et al., 2015; Scanes et al., 2020). Such stressors threaten estuarine organisms, including sediment-dwelling ectothermic invertebrates (Birchenough et al., 2015; Fujii, 2012; Lacoste et al., 2023). These benthic organisms play critical roles in estuarine ecosystems, contributing to sediment modification, organic matter cycling, and energy transfer in food webs (Herman et al., 1999; Pinto et al., 2014; Reiss and Kröncke, 2005). To maintain the integrity of estuaries (Borja et al., 2011), understanding the effects of sediment contamination and seawater warming on estuarine benthos is urgently needed.

The ragworm *Hediste diversicolor* is a common infaunal species with ecological and commercial importance in European estuaries (Beyer and Sundt, 2006; Scaps, 2002; Teixeira et al., 2022; Wang et al., 2020). This species is also the most studied polychaete in ecotoxicology, and thus an ideal model to examine the toxic effects of both conventional and emerging contaminants (Medeiros Aguiar et al., 2023; Pires et al., 2022; Silva et al., 2020). However, our survey of original articles on the effects of contaminated sediments on *H. diversicolor* showed that most studies (65%) were observational, while fewer studies were experimental, using either spiked sediments (24%) or field-contaminated sediments (11%) (Table S1). Observational studies may fail to identify causal effects of contaminated sediments due to confounding bias from environmental variables at the study sites (Hatami, 2019). Meanwhile, experimental studies with a single contaminant in the spiked sediments may not represent realistic exposures (Chapman, 2002; Weis and Palmquist, 2021). A more balanced approach can be obtained by experimental studies with field-contaminated sediments, but such studies are unfortunately underrepresented. Studies examining the effects of

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contaminated sediments in the context of seawater warming are even rarer (Moreira et al., 2006), despite potential interactions between the two stressors (e.g., synergism and antagonism, Burton and Johnston, 2010; Crain et al., 2008; Schiedek et al., 2007).

Here we investigated the responses of ragworms to combined exposures to contaminated sediments from the Oder estuary and to warming under laboratory conditions. The Oder River originates in the Czech Republic and flows through Poland and Germany before reaching the Baltic Sea (Fig. 1). The Oder catchment area is home to many populated cities (e.g., Wrocław and Szczecin), industrial complexes (e.g., mining, metallurgy, petrochemicals, and shipbuilding), and agricultural lands (e.g., cereal production and livestock farming) (Müller et al., 2002). Consequently, wastewater and land discharges have contaminated the estuarine waters and sediments with various metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and pharmaceuticals (Fisch et al., 2017, 2021; Glasby et al., 2004; Kowalewska et al., 2003; Kucharski et al., 2022a). Due to the dilution effect, the contamination level of the Oder estuary sediments was reported to decrease towards the open sea (El Toum et al., 2023; Kucharski et al., 2022b; Szefer et al., 2009). Thus, we collected sediments along this presumed pollution gradient and exposed ragworms to these sediments at 10 and 20 °C. The 10 °C approximates the mean annual temperature in the Oder estuary (Table S2), and the 20 °C simulates the projected warming of northern European estuaries (up to ~5 °C increase by 2100, plus a monthly temperature range of ~5 °C, IPCC, 2021; Meier et al., 2022). To assess the combined effects of contamination and warming on ragworms, we measured a battery of general biomarkers related to detoxification and antioxidant defense (glutathione *S*-transferase activity, glutathione reductase activity, and total antioxidant capacity), oxidative and dicarbonyl stress (malondialdehyde and methylglyoxal levels), and bioenergetics (carbohydrate, lipid, and protein levels and mitochondrial electron transport system activity) (Colas and Le Faucheur, 2024; Mouneyrac and Amiard-Triquet, 2013; Sokolova, 2013).

2. Materials and methods

2.1. Sediment collection and characterization

Surface sediments were sampled at eight sites in the German part of the Oder estuary (Fig. 1, Table S3), representing three regions with reported increasing contamination levels, i.e., the Pomeranian Bay (least contaminated, control), Peenestrom (moderately contaminated), and Szczecin Lagoon (most contaminated) (El Toum et al., 2023). The top 10 cm of sediments were collected with Van Veen grabs in May–July 2021 and transported to the laboratory in plastic buckets. Physicochemical properties of sediments, including dry bulk density, total organic matter content, C:N mass ratio, and grain size distribution were characterized following standard protocols (Blake, 1965; Davies, 1974; Robertson et al., 1984; Verardo et al., 1990). To verify the contamination gradient, sediments were analyzed for common pesticides (Liess et al., 2022; Wick et al., 2019) using supported liquid extraction (SLE, Chem Elut, Agilent, USA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS, 1290 Infinity II, Agilent and QTRAP 6500+, SCIEX, USA) (Trau et al., 2023). Details are provided in the Supplementary material.

2.2. Animal collection

For a fair comparison of sediment effects, we did not use ragworms

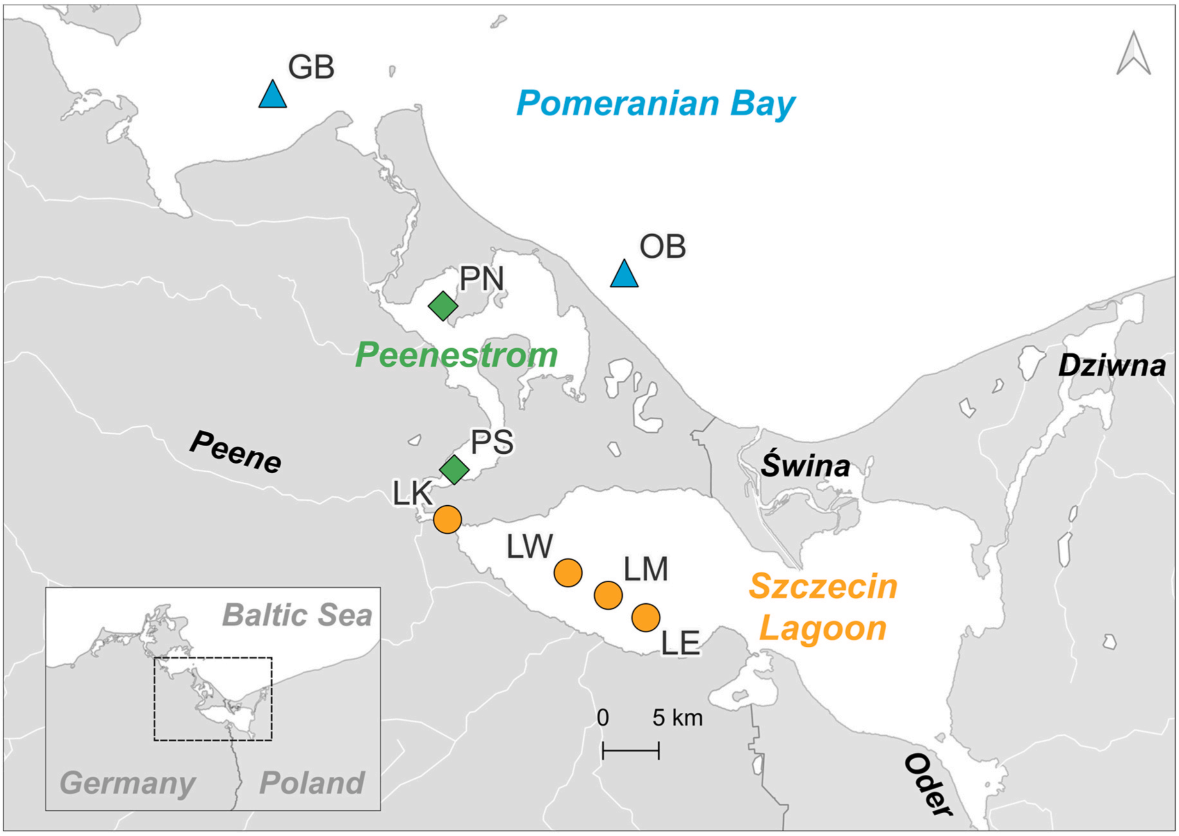

Fig. 1. Sediment sampling sites in the German part of the Oder (Odra) estuary. The Oder River drains into the Szczecin Lagoon and then flows through the Peenestrom, Świna, and Dziwna straits into the Pomeranian Bay, southwestern Baltic Sea. Eight sites were selected along a reported contamination gradient (El Toum et al., 2023): GB – Greifswald Bay and OB – Oder Bay in the Pomeranian Bay (triangles, least contaminated), PN – Peenestrom North and PS – Peenestrom South in the Peenestrom (diamonds, moderately contaminated), and LK – Lagoon Karnin, LW – Lagoon West, LM – Lagoon Middle, and LE – Lagoon East in the Szczecin Lagoon (circles, most contaminated). Basemap from DIVA-GIS.

from any of the Oder estuary populations for the exposure, even though all three study regions are natural habitats of *H. diversicolor* (Gogina et al., 2017; Gosselck and Schabelon, 2007; Obolewski et al., 2009). Instead, ragworms were collected in September 2021 from the nearby Warnow estuary, Germany (54.1728, 12.1414), which is reportedly less contaminated than the Oder (Fisch et al., 2017; Müller and Heininger, 1999) but has a similar temperature regime (Table S2). Ragworms were depurated for ten days in clean, sandy sediment (Pham et al., 2023) and aerated artificial seawater (Pro-Reef, Tropic Marin, Germany) at 15 ± 0.5 °C and salinity 10, similar to their habitat conditions at the time of collection. The worms were fed *ad libitum* with fish food (Marine Flakes, Tetra, Germany) and the seawater was renewed daily.

2.3. Sediment and warming exposures

A two-factor experiment was conducted with an 8 × 2 design, corresponding to eight collected sediment samples and two temperatures, 10 °C (control) and 20 °C (warming). For each treatment combination, three 1-L beakers were prepared, each containing ~400 mL of field sediment and 600 mL of overlying artificial seawater at salinity 10. The beakers were either kept in an environmental chamber at 10 ± 0.5 °C or placed in a water bath at 20 ± 0.5 °C maintained by a circulator (HAAKE C1–K15, Thermo Fisher Scientific, Germany). After one month of sediment incubation, ragworms were randomly allocated to the 16 treatment groups with four worms per beaker (n = 12 per group). The exposure lasted three weeks without food supplementation and seawater renewal (to avoid the introduction or removal of contaminants). The overlying water was aerated to maintain dissolved oxygen saturation (~10.0 mg L^{-1} at 10 °C and ~8.3 mg L^{-1} at 20 °C, pH ~8.4), and deionized water was added daily to compensate for evaporation and maintain salinity at 10. After the exposure, surviving worms were retrieved and cleaned in seawater, then shock-frozen in liquid nitrogen and stored at −80 °C for subsequent biomarker measurements.

2.4. Colorimetric assays

After wet mass (WM) was recorded, individual worms were homogenized in ice-cold buffer using Potter-Elvehjem tissue grinders (Pham et al., 2023). Total antioxidant capacity (TAC) and the levels of malondialdehyde (MDA), methylglyoxal (MGO), carbohydrates (CARB), lipids (LIP), and proteins (PRO) were measured in the homogenates using colorimetric endpoint assays (Bradford, 1976; Buege and Aust, 1978; Folch et al., 1957; Masuko et al., 2005; Mitchel and Birnboim, 1977; Re et al., 1999; Van Handel, 1985). The activities of glutathione *S*-transferase (GST), glutathione reductase (GR), and the mitochondrial electron transport system (ETS) were measured using kinetic assays at 25 °C (De Coen and Janssen, 1997; Habig et al., 1974; Mannervik, 1999). Details are provided in the Supplementary material.

2.5. Data analyses

Differences in sediment characteristics among sites were explored using average linkage hierarchical clustering with Euclidean distance based on the standardized data (Kindt and Coe, 2005). Worm survival, wet mass, and biomarker responses were analyzed using (generalized) linear mixed-effects models (Bates et al., 2015), with sediment and temperature as interacting fixed effects and experimental beaker as a random effect (Colegrave and Ruxton, 2018). A binomial distribution with logit link was used to model the survival, and wet mass was included as a covariate to model biomarker responses (Durou et al., 2005; Kalman et al., 2010; Stomperudhaugen et al., 2009). Model estimated marginal means and 95% bootstrap confidence intervals were reported for each treatment group (Garofalo et al., 2022; Lenth, 2023; Lüdecke et al., 2020), along with Cohen's d effect size (Cohen, 1962; Pham and Sokolova, 2023). The effects were considered small, medium, and large when $|d| \geq 0.2$, 0.5, and 0.8, respectively (Cohen, 1962). Permutation tests for multi-factor analysis of variance were applied to the models (Anderson and Braak, 2003; Ernst, 2004; Manly, 2007; Pham et al., 2022). When the permutation p-values ≤0.001, 0.01, 0.05, or 0.1, evidence against the null hypotheses was considered very strong, strong, moderate, or weak, respectively (Muff et al., 2022). As recommended by Kozak and Powers (2017), post-hoc tests were not conducted.

We performed both analyses with the sediment factor categorized by site (eight levels) and region (three levels). Given their similar results, only analyses using regions as factor levels are presented here for brevity. Analyses using sites as factor levels are available in the Supplementary material (Fig. S3). All analyses were conducted in R v4.3.1 (R Core Team, 2023) with codes adapted from Pham et al. (2023).

3. Results

3.1. Sediment characteristics

The cluster analysis showed that sediments from the same region were more similar in physicochemical properties and pesticide levels (Fig. S1). Sediments in the Pomeranian Bay were characterized by high densities (~1.4 g cm^{-3}) and large proportions of fine sands (125–250 µm) (Fig. 2a–c). The Peenestrom sediments had lower densities (~0.3 g cm^{-3}) and contained mainly very fine sands (63–125 µm), while the Szczecin Lagoon sediments were the lightest (<0.2 g cm^{-3}) and muddiest (<63 µm fraction of 62–76%). Sediments in the Szczecin Lagoon were also the richest in organic matter (19–34%), followed by the Peenestrom sediments (9–10%), while the Pomeranian Bay sediments had little organic matter (<1%) (Fig. 2b). The C:N ratios in organic matter of the Pomeranian Bay sediments were lower (~9) than those of the Peenestrom and Szczecin Lagoon sediments (10–12).

A total of 14 pesticides and pesticide metabolites were detected in the Oder estuary sediments (Fig. S2), of which only one was found in the Pomeranian Bay sediments (dinoterb), while up to ten compounds were found in a sediment from the Szczecin Lagoon (Fig. 2d). Total pesticide contents in the Szczecin Lagoon and Peenestrom sediments were ~11 and ~3 times higher, respectively, than those in the Pomeranian Bay sediments. The most abundant compounds in the sediments were terbutylazine-2-hydroxy (max. 0.47 µg kg^{-1}), carbendazim (max. 0.31 µg kg^{-1}), and dinoterb (max. 0.70 µg kg^{-1}) (Fig. S2).

3.2. Survival and mass

Worm survival in 16 treatment groups at the end of the three-week exposure was 78.6% (SD = 11.4%, Table S4). There was no evidence that survival was affected by sediment or temperature (p > 0.1, Table S5).

No evidence was found for the effect of temperature on worm wet mass, but moderate evidence was found for the effect of sediment (Fig. 3a). Worms exposed to Peenestrom and Szczecin Lagoon sediments had higher wet mass than worms exposed to Pomeranian Bay sediments (Fig. 5).

3.3. Detoxification and antioxidant defense

There was no evidence for the effects of sediment and temperature on GST activity (Fig. 3b). While no evidence was found for the effect of sediment on GR activity, very strong evidence was found for the effect of temperature, with worms exposed to 20 °C having lower GR activity than those exposed to 10 °C (Fig. 3c and 5). Regarding TAC, there was no evidence for the effect of temperature but weak evidence for the effect of sediment, in which worms exposed to Szczecin Lagoon sediments had lower TAC than those exposed to Pomeranian Bay sediments (Fig. 3d and 5).

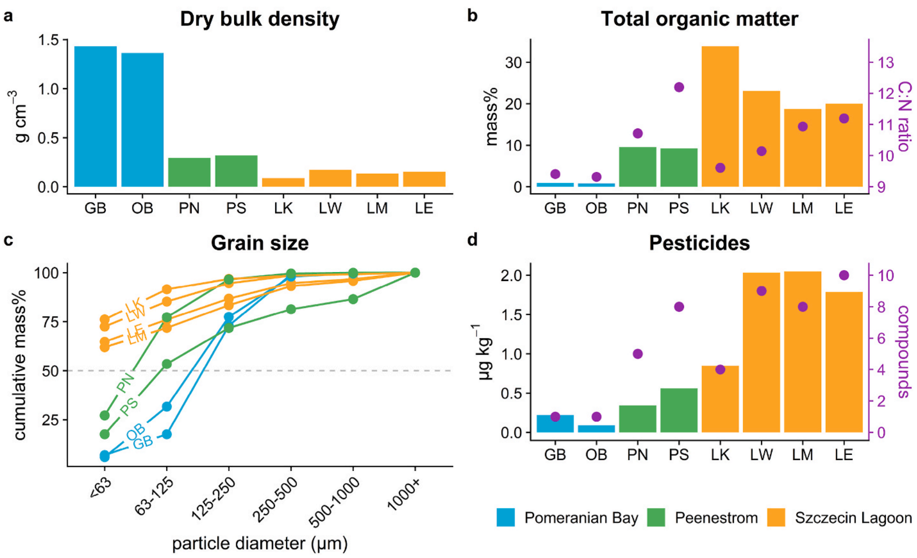

Fig. 2. Physicochemical properties (a–c) and pesticide levels (d) of sediments collected at eight sites in three regions of the Oder estuary (Fig. 1). C:N ratio and number of pesticides (b, d) are represented by dots and secondary y-axes. Grain size classes (c) include 1000+ µm – very coarse sand, 500–1000 µm – coarse sand, 250–500 µm – medium sand, 125–250 µm – fine sand, 63–125 µm – very fine sand, and <63 µm – mud (Wentworth, 1922). Detailed pesticide profiles (d) are shown in Fig. S2.

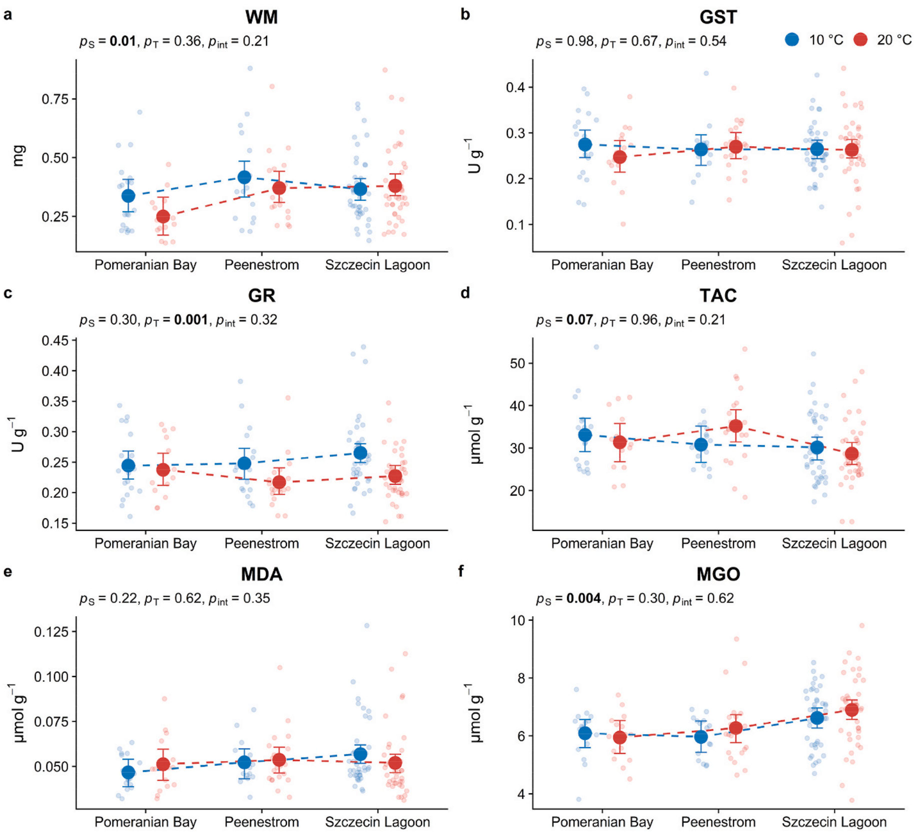

Fig. 3. Effects of Oder estuary sediments and temperature on wet mass (WM), glutathione *S*-transferase (GST) and glutathione reductase (GR) activities, total antioxidant capacity (TAC), and malondialdehyde (MDA) and methylglyoxal (MGO) levels of *Hediste diversicolor*. Data are presented with individual observations, estimated marginal means, and 95% bootstrap confidence intervals. Permutation *p*-values are given for sediment (S), temperature (T), and their interaction (int). Corresponding *F*-statistics are reported in Table S6.

3.4. *Oxidative and dicarbonyl stress*

No evidence was found for the effect of sediment and temperature on MDA levels (Fig. 3e). There was no evidence for the effect of temperature but strong evidence for the effect of sediment on MGO levels, with worms exposed to Szczecin Lagoon sediments having higher MGO levels than those exposed to Pomeranian Bay sediments (Fig. 3f and 5).

3.5. Bioenergetics

There was no evidence that carbohydrate content was affected by sediment or temperature (Fig. 4a). No evidence was found for the effect of sediment, but strong and weak evidence was found for the effect of temperature on lipid and protein contents, respectively (Fig. 4b and c). Worms exposed to 20 °C had higher lipid and protein levels than those exposed to 10 °C (Fig. 5). There was no evidence for the effects of sediment and temperature on ETS activity (Fig. 4d).

3.6. Response patterns

There was no evidence for the non-additive effect of sediment and temperature on any of the responses examined ($p > 0.1$, Figs. 3 and 4). The biomarkers under investigation were often influenced by only one factor, either sediment or temperature, but not by both simultaneously. Also, only small and medium effect sizes ($|d| < 0.8$) were observed for biomarker responses (Fig. 5).

4. Discussion

4.1. Sediment contamination in the Oder estuary

The decreasing contamination of the Oder River as it flows towards the Baltic Sea has been well documented. Szefer et al. (2009) reported higher sediment enrichment of heavy metals in the Szczecin Lagoon than in the Pomeranian Bay. Similar trends were observed for pharmaceuticals and personal care products, with water and sediment samples collected in the Oder River before entering the Szczecin Lagoon being more contaminated than those taken near the Peenestrom, Świna, and Dziwna straits (Fisch et al., 2017; Kucharski et al., 2022b). El Toum et al. (2023) analyzed sediments collected at the same sites as in our study and found the increasing contents of heavy metals and PAHs in the order: Pomeranian Bay < Peenestrom < Szczecin Lagoon. Our data on pesticide levels are consistent with these findings, and together they confirm the existence of a gradient for multiple contaminant classes in the Oder estuary.

This contamination gradient has been attributed to the dilution effect (Fisch et al., 2017), in which seawater and marine sediments reduce the concentration of contaminants from the freshwater sources. Our data on the physicochemical properties of the Oder estuary sediments also

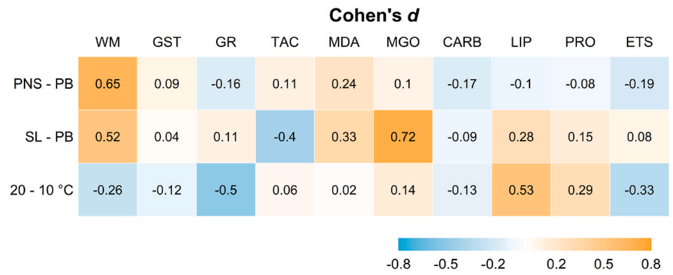

Fig. 5. Responses of *Hediste diversicolor* to sediment contamination and seawater warming summarized by Cohen's *d* effect size. The heatmap shows the standardized mean differences between worms exposed to Peenestrom (PNS) or Szczecin Lagoon (SL) sediments and Pomeranian Bay (PB) sediments, and between worms exposed to 20 °C and 10 °C. Small, medium, and large effect sizes correspond to $|d| \geq 0.2$, 0.5, and 0.8 (Cohen, 1962).

support this idea. Towards the Baltic Sea, the sediments had coarser grains and less organic matter, and thus a lower affinity for heavy metals and organic contaminants (Kersten and Smedes, 2002; Shelton and Capel, 1994). The C:N ratio of organic matter generally decreased downstream, reflecting the increased marine influence (Leng and Lewis, 2017). The elevated C:N ratio in the Peenestrom sediments, which was an exception to this trend, may indicate an additional input of terrigenous components from the Peene River (Fig. 1).

Our study also showed the prevalence of terbutylazine-2-hydroxy, carbendazim, and dinoterb in the Oder estuary sediments (Fig. S2). Terbutylazine-2-hydroxy is a metabolite of the herbicide terbuthylazine, which is widely used for weed control in maize production (Krier et al., 2022; Schulte et al., 2012). The fungicide carbendazim has been banned for use in plant protection products in the European Union (EU) since 2016, but it is still used as a biocide in firm and building material preservatives (European Food Safety Authority (EFSA) et al., 2021; Merel et al., 2018). The herbicide dinoterb, on the other hand, has been banned from the EU market since 2003 (Becker et al., 2023, EU Pesticides Database v3.1). While terbuthylazine and carbendazim have been frequently detected in surface waters of the Oder estuary and the Baltic Sea (Fisch et al., 2021; Nödler et al., 2013; Schulte-Oehlmann et al., 2011), there are no recent similar records for dinoterb. Therefore, its presence in sediments is likely the result of historical releases and high environmental persistence (Beulke and Malkomes, 2001).

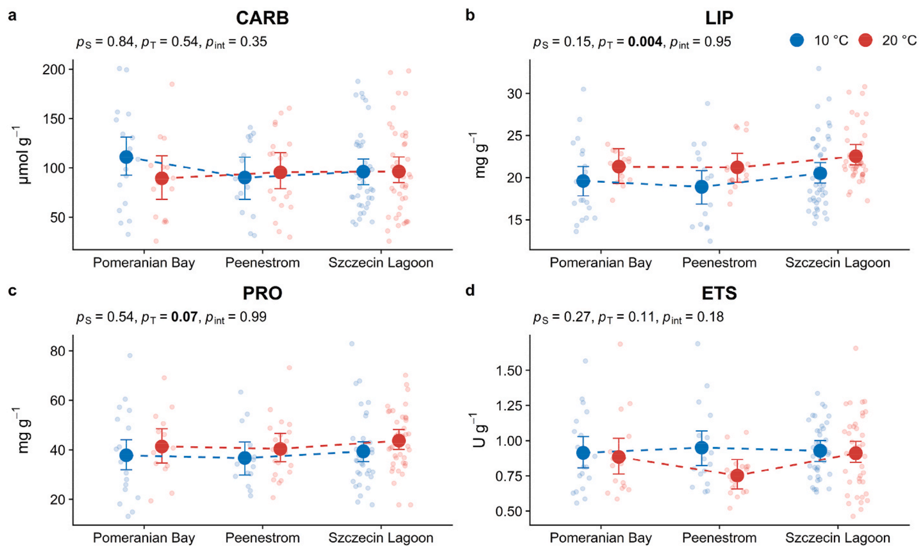

Fig. 4. Effects of Oder estuary sediments and temperature on carbohydrate (CARB), lipid (LIP), and protein (PRO) contents, and mitochondrial electron transport system (ETS) activity of *Hediste diversicolor*. Data are presented with individual observations, estimated marginal means, and 95% bootstrap confidence intervals. Permutation *p*-values are given for sediment (S), temperature (T), and their interaction (int). Corresponding *F*-statistics are reported in Table S6.

Unfortunately, the lack of sediment quality guidelines (SQGs) for these pesticides makes it difficult to predict their effects based on the measured contents in sediments (Birch, 2018; Burton, 2002).

4.2. Effects of sediments on ragworms

In an attempt to reduce both confounding and artificiality, we conducted a laboratory exposure using directly sediments from the Oder estuary. Despite varying contamination levels, we recorded unaltered activities of the phase II enzyme GST, which detoxifies xenobiotics by glutathione conjugation (Domingues et al., 2010), and the enzyme GR, which regenerates glutathione to defend against reactive oxygen species (ROS) from redox-cycling chemicals (Couto et al., 2016). TAC, which indicates the ability to neutralize ROS by a wide range of antioxidants (Cano et al., 2023; Floegel et al., 2011; Regoli et al., 2002), decreased only in worms exposed to the most contaminated sediments (Szczecin Lagoon). MDA levels, a marker of ROS-induced oxidative damage to membrane lipids (Ayala et al., 2014), were similar in all groups. These results suggest that exposure to the more contaminated sediments did not cause significant detoxification enhancement or oxidative stress in ragworms.

Worms exposed to the most contaminated sediments experienced medium dicarbonyl stress as indicated by increased levels of MGO, a deleterious metabolic by-product (Allaman et al., 2015). This could be a result of the reduced TAC, where less glutathione may be available for MGO removal by the glyoxalase system (Rabbani et al., 2020). However, we did not observe sediment-associated differences in cellular energy reserves (carbohydrates, lipids, and proteins) and energy demand as proxied by ETS activity (De Coen and Janssen, 1997; Sokolova, 2021). These results imply that dicarbonyl stress did not impose significant cellular maintenance costs in ragworms.

With the current study design, it is difficult to separate the effects of contaminants from other sediment characteristics. Field sediments with finer grains and more organic matter were found to be associated with higher biomass of ragworms (Van Colen et al., 2014), which is not surprising given that these sediments tend to have higher food availability (Mouneyrac et al., 2010). In contrast, Wiesebron et al. (2021) reported that neither sediment density nor grain size influenced worm survival and respiration rates. These findings are consistent with our observation that worms exposed to Peenestrom and Szczecin Lagoon sediments had higher mass but did not differ in their survival rates or tissue oxygen demand as measured by ETS activity (Fanslow et al., 2001).

4.3. Temperature effects and interaction with sediments

One of the few endpoints affected by temperature was GR activity. It is important to note that enzyme activities were measured at 25 °C, but not at the exposure temperatures of 10 and 20 °C (due to the limited cooling capacity of the instrument). Assuming a constant Q_{10} coefficient across the temperature range (10–25 °C, Galasso et al., 2018), the lower GR activity in worms exposed to 20 °C could be translated into the reduced GR production in response to warming or increased inhibition or denaturation of the enzyme by high temperature. The latter explanation is less likely, as no negative effects of warming on energy reserves were observed. In fact, the content of lipids and proteins increased with high temperature, suggesting higher energy assimilation and biosynthesis (Sokolova, 2021). Such positive effects of warming have also been observed in several reports (Aguado-Giménez et al., 2023; Bhuiyan et al., 2021).

In our study, all combined effects of sediment and temperature were additive, which is consistent with previous reports on the lack of interaction between sediment contamination and seawater warming on cellular responses of *H. diversicolor* (Moreira et al., 2006; Pham et al., 2023). This is understandable given that simultaneous effects of the two stressors were missing and no large effects of either stressor were recorded.

4.4. Conclusions and outlook

Despite considerable differences in physicochemical properties and contamination levels, the Oder estuary sediments did not differ greatly in their effects on ragworms. Given limited budget, we could not assess the bioaccumulation of contaminants in ragworms. However, our chosen biomarker battery provided an integrated assessment of sediment effects with higher ecological relevance than the chemical data (Apitz, 2011; Heise et al., 2020). Notably, elevated temperature neither aggravated nor alleviated the sediment effects, but had some positive effects on the worm's energy storage. Our study suggests that ragworms are relatively resilient to sediment contamination and seawater warming, even when exposed to both stressors simultaneously. Future studies could use ragworms from a different population or focus on other stressors such as hypoxia and acidification.

CRediT authorship contribution statement

Duy Nghia Pham: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Anja Ruhl:** Writing – review & editing, Methodology, Investigation, Data curation. **Kathrin Fisch:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Safia El Toum:** Writing – review & editing, Investigation, Data curation. **Susanne Heise:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Data curation. **Inna M. Sokolova:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Research data are publicly available on Zenodo at https://doi.org/10.5281/zenodo.8384183.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2024.108702.

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Effects of contamination and warming on ragworms *Hediste diversicolor*: A laboratory experiment with Oder estuary sediments

Supplementary material

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1. Introduction

Table S1. Summary of original research on the effects of contaminated sediments on *Hediste diversicolor*, sorted by study type (Ernst, 2004; Pinquart, 2021), study areas, and contaminants of interest. Study types: OBS – observational studies, EXP-SPIKED – experimental studies using sediments spiked with contaminants, EXP-FIELD – experimental studies using directly field-contaminated sediments. Data from Scopus using the query *(TITLE-ABS-KEY(sediment AND hediste AND diversicolor) AND TITLE-ABS-KEY(pollution OR contamination)) AND PUBYEAR > 1999 AND PUBYEAR < 2024*, dated Aug 2023. Of 83 results, 37 publications match the above scope.

Study type	Countries	Ecosystems	Contaminants	References
OBS	Egypt	Burullus Lake	pesticide (malathion)	Hamdy et al. (2022)
OBS	France	Gravelines, Boulogne Harbor	phthalates, metals	Cuvillier-Hot et al. (2018)
OBS	France	Seine, Authie Estuaries	metal (lead)	Philippe et al. (2008)
OBS	France, UK	Seine Estuary, Boulogne Harbor, Restronguet Creek	metals	Berthet et al. (2003)
OBS	Italy	Pialassa Baiona Lagoon	metal (mercury)	Virgilio et al. (2003)
OBS	Italy	Venice Lagoon	dioxins, dibenzofurans	Picone et al. (2020)
OBS	Italy	Venice Lagoon	metals	Frangipane et al. (2005)
OBS	Italy, Netherlands	North Adriatic Coast, Schelde Estuary	microplastics	Piarulli et al. (2020)
OBS	Morocco	Bouregreg Estuary	metals	Khamar et al. (2018)
OBS	Portugal	Aveiro Lagoon	metal (mercury)	Nunes et al. (2008)
OBS	Portugal	Sado Estuary	metal (mercury)	Lillebø et al. (2011)
OBS	Spain	Guadalquivir Estuary	metals	Tornero et al. (2014)
OBS	Tunisia	Gulfs of Tunis, Hammamet, Gabes	microplastics	Missawi et al. (2020)
OBS	Turkey	Bafa Lake	metals	Aydin-Onen et al. (2015)
OBS	UK	Colne Estuary	pharmaceuticals, recreational drugs, pesticides	Miller et al. (2021)

Study type	Countries	Ecosystems	Contaminants	References
OBS	UK	Exe, Kingsbridge, Plym Estuaries	microplastics	Porter et al. (2023)
OBS	UK	Humber Estuary	cyclic volatile methyl siloxane	Kierkegaard et al. (2011)
OBS	UK	Humber Estuary	metals, organochlorines, xenoestrogens	García-Alonso et al. (2011)
OBS	UK	Milford Haven Waterway	metals, organotins, PAHs, PCBs	Langston et al. (2012)
OBS	UK	Plym Estuary	antifouling paint particles	Muller-Karanassos et al. (2019)
OBS	UK	Plym, Fal Estuaries	metal (thallium)	Turner et al. (2013)
OBS	UK	Restronguet Creek, Mylor Bridge, Cowlands Creek	metal (copper)	McQuillan et al. (2014)
OBS	UK	Severn Estuary, Bristol Channel	metals	Bird et al. (2011)
OBS	USA	Goose Pond (Penobscot Bay)	metals	Breton and Prentiss (2019)
EXP-SPIKED	Denmark	Roskilde Fjord	silver nanoparticles	Cong et al. (2014)
EXP-SPIKED	France	Bay of Bourgneuf	microplastics	Revel et al. (2020)
EXP-SPIKED	Germany	Warnow Estuary	metal (copper)	Pham et al. (2023)
EXP-SPIKED	Italy	Magra Estuary	metal (arsenic)	Gaion et al. (2014)
EXP-SPIKED	Norway	Oslofjord	tributyltin, perfluorononanoic acid	Stomperudhaugen et al. (2009)
EXP-SPIKED	Poland	Puck Bay	microplastics	Urban-Malinga et al. (2022)
EXP-SPIKED	Spain	Bay of Cádiz	pharmaceuticals	Maranho et al. (2014)
EXP-SPIKED	Spain	Bay of Cádiz	pharmaceuticals	Maranho et al. (2015)
EXP-SPIKED	UK	Yealm Estuary	tyre particles	Garrard et al. (2022)
EXP-FIELD	Ghana	Tema Harbour	metals	Botwe et al. (2017)
EXP-FIELD	Norway	Åsefjorden	flame retardant (hexabromocyclododecane)	Haukås et al. (2010)
EXP-FIELD	Portugal	Sado Estuary	metals	Moreira et al. (2006)
EXP-FIELD	Tunisia	Gulf of Gabes	metals, PAHs	Ghribi et al. (2019)

Table S2. Average monthly sea surface temperatures (°C) at Usedom (representative of the German part of the Oder estuary) and Warnemünde (representative of the Warnow estuary). Data from the National Oceanic and Atmospheric Administration (NOAA, seatemperature.org) and the Deutscher Wetterdienst (DWD, weather-atlas.com).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Mean
Usedom													
NOAA	2.8	2.8	2.8	6.1	10.6	15.9	18.5	18.8	17.2	13.2	9.2	5.6	10.3
DWD	2.8	2.8	2.8	6.1	10.6	16	18.5	18.8	17.3	13.2	9.2	5.7	10.3
Warnemünde													
NOAA	2.6	2.7	2.9	6.3	11.0	15.8	17.8	17.9	16.6	12.8	8.9	5.4	10.1
DWD	2.8	2.8	3.0	6.3	11.0	15.8	17.8	17.9	16.7	13.0	8.9	5.6	10.1

2. Materials and methods

2.1. Sediment collection and characterization

Table S3. Sediment sampling sites along a reported contamination gradient (El Toum et al., 2023) in the German part of the Oder estuary. Sediment samples were named after their sites.

Sediment	Site	Region	Latitude	Longitude	Collection date
GB	Greifswald Bay	Pomeranian Bay	54.185252	13.607864	19.05.2021
OB	Oder Bay	Pomeranian Bay	54.045356	14.093766	19.05.2021
PN	Peenestrom North	Peenestrom	54.016483	13.846550	22.07.2021
PS	Peenestrom South	Peenestrom	53.884833	13.865450	22.07.2021
LK	Lagoon Karnin	Szczecin Lagoon	53.844733	13.857317	20.05.2021
LW	Lagoon West	Szczecin Lagoon	53.803250	14.022483	18.05.2021
LM	Lagoon Middle	Szczecin Lagoon	53.785567	14.077633	19.05.2021
LE	Lagoon East	Szczecin Lagoon	53.767983	14.129067	19.05.2021

For pesticide analysis, sediment samples were lyophilized and homogenized. Up to 5 g of dried sample was spiked with 0.2 μg μL^{-1} of surrogate standards (atrazine-d5, chlorpyrifos-d10, and pirimicarb-d6). Then 30 mL of 2:1 acetone/water (v/v) was added, allowed to stand for 30 min, then shaken at 200 rpm for 1 h and centrifuged at 3000 rpm for 5 min. A 15-mL aliquot was mixed with 5 mL of 20% NaCl, followed by a SLE (Chem Elut 20 mL, unbuffered, Agilent, USA). After 15 min, the sample was extracted with 2 × 50 mL of dichloromethane. The eluate was evaporated to dryness, resuspended in 1 mL of 1:1 methanol/water (v/v) spiked with isoproturon-d6, 2,4,5-trichlorophenoxyacetic acid, and prochloraz-d4 as internal standards, and ultrasonicated for 30 s.

The samples were analyzed by LC-MS/MS (1290 Infinity II, Agilent, USA and QTRAP 6500+, SCIEX, USA) on either a Zorbax Eclipse C18 column (50 × 2.1 mm, 1.8 µm, Agilent, USA) or a Kinetex C18 (50 × 2.1 mm, 2.6 µm, Phenomenex, USA). Of each sample, 2 µL was injected and a gradient program was run at a flow rate of 0.5 mL min^{-1}, A: 95% for 0.5 min, 5% to 3.5 min, kept for 1.5 min, 95% to 5.1 min, kept until 6 min. The mobile phase consisted of either A: water + 1 mmol ammonium fluoride, B: 65:35 methanol/acetonitrile (v/v) or A: water + 0.5% formic acid + 5 mmol ammonium formate, B: methanol + 0.5% formic acid + 5 mmol ammonium formate. The MS/MS parameters were set as follows: 5500 V, 400 °C for positive mode and -4500 V, 300 °C for negative mode, each with a curtain gas of 35.

2.4. Colorimetric assays

Briefly, total antioxidant capacity (TAC) in Trolox equivalents was evaluated by the reduction of 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•+) measurable at 734 nm (Re et al., 1999). Malondialdehyde (MDA) content was evaluated by the reaction with thiobarbituric acid, which forms a 530-nm absorbing product with the extinction coefficient of 156 mM^{-1} cm^{-1} (Buege and Aust, 1978). Methylglyoxal (MGO) content was evaluated by the reaction with Girard's reagent T in borax solution using MGO standards, which produces a disubstituted compound measurable at 325 nm (Mitchel and Birnboim, 1977). Carbohydrate content in glucose equivalents was determined by the reaction with phenol-sulfuric acid, which forms a 492-nm absorbing product (Masuko et al., 2005). Lipid content in the chloroform-methanol extraction of homogenates (Folch et al., 1957) was assessed by the sulfo-phospho-vanillin reaction, which produces a 490-nm absorbing compound (Van Handel, 1985). Protein content in bovine serum albumin equivalents was quantified by the Bradford assay, which produces a 595-nm absorbing protein-dye complex (Bradford, 1976). Glutathione *S*-transferase (GST) activity was quantified by the reaction between reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) monitored at 340 nm using the extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig et al., 1974). Glutathione reductase (GR) activity was assessed by the NADPH-dependent reduction of glutathione disulfide to GSH monitorable at 340 nm using the extinction coefficient of 6.2 mM^{-1} cm^{-1} (Mannervik, 1999). Mitochondrial electron transport system (ETS) activity, expressed as oxygen consumption equivalents, was assessed by the NAD(P)H-dependent reduction of iodonitrotetrazolium chloride (INT) to formazan monitorable at 490 nm using the extinction coefficient of 15.9 mM^{-1} cm^{-1} (De Coen and Janssen, 1997). All measurements were performed in 96-well plates using a microplate reader (SpectraMax iD3, Molecular Devices, Germany).

3. Results

3.1. Sediment characteristics

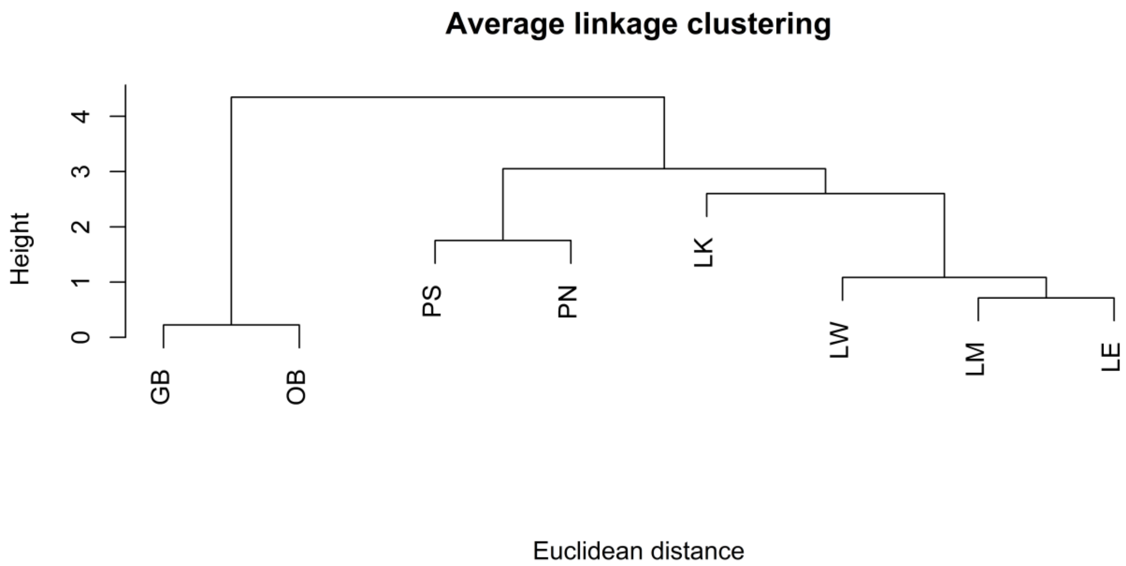

Fig. S1. Dendrogram for cluster analysis of sediments in the Oder estuary based on sediment density, total organic matter content, C:N ratio, <63 µm fraction content, number of pesticides detected, and total pesticide content.

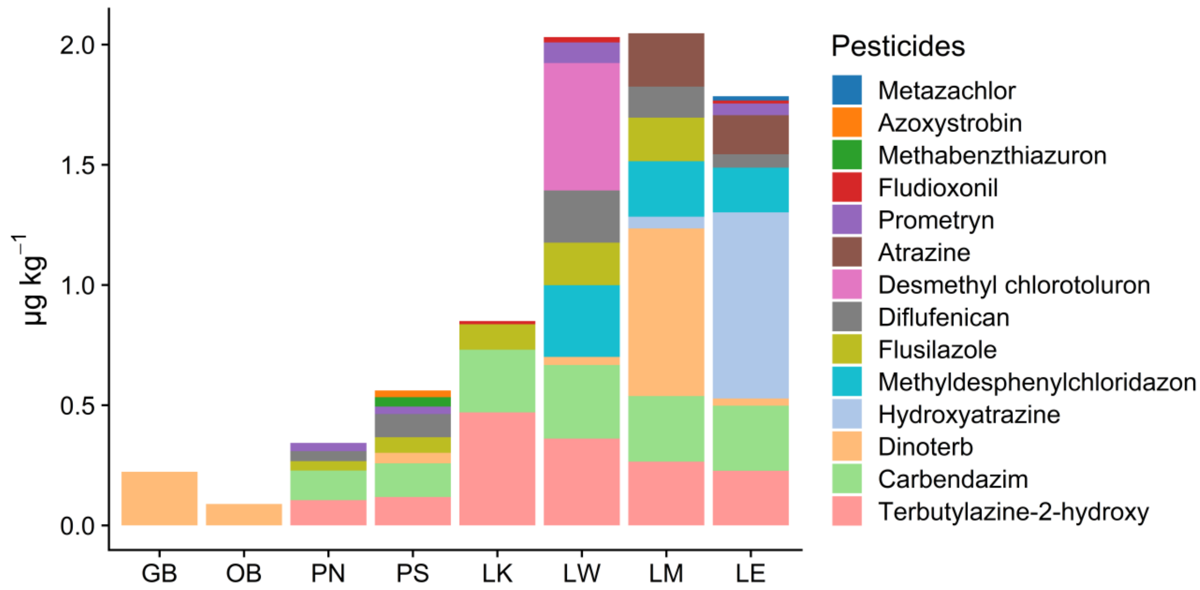

Fig. S2. Pesticide profiles of sediments in the Oder estuary. Out of 132 pesticides and pesticide metabolites screened, 14 compounds were detected in the sediments. The compounds are listed in increasing order of the average content in eight sediment samples.

3.2-5. Survival and mass-Bioenergetics

Table S4. Survival of *H. diversicolor* at the end of the three-week exposure (n = 12).

Number of lives	10 °C	20 °C
GB	9	7
OB	10	8
PN	8	10
PS	8	10
LK	10	10
LW	9	10
LM	11	12
LE	11	8

Table S5. Permutation tests for the effects of sediment, temperature, and their interaction on the survival of *H. diversicolor* at the end of the exposure with sediment factor categorized by site and region[1].

Sediment levels	Factor	df_{num}	F	p
Sites	Sediment	7	0.41	0.90
	Temperature	1	0.06	0.79
	Interaction	7	0.65	0.55
Regions	Sediment	2	1.55	0.22
	Temperature	1	0.02	0.90
	Interaction	2	1.23	0.34

[1] df_{den} are not available for generalized linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

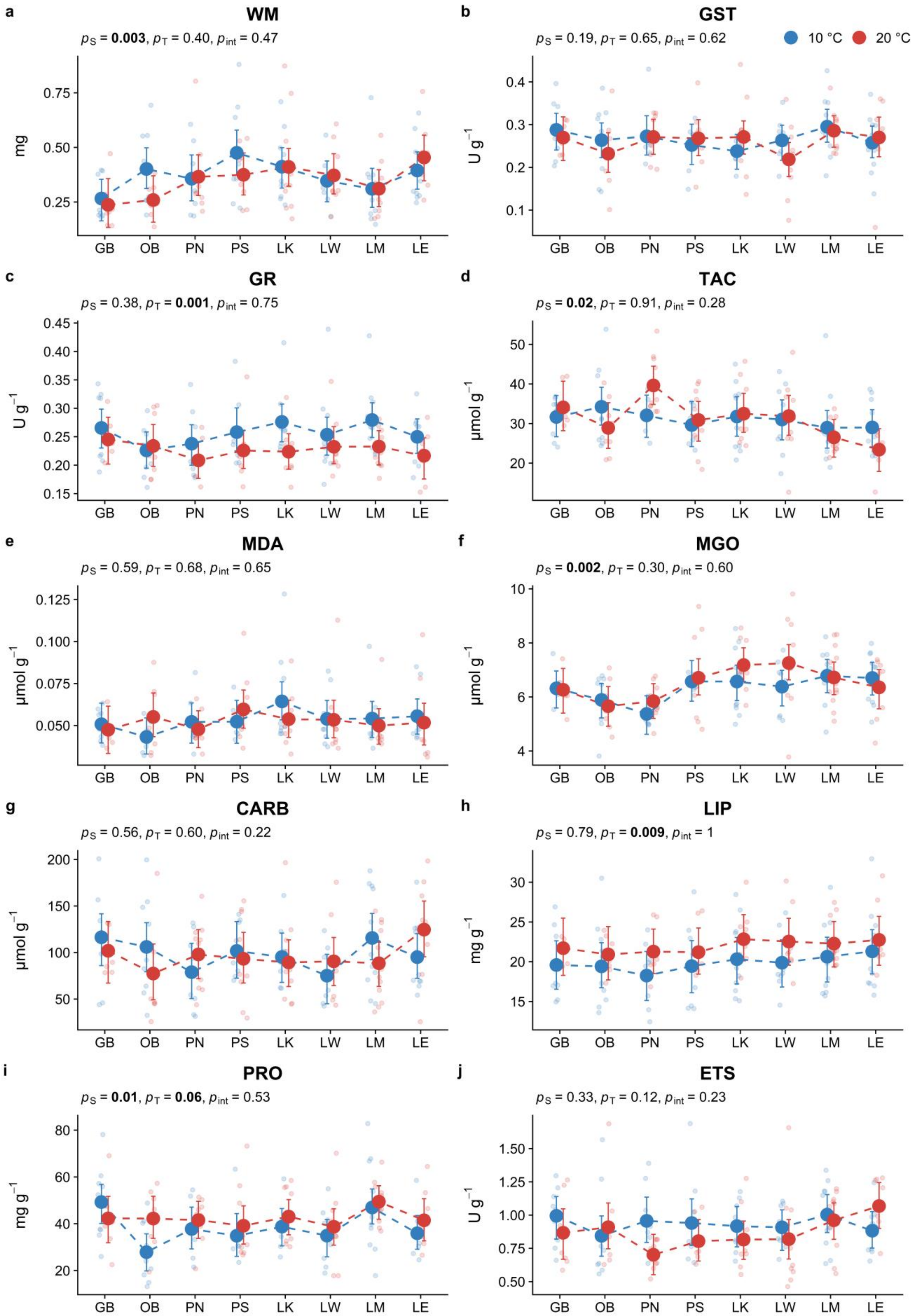
a
WM
p_S = **0.003**, p_T = 0.40, p_{int} = 0.47
mg
0.75
0.50
0.25
b
GST
p_S = 0.19, p_T = 0.65, p_{int} = 0.62
10 °C
20 °C
U g^{-1}
0.4
0.3
0.2
0.1
c
GR
p_S = 0.38, p_T = **0.001**, p_{int} = 0.75
U g^{-1}
0.45
0.40
0.35
0.30
0.25
0.20
0.15
d
TAC
p_S = **0.02**, p_T = 0.91, p_{int} = 0.28
µmol g^{-1}
50
40
30
20
e
MDA
p_S = 0.59, p_T = 0.68, p_{int} = 0.65
µmol g^{-1}
0.125
0.100
0.075
0.050
f
MGO
p_S = **0.002**, p_T = 0.30, p_{int} = 0.60
µmol g^{-1}
10
8
6
4
g
CARB
p_S = 0.56, p_T = 0.60, p_{int} = 0.22
µmol g^{-1}
200
150
100
50
h
LIP
p_S = 0.79, p_T = **0.009**, p_{int} = 1
mg g^{-1}
30
25
20
15
i
PRO
p_S = **0.01**, p_T = **0.06**, p_{int} = 0.53
mg g^{-1}
80
60
40
20
j
ETS
p_S = 0.33, p_T = 0.12, p_{int} = 0.23
U g^{-1}
1.50
1.25
1.00
0.75
0.50
GB OB PN PS LK LW LM LE

Fig. S3. Effects of Oder estuary sediments and temperature on wet mass (WM), glutathione *S*-transferase (GST) and glutathione reductase (GR) activities, total antioxidant capacity (TAC), malondialdehyde (MDA) and methylglyoxal (MGO) levels, carbohydrate (CARB), lipid (LIP), and protein (PRO) contents, and mitochondrial electron transport system (ETS) activity of *Hediste diversicolor*. Data are presented with individual observations, estimated marginal means, and 95% bootstrap confidence intervals. Permutation *p*-values are given for sediment (S), temperature (T), and their interaction (int). Corresponding *F*-statistics are reported in Table S6.

Table S6. *F*-statistics in permutation tests for the effects of sediment, temperature, and their interaction on the responses of *Hediste diversicolor* with sediment factor categorized by site and region[2].

Response	Sediment levels	Sediment	Temperature	Interaction
WM	Sites	2.70	0.68	0.90
	Regions	3.75	0.85	1.46
GST	Sites	1.33	0.19	0.73
	Regions	0.03	0.18	0.62
GR	Sites	1.06	11.64	0.57
	Regions	1.24	12.16	1.10
TAC	Sites	2.76	0.01	1.33
	Regions	2.88	0.00	1.56
MDA	Sites	0.80	0.16	0.72
	Regions	1.51	0.24	1.07
MGO	Sites	3.84	1.12	0.80
	Regions	7.00	1.12	0.50
CARB	Sites	0.78	0.25	1.20
	Regions	0.16	0.34	1.02
LIP	Sites	0.53	7.22	0.07

[2] df_{num} are the same as in Table S5, df_{den} are not available for linear mixed-effects models (Bates, Fri May 19 22:40:27 CEST 2006).

Response	Sediment levels	Sediment	Temperature	Interaction
	Regions	1.97	9.46	0.05
PRO	Sites	2.60	3.58	0.85
	Regions	0.62	3.50	0.01
ETS	Sites	1.08	2.33	1.37
	Regions	1.31	2.44	1.75

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Bates, D., Fri May 19 22:40:27 CEST 2006. [R] lmer, p-values and all that.

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5.3 Publication 3

Pham, **D.N.**, El Toum, S., Martineau, R., Heise, S., Sokolova, I.M., 2025. Sediment contamination in two German estuaries: A biomarker-based toxicity test with the ragworm *Hediste diversicolor* under intermittent oxygenation. Environmental Research 265, 120451. https://doi.org/10.1016/j.envres.2024.120451

Declaration of personal contributions

I hereby declare that my personal contributions, as defined in the Contributor Role Taxonomy (CRediT), to **Publication 3** are as follows:

- I took a leading role in **Methodology**, **Investigation**, and **Data curation**.
- I was solely responsible for **Software**, **Formal Analysis**, **Visualization**, and **Writing – original draft**.

Rostock, 2025-04-06

Duy Nghia PHAM

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Sediment contamination in two German estuaries: A biomarker-based toxicity test with the ragworm *Hediste diversicolor* under intermittent oxygenation

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A B S T R A C T

Toxicity testing is an important tool for risk assessment of sediment contamination in estuaries. However, there has been a predominant focus on fitness parameters as toxic endpoints and on crustaceans as test organisms, while effects at the sub-organismal level and on other benthic taxa have received less attention. Also, interactions between sediment contamination and natural stressors such as oxygen are often neglected in traditional toxicity tests. Here we conducted a toxicity test of sediments from the Elbe and Oder (Odra) estuaries under three weeks of continuous and intermittent oxygenation, using biomarkers in an annelid, the ragworm *Hediste diversicolor*. Contaminated sediments affected worm survival and some biomarkers of antioxidant defense, electrophilic stress, and energy status with response ratios of above 20%. Toxic effects were most pronounced in sediments from the upper Elbe estuary, which contained high levels of heavy metals and organic chemicals. Oxygen regimes hardly changed the sediment effects, suggesting the robustness of the biomarker-based toxicity test with ragworms.

1. Introduction

More than 50% of the world's population lives in river basins and coastal areas (Hongtao and Ting, 2021; Kummu et al., 2011). Increased development in these regions puts immense pressure on aquatic ecosystems, including estuaries (Freeman et al., 2019). Among various anthropogenic stressors, chemical contamination is often considered the greatest threat (Borgwardt et al., 2019). Many heavy metals and organic chemicals released into the water tend to accumulate and persist in estuarine sediments (Burton, 2002; Chapman and Wang, 2001), potentially affecting the health and functioning of local biota, particularly benthic organisms (Pinto et al., 2009). The effects of contaminated sediments can also extend beyond their original location, for example, due to dredging and disposal of dredged material, or by extreme flood events (Crawford et al., 2022; Roberts, 2012). Therefore, the risk assessment of sediment contamination in estuaries is an important task (Apitz, 2011).

An experimental approach to sediment pollution assessment is toxicity testing, which aims to determine the causal effects of contaminated sediments on test organisms under controlled conditions (Chapman, 1990; Simpson et al., 2016). Traditional toxicity tests (bioassays) typically focus on fitness endpoints such as survival, growth, and reproduction (Heise et al., 2020; Leppanen et al., 2024). However, adverse effects on fitness often take time to manifest, and in these cases, sub-organismal changes (biomarkers) can be useful early warning signs (Hagger et al., 2006; Newman, 2015). In terms of test species, current toxicity tests are biased towards crustaceans (e.g., amphipods and copepods), while other important estuarine taxa such as mollusks and annelids are underrepresented (Chapman et al., 2013; Simpson et al., 2016). Also, not many tests have examined the effects of contaminated sediments in combination with natural stressors such as salinity, temperature, and oxygen (Elliott and Quintino, 2007; Pham et al., 2024), despite concerns about their potential interactions (Burton and Johnston, 2010; Sokolova and Lannig, 2008). For example, oxygen depletion may

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limit the energy available for detoxification and damage repair, thereby enhancing contaminant toxicity (Sokolova, 2013, 2021).

To fill these gaps, we conducted a toxicity test of sediments under two oxygen regimes with a focus on biomarker responses of an estuarine annelid. We collected sediments from two German estuaries, the Elbe and the Oder, both contaminated by multiple urban, industrial, and agricultural sources in their catchments (Förstner et al., 2004; Kowalewska et al., 2003; Müller et al., 2002; Wetzel et al., 2013). The sediment toxicity test was set up for three weeks under continuous oxygenation, which is standard practice for laboratory exposures (Simpson et al., 2016), and intermittent oxygenation, which simulates more realistically oxygen fluctuations in the field (Fusi et al., 2023; Jarvis et al., 2023). The ragworm *Hediste diversicolor*, a common benthic organism in European estuaries (Beyer and Sundt, 2006; Scaps, 2002), was used as the test species. By building complex galleries in the sediment, aerating, and feeding, ragworms significantly influence the fluxes of nutrients, organic matter, oxygen, and contaminants across the sediment-water interface, making them important ecosystem engineers (Laing et al., 2022; Porter et al., 2023; Zhu et al., 2016). To assess the toxic effects on ragworms, we measured two fitness endpoints (survival and mass) and a suite of biomarkers related to detoxification and antioxidant defense, electrophilic stress, and energy status (Hampel et al., 2016; Pham et al., 2022; Sokolova et al., 2012).

2. Materials and methods

2.1. Estuarine regions and sediment collection

The Elbe and Oder rivers originate in the Czech Republic and flow into the North Sea and the Baltic Sea, respectively (Fig. 1). Geologically, the Elbe river mouth is a tidal, coastal plain estuary (Amann et al., 2012; Rewrie et al., 2023). It is often divided into three regions based on salinity, the Limnic Elbe (salinity <1), the Transitional Elbe (salinity 1–20), and the Coastal Elbe (salinity >20) (Carstens et al., 2004; Geerts et al., 2012). In contrast, the Oder (Odra) river mouth is a non-tidal, bar-built estuary (Osadczuk et al., 2007). It consists of three geographic regions, the Szczecin Lagoon (salinity <2), connecting straits such as the Peenestrom (salinity 2–8), and the Oder Bay (salinity 8) (Mohrholz and Lass, 1998; Schernewski et al., 2012).

Contamination in the coastal regions of both estuaries is of minor concern due to the dilution effect of seawater and marine sediments (Pham et al., 2024). Therefore, we only investigated sediments in four regions, the Transitional Elbe, Limnic Elbe, Peenestrom, and Szczecin Lagoon (Fig. 1). In each region, surface sediments (top 10 cm) were sampled with Van Veen grabs or spades at two or three sites in October 2021 (one sediment sample per site). The sediments were transported to the laboratory in sealed plastic buckets and stored in a cold, dark chamber at 4 °C.

2.2. Sediment characterization

Total organic matter content and C:N mass ratio were measured in-house following established protocols (Davies, 1974; Verardo et al., 1990). Analyses of dry matter content (total solids), grain size distribution, and contaminants were conducted by an accredited laboratory (GBA Gesellschaft für Bioanalytik, Germany) using standard methods (Otte et al., 2013; Wetzel et al., 2013). A large number of persistent contaminants commonly included in national monitoring programs were analyzed (German Environment Agency, 2024). These were eight heavy metals (Hg, Pb, Cd, As, Cr, Ni, Cu, and Zn), 16 polycyclic aromatic hydrocarbons (PAHs), two chlorobenzenes (CLBs), seven polychlorinated biphenyl congeners (PCBs), five hexachlorocyclohexane isomers (HCH), and dichlorodiphenyltrichloroethane (DDT) along with its metabolites (collectively referred to as DDX). Heavy metals were measured in the fine fraction (<20 μm) of the sediments, while organic contaminants were measured in the gravel-excluded fraction (<2 mm). A list of organic contaminants and details of the standard methods and measurement uncertainties are provided in the Supplementary material.

2.3. Test species and control sediment collection

The ragworm *Hediste diversicolor* inhabits all regions of the Elbe and Oder estuaries (Gosselck and Schabelon, 2007; Krieg, 2007; Zauke, 1977) and is therefore a highly representative test species (US EPA, 1994). However, to ensure the sensitivity of the test subjects, we obtained ragworms from a nearby estuary with a presumably lower

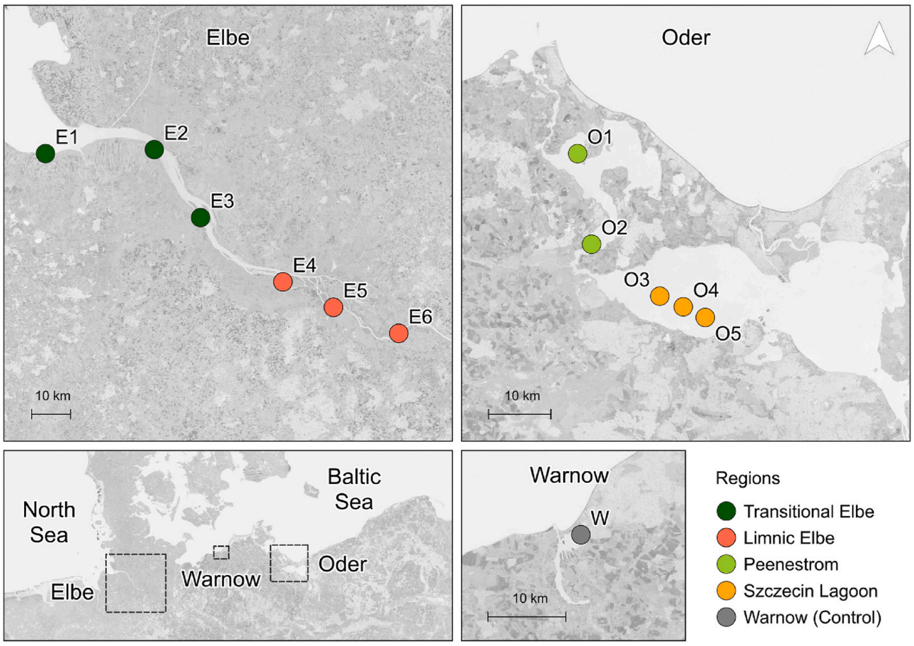

Fig. 1. Sediment sampling sites in the Elbe and Oder estuaries. Six sites (E1 to E6) were selected in two regions of the Elbe estuary and five sites (O1 to O5) were selected in two regions of the Oder estuary. Ragworms *Hediste diversicolor* and control sediment were collected from a site (W) in the Warnow estuary. The coordinates of the sampling sites are given in Table S1. Basemap from Microsoft Bing.

contamination level (Fisch et al., 2017), the Warnow in the Baltic Sea (Fig. 1). The worms were collected by sediment sieving and transported to the laboratory in plastic drums together with field sediments and seawater. The drums were maintained for one week in an environmental room at 17.5 °C and with a 16:8 h light-dark cycle. The overlying water was continuously aerated, supplemented with fish food (Marine Flakes, Tetra, Germany), and renewed daily with artificial seawater at salinity 10 (Pro-Reef, Tropic Marin, Germany). These temperature, light, and salinity conditions approximated those in the field on the day of collection and were also used during the sediment incubation and exposure described later. To examine the temporal changes in test endpoints, a pre-exposure group of 21 worms was collected, shock-frozen in liquid nitrogen, and stored at −80 °C for biomarker measurements.

Sediment was also collected from the same site in the Warnow estuary to serve as a control in the toxicity test (Fig. 1). Its collection and characterization followed the same procedures as the sediments from the Elbe and Oder estuaries.

2.4. Sediment exposure and oxygen regimes

A two-factor experiment was conducted with a 12 × 2 design, corresponding to 12 sediment samples (Fig. 1) and two oxygen regimes (continuous and intermittent oxygenation). For each of the 24 experimental groups, three 1-L glass bottles (ROTILABO, Carl Roth, Germany) were prepared and incubated for one month, each containing 400 mL of sediment and 600 mL of artificial seawater at salinity 10. The overlying water was continuously aerated with an air diffuser, and seawater or deionized water was added daily to compensate for evaporation while maintaining the salinity.

The three-week exposure began with the random assignment of seven ragworms to each bottle ($n = 21$ worms per experimental group). Bottles in the continuous oxygenation groups were left as they were, with saturated oxygen in the overlying water at ~8.9 mg L^{-1}. In contrast, bottles in the intermittent oxygenation groups were aerated and then closed with airtight plastic caps for a total of 12 cycles (Fig. 2). In each closed period, three bottles were randomly selected for oxygen depletion monitoring using modified caps with embedded probes (LDO101 with HQ40d portable meter, Hach, USA). The closed periods were started arbitrarily but ended when the experimenter noticed that at least one of the selected bottles had reached the hypoxia with oxygen in the overlying water at <2 mg L^{-1}. After the exposure, surviving worms were retrieved, shock-frozen in liquid nitrogen, and stored at −80 °C for biomarker measurements.

2.5. Biomarker measurements

After recording the wet mass of each worm, multiple biomarkers were measured using established protocols. Detoxification and antioxidant defense were assessed by the activities of carboxylesterase (CES, Hosokawa and Satoh, 2001), glutathione *S*-transferase (GST, Habig et al., 1974), and glutathione reductase (GR, Mannervik, 1999) and total antioxidant capacity (TAC, Re et al., 1999). Electrophilic stress was evaluated by the levels of methylglyoxal (MGO, Mitchel and Birnboim, 1977), malondialdehyde (MDA, Buege and Aust, 1978), and protein carbonyls (PC, Levine et al., 1990). Energy status was examined by the contents of carbohydrates (CAR, Masuko et al., 2005), lipids (LIP, Van Handel, 1985), and proteins (PRO, Bradford, 1976), mitochondrial electron transport system activity (ETS, De Coen and Janssen, 1997), and the adenylate levels (ATP, ADP, and AMP, Crouch et al., 1993; Jaworek et al., 1974). ATP, ADP, and AMP were measured in a set of ~6 worms per experimental group, while the other biomarkers were measured in another set of ~10 worms per group. All measurements were performed spectrophotometrically on a microplate reader (SpectraMax iD3, Molecular Devices, Germany) at 25 °C.

Several composite biomarkers of energy status were also constructed. Carbohydrate, lipid, and protein contents were converted to energy equivalents using the specific enthalpies of 17.5, 39.5, and 24 J mg^{-1} (Gnaiger, 1983) and summed to obtain the total energy available (Ea). ETS activity was transformed to the energy consumption rate (Ecr) using the oxyenthalpic equivalent of 484 kJ mol^{-1} O_2 (De Coen and Janssen, 1997). Cellular energy allocation (CEA, Verslycke et al., 2004) was then calculated as Ea/Ecr. Also, adenylate energy charge (AEC, Atkinson and Walton, 1967) was computed as (ATP + 0.5 ADP)/(ATP + ADP + AMP), where the denominator represents the total adenylates (collectively referred to as AXP).

For the sediment toxicity assessment based on biomarkers, we considered increases in CES, GST, GR, and ETS activities and increases in MGO, MDA, and PC levels as adverse effects on ragworms. Decreases in TAC, Ea, CEA, AXP, and AEC were also considered adverse effects.

2.6. Data analyses

Survival, mass, and biomarker responses of post-exposure worms were analyzed using (generalized) linear mixed models (Bates et al., 2015), with sediment and oxygen as interacting fixed effects and experimental bottle as a random effect. Model predicted means (Lenth et al., 2024) were calculated for 12 sediment samples and two oxygen regimes, as well as 24 combined experimental groups. The response

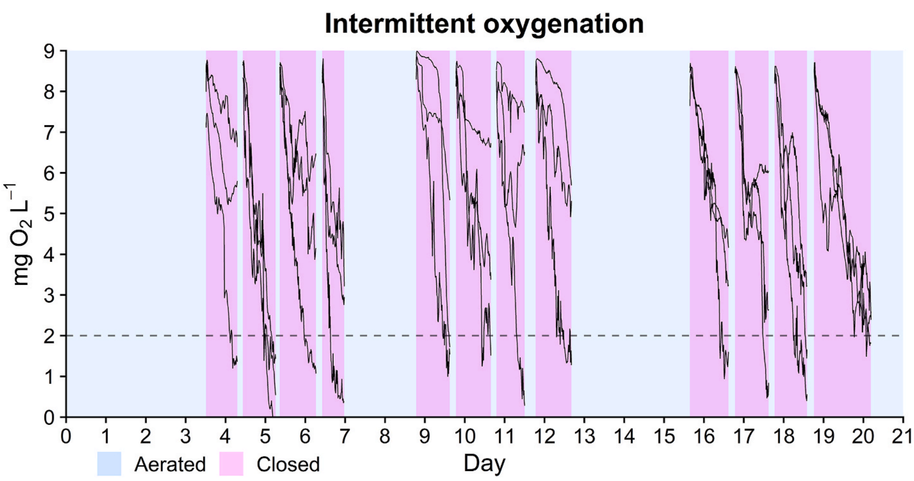

Fig. 2. Effects of intermittent oxygenation on dissolved oxygen in the overlying water of test bottles. The bottles were aerated and closed, and this process was repeated for 12 cycles. Due to the limited number of oxygen probes, only three random bottles were monitored during each closed period. The dashed line marks the hypoxic level that triggered the reoxygenation of the bottles.

ratio (RR, Friedrich et al., 2008; Pham and Sokolova, 2023) was also calculated with the Warnow estuary sediment and continuous oxygenation as controls. Two-factor permutational analysis of variance (Ernst, 2004; Luo and Koolaard, 2024) was applied to the fitted models to test for the effects of sediment, oxygen, and their interaction. Evidence against the null hypotheses was considered very strong, strong, or moderate if the permutation p-values ≤ 0.001, 0.01, or 0.05 (Muff et al., 2022). In these cases of statistical significance, we focused on the means and RR for interpretation instead of performing post-hoc tests (Kozak and Powers, 2017). Specifically, we considered a relative change of at least 20% compared with the controls (RR $\leq$ 0.8 or RR $\geq$ 1.2) to be of toxicological significance (Chapman, 2007, 2016; Neumann-Hensel and Melbye, 2006; Simpson et al., 2016).

Correlations between all test endpoints and between significant endpoints and contaminant levels were assessed using Pearson's and Spearman's coefficients. When $|r|$ or $|r_S| \geq 0.7$, the relationship was considered strong (Akoglu, 2018). Temporal changes in test endpoints were also examined by comparing the pre-exposure worms with the worms exposed to the Warnow estuary sediment under continuous oxygenation. All analyses were conducted in R v4.3.3 (R Core Team, 2024) with codes adapted from Pham et al. (2024).

3. Results

3.1. Sediment characteristics

Sediments in the Warnow estuary and the Transitional Elbe had high dry matter contents (~70%), low amounts of fine grains, and little organic matter (Fig. 3). In contrast, the Szczecin Lagoon sediments had low dry matter contents (<20%) and were rich in fine fraction (~70%) and organic matter (~15%). Sediments in the Limnic Elbe and the Peenestrom both contained medium contents of dry matter and medium amounts of organic matter (except for site E6), but the Limnic Elbe sediments had more fine grains. The C:N ratio of organic matter in all sediment samples varied between 8 and 12 (Fig. S1).

Most heavy metals were found with the highest contents in sediments E5 and E6 from the Limnic Elbe (Fig. 4 and S1). The Limnic Elbe sediments (E4, E5, and E6) also contained the highest levels of most organic contaminants, including total CLBs, PCBs, HCH, and DDX. An exception was total PAHs, which were highest in the Szczecin Lagoon sediments (O3, O4, and O5). Contents of contaminants were generally lower in the Transitional Elbe compared with the Limnic Elbe. Similarly, contaminant levels were often lower in the Peenestrom compared with the Szczecin Lagoon. For most contaminants, the Warnow estuary sediment had lower contents than those from the Elbe and Oder estuaries.

3.2. Sediment-oxygen interactions

There was no evidence for the interactive effects of sediment and oxygen on most of the test endpoints. The exceptions were protein content and Ea, which are described later. Given the lack of interaction, the figures in the next sections show the effects of sediment and oxygen separately.

3.3. Survival and mass

Moderate evidence was found for the effects of sediment and oxygen on worm survival (Fig. 5). Worms exposed to sediments E1, E5, and E6 had lower survival probabilities than worms in the control sediment (RR = 0.76–0.78), while worms in sediments O1 and O2 had higher survival probabilities (RR = 1.18–1.21). Worms kept under intermittent oxygenation also had a higher survival rate than worms in continuous oxygenation (RR = 1.13).

While there was no evidence for the effect of sediment on worm mass, very strong evidence was found for the effect of oxygen, with worms exposed to intermittent oxygenation having higher mass than worms under continuous oxygenation (RR = 1.21, Fig. 5).

3.4. Detoxification and antioxidant defense

There was no evidence for the effect of sediment on CES activity but very strong evidence for the effect of oxygen, with worms exposed to intermittent oxygenation having lower CES activity (RR = 0.86, Fig. 6). For GST and GR activities, no evidence was found for the effects of sediment and oxygen (Fig. S2).

Strong and very strong evidence was found for the effects of sediment and oxygen on TAC (Fig. 6). Worms exposed to sediments from the Elbe and Oder estuaries often had lower TAC than those in the control sediment, with the lowest TAC in sediment O5 (RR = 0.77). Worms kept under intermittent oxygenation also had a lower TAC than worms under continuous oxygenation (RR = 0.9).

3.5. Electrophilic stress

There was very strong evidence for the effect of sediment on MGO levels, but no evidence for the effect of oxygen (Fig. 6). Compared with the control sediment, all sediments from the Elbe and Oder estuaries resulted in higher MGO levels. Notably, the MGO levels were highest in

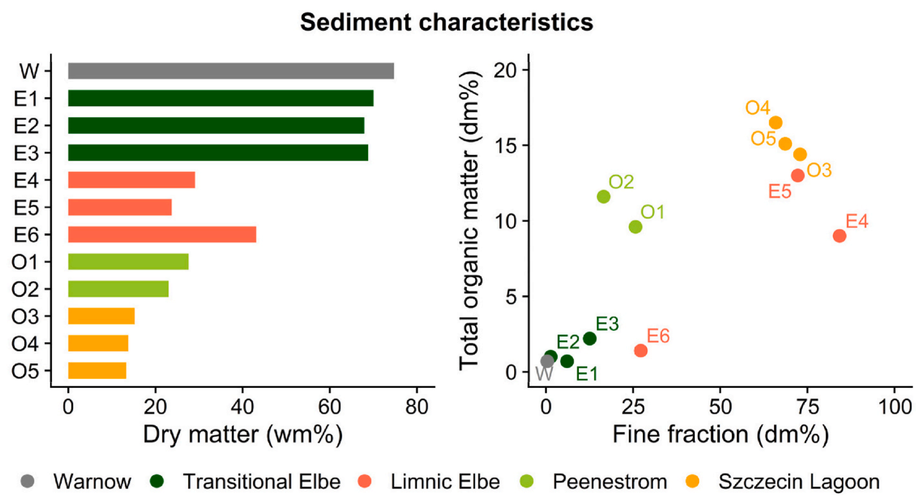

Fig. 3. Physicochemical properties of sediments collected at 12 sites in the Warnow, Elbe, and Oder estuaries. Units are wm% - percent wet mass or dm% - percent dry mass. C:N ratio is shown in Fig. S1.

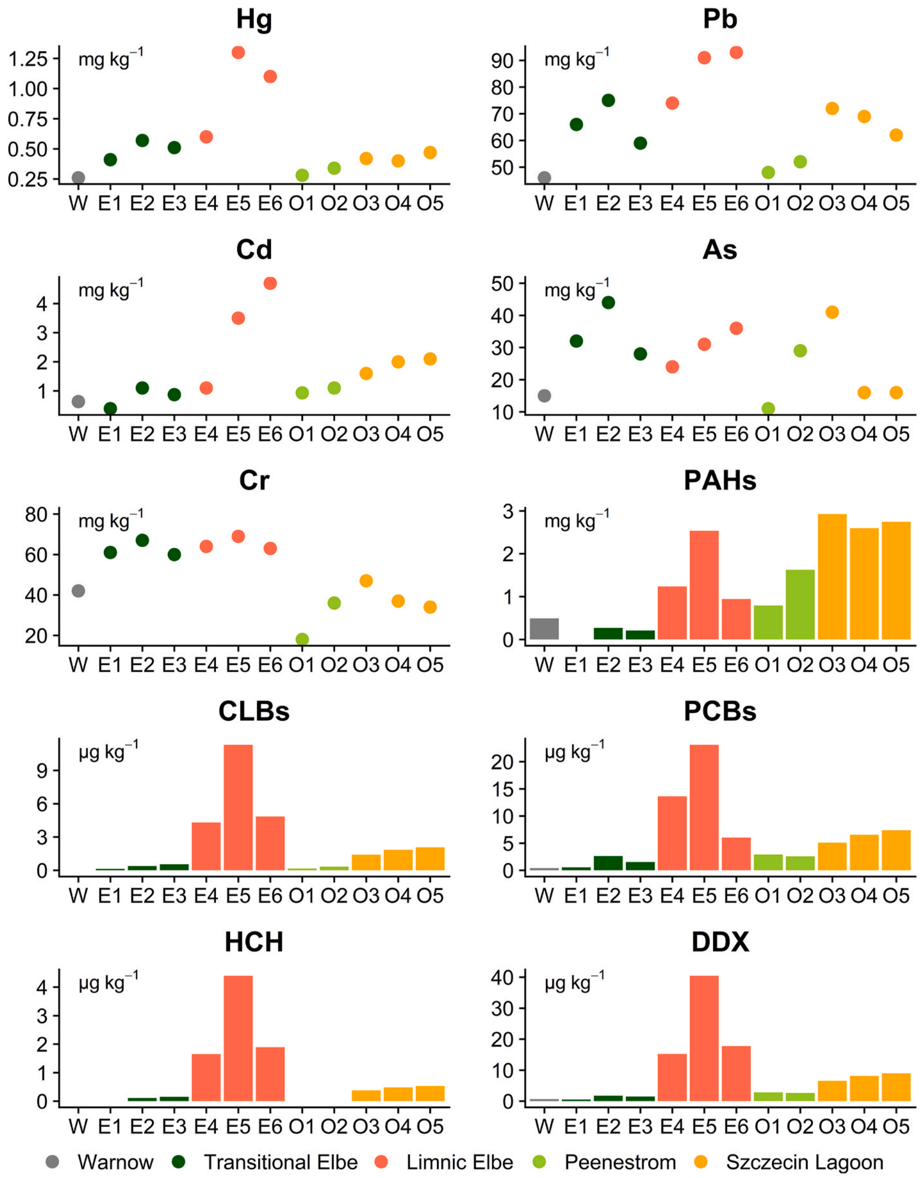

Fig. 4. Contents of heavy metals and organic contaminants in sediments collected at 12 sites in the Warnow, Elbe, and Oder estuaries. Total contents are reported for five groups of organic contaminants, PAHs - polycyclic aromatic hydrocarbons, CLBs - chlorobenzenes, PCBs - polychlorinated biphenyls, HCH - hexachlorocyclohexane, and DDX - dichlorodiphenyltrichloroethane (DDT) and metabolites. Ni, Cu, and Zn are shown in Fig. S1. Because heavy metals have a geological background, dot plots are used instead of bar plots to highlight relative differences between sites.

worms exposed to the Limnic Elbe sediments (RR = 1.33–1.44).

No evidence was found for the effects of sediment and oxygen on MDA and PC levels (Fig. S3).

3.6. Energy status

There was no evidence for the effects of sediment and oxygen on carbohydrate and lipid contents (Fig. S4). Very strong evidence was found for the interactive effect of sediment and oxygen on protein content (Fig. S4). In most sediments, worms exposed to intermittent oxygenation had higher protein contents than those under continuous oxygenation. The exceptions were sediments E1, E5, and E6, in which worms under continuous oxygenation had higher protein levels.

Very strong evidence was also found for the interactive effect of sediment and oxygen on Ea (Fig. 7). In most sediments, worms exposed to continuous and intermittent oxygenation had similar Ea values. Some exceptions were sediments E2 and E6, where the differences between two oxygen regimes were large. However, worms in sediments from the Elbe and Oder estuaries generally had higher Ea than those in the control sediment.

There was very strong evidence for the effects of sediment and oxygen on ETS activity (Fig. 7). Most sediments from the Elbe and Oder estuaries resulted in higher ETS activity compared with the control sediment. Notably, the ETS activity was highest in worms exposed to sediments E5 and E6 (RR = 1.21–1.29). Worms exposed to intermittent oxygenation had lower ETS activity than those in continuous oxygenation (RR = 0.91). There was no evidence for the effect of sediment on CEA, but moderate evidence was found for the effect of oxygen, in which worms exposed to intermittent oxygenation had higher CEA (RR = 1.07, Fig. 7).

No evidence was found for the effects of sediment and oxygen on AMP levels, but there was moderate to strong evidence for the effects of sediment and oxygen on ATP and ADP levels (Fig. S5). Most sediments from the Elbe and Oder estuaries resulted in elevated ATP levels compared with the control sediment (RR up to 1.44), while ADP levels were more variable (RR = 0.84–1.26). Both ATP and ADP levels of

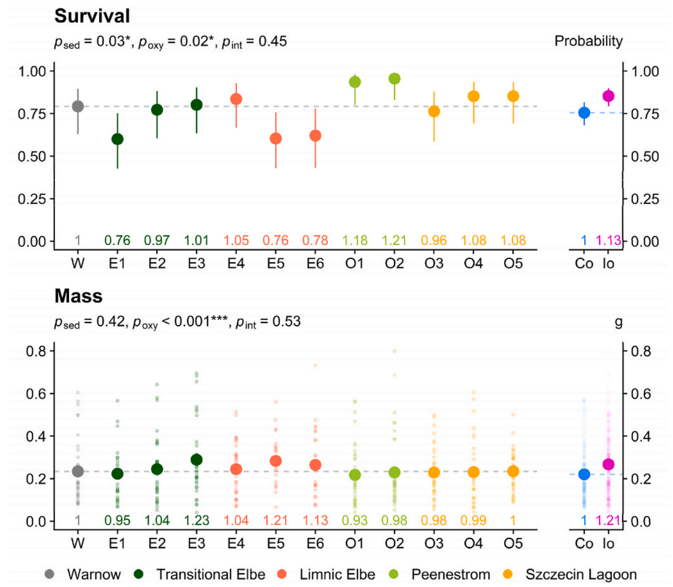

Fig. 5. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on survival and mass of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots. For survival, 95% confidence intervals are shown (n = 42 worms per sediment group and 252 worms per oxygen group). Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation p-values are given for sediment (sed), oxygen (oxy), and their interaction (int).

worms under intermittent oxygenation were lower than those in continuous oxygenation (RR = 0.89 and 0.92).

There was very strong evidence for the effects of sediment and oxygen on total adenylates, with elevated levels in most sediments (RR up to 1.22) but a reduced level under intermittent oxygenation (RR = 0.9, Fig. 8). However, no evidence was found for the effects of sediment and oxygen on AEC (Fig. 8).

3.7. Endpoint correlations and temporal changes

Strong correlations ($|r| \geq 0.7$) occurred only between composite biomarkers and their constituent biomarkers (Fig. S6). In particular, Ea was positively correlated with lipid content ($r = 0.78$). AXP was also positively correlated with ATP content ($r = 0.87$), while AEC was positively correlated with ATP content ($r = 0.79$) but negatively correlated with AMP content ($r = -0.88$).

There was no evidence for the differences in mass and biomarker responses between pre-exposure worms and post-exposure control worms (Table S2).

3.8. Toxicity assessment of sediments

In summary, evidence was found for the effects of sediment on worm survival, TAC, MGO content, and ETS activity. Based on these endpoints, sediment samples that were considered toxic are listed in Table 1. Toxic effects were most frequently observed in sediments E5 and E6.

Strong correlations ($|r_S| \geq 0.7$) were found between these endpoints and several contaminants (Fig. S7). Notably, survival was negatively correlated with Cr and Ni. MGO was positively correlated with Hg, Pb, Ni, Zn, CLBs, HCH, and DDX. Also, ETS was positively correlated with Pb, As, and Zn.

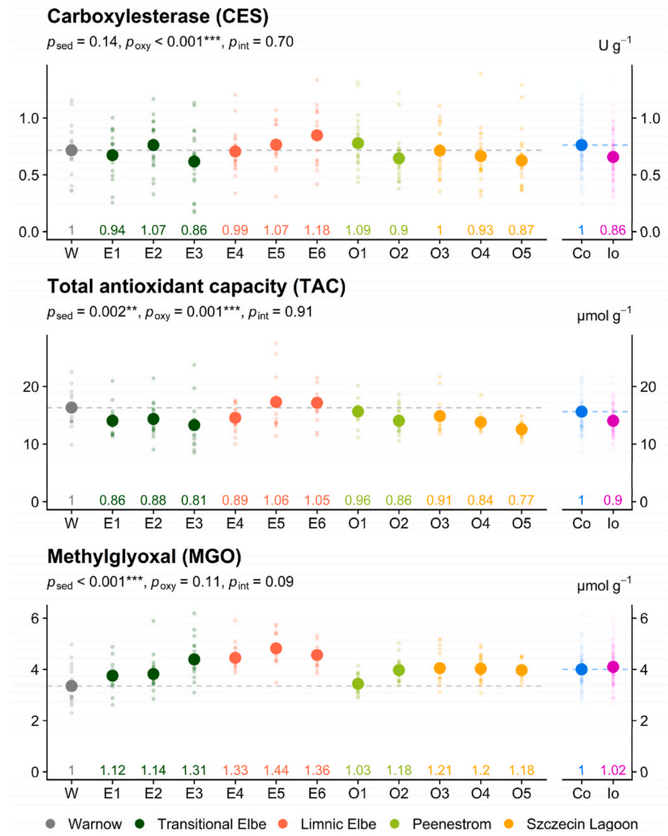

Fig. 6. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on carboxylesterase (CES) activity, total antioxidant capacity (TAC), and methylglyoxal (MGO) content of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots. Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation p-values are given for sediment (sed), oxygen (oxy), and their interaction (int).

4. Discussion

4.1. Sediment contamination in the Elbe and Oder estuaries

In this study, we considered only certain classes of persistent contaminants that are commonly used in monitoring programs (*German Environment Agency, 2024*). However, we expect that the observed patterns shared by the analyzed chemicals could be generalized to many other unmeasured substances.

Our data suggest that contamination gradients exist in both estuaries, with upstream sediments typically being more contaminated. This could be explained by the low-energy environment in the upper estuarine regions (*Kowalewska et al., 2003*; *Wetzel et al., 2013*), which favored the deposition of fine grains and organic matter. Due to the affinity of these sediment fractions for heavy metals and organic chemicals (Burton, 2002), the Limnic Elbe and the Szczecin Lagoon became effective sinks for contaminants. Towards the North Sea and the Baltic Sea, these contaminants were diluted by seawater and marine sediments (*Pham et al., 2024*), resulting in lower contamination status in the Transitional Elbe and the Peenestrom. Notably, the influence of the marine environment can also be found in upstream sampling sites, as indicated by the C:N ratios in sediments between 8 and 12, typical for organic matter of mixed marine and terrestrial origins (*Tang et al., 2020*).

In general, the Elbe estuary had higher levels of contaminants than the Oder. Several factors may contribute to this trend, such as differences in contaminant loads from their catchment areas or simply the

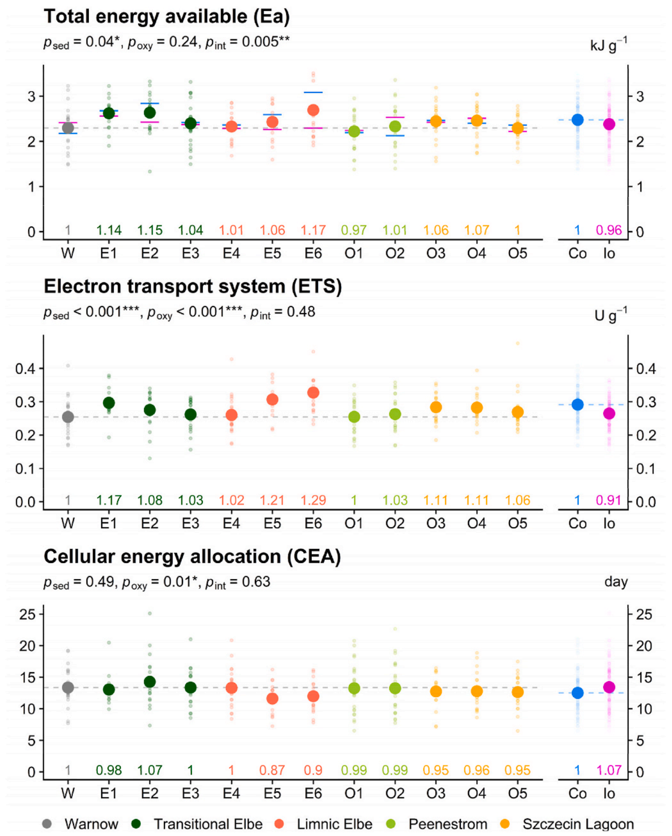

Fig. 7. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on total energy available (Ea), electron transport system (ETS) activity, and cellular energy allocation (CEA) of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots. Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation *p*-values are given for sediment (sed), oxygen (oxy), and their interaction (int). To illustrate the interactive effect on Ea, two short segments indicating predicted means of oxygen regimes are additionally shown for each sediment sample.

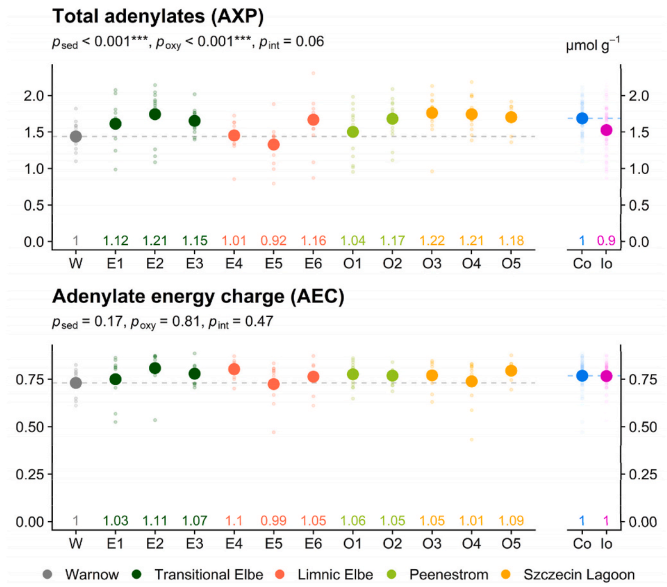

Fig. 8. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on total adenylates (AXP) and adenylate energy charge (AEC) of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots. Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation *p*-values are given for sediment (sed), oxygen (oxy), and their interaction (int).

proximity of sampling sites to the upstream contamination sources. An exception among the analyzed chemicals was PAHs, which were more abundant in the Oder estuary sediments. As by-products of the incomplete combustion of organic matter, elevated PAH contents possibly reflect the intensive industrial activities such as coal mining and metallurgy in the Oder headwaters in the past (Kowalewska et al., 2003; Müller et al., 2002).

Compared with the 1980s, water and sediment quality in the Elbe and Oder rivers have reportedly improved as a result of European and national environmental measures, such as emission reductions and the construction of wastewater treatment plants (Ciszewski, 2003; Netzband et al., 2002). Although we did not investigate the temporal changes in sediment contamination, our study found similar ranges of heavy metals and organic contaminants as previous surveys in the Elbe and Oder estuaries (Kowalewska et al., 2003; Wetzel et al., 2013). This may signal a slowdown in the sediment quality improvement in recent years.

The presence of synthetic organic chemicals or the elevated contents of naturally occurring substances such as heavy metals in sediments only indicates contamination. Pollution, on the other hand, is the extent to which contamination causes adverse biological effects (Chapman, 2007). For sediment pollution assessment, chemical data are often compared with regulatory benchmarks, such as Sediment Quality Guidelines (SQG) or European Environmental Quality Standards (EQS). However, these benchmarks are only provided for a limited number of chemicals (Birch, 2018; Burton, 2002) and usually not tailored to estuarine sediments (Chapman, 2002; Chapman and Wang, 2001). Among the analyzed contaminants in our study, EQS are only available for As, Cr, Cu, and Zn (40, 640, 160, 800 mg kg^{-1} of the <63 μm fraction, respectively), and PCBs (20 μg kg^{-1} of the <2 mm fraction for each congener) (German Environment Agency, 2017). While the PCBs content in all sediment samples did not exceed the threshold value, a comparison of heavy metal contents with EQS is unfortunately not possible because our measurements were made in the <20 μm fraction. Given the limitations of chemical-based pollution assessment, it is often used in combination with other approaches such as sediment toxicity testing (Chapman, 1990), as was done in our study.

4.2. Sediment toxicity to ragworms

The most ecologically relevant endpoint in our toxicity test was survival. A significant decrease in worm survival was found in sediments E5 and E6 from the Limnic Elbe, which is not surprising as these sediments were the most contaminated. However, survival was also low in sediment E1 from the Transitional Elbe, which did not show high levels of the analyzed contaminants. It is possible that the observed toxic effect was caused by unmeasured chemicals in the sediment. In this case, Toxicity Identification Evaluation (TIE) procedures could be performed to find the causative chemicals (Ho and Burgess, 2009). Notably, worm survival was high in the Peenestrom sediments, which could be the combined result of low contamination and adequate organic matter supply in the samples. Given the differences in worm survival between sediment samples, survival bias may be present in other endpoints.

We did not observe increased activities of the biotransformation enzymes CES, GST, and GR in worms. CES and GST detoxify contaminants by hydrolysis and glutathione conjugation, respectively (Parkinson et al., 2022), while GR is responsible for glutathione regeneration (Couto et al., 2016). TAC, which measures the amount of enzymatic and non-enzymatic antioxidants to neutralize reactive oxygen species (ROS) (Regoli et al., 2002), was significantly reduced in only one sample (sediment O5 in the Szczecin Lagoon). These results may indicate that

Table 1
Sediment toxicity assessment based on test endpoints. Only endpoints showing the sediment effect with p-value ≤ 0.05 were included. Sediment samples with RR $\leq$ 0.8 or RR $\geq$ 1.2 were identified as toxic and are marked with "+".

	E1	E2	E3	E4	E5	E6	O1	O2	O3	O4	O5
Survival	+				+	+					
TAC											+
MGO			+	+	+	+			+	+	
ETS					+	+					

the contamination levels of most sediments from the Elbe and Oder estuaries were not high enough to stimulate detoxification and antioxidant defense in ragworms.

We also examined three biomarkers of electrophilic stress, MGO, MDA, and PC. MGO is formed as a by-product of glycolysis and many other metabolic pathways (*Lai* et al., *2022*), while MDA is an end product of ROS-induced lipid peroxidation (*Ayala* et al., *2014*). MGO and MDA are both reactive carbonyl species (RCS) that irreversibly modify proteins and lead to the formation of PC (*Rodríguez-García* et al., *2020*). Here we found elevated levels of MGO in worms in more than half of the sediment samples, but no changes in MDA and PC levels, suggesting substantial, but not very high, electrophilic stress.

Significantly elevated ETS activity was observed in worms exposed to sediments E5 and E6 from the Limnic Elbe, indicating increased cellular maintenance costs to cope with contaminants (*Fanslow* et al., *2001*; *Sokolova, 2021*). However, worms in most sediment samples were able to maintain high total energy available in their carbohydrate, lipid, and protein reserves, as well as high levels of ATP and total adenylates. Consequently, both CEA and AEC were maintained at the normal levels. CEA can be thought of as the time that worms can survive on their energy reserves after the energy input is stopped (*Pham* et al., *2023*), while AEC indicates the amount of useable metabolic energy stored in the adenylate pool (*Fuente* et al., *2014*). Worm mass, as a proxy for growth, also did not differ between sediment samples. Taken together, these results suggest that the Elbe and Oder estuary sediments did not cause significant impairment of worm energy status, probably due to the fact that more contaminated sediments often have more food supply in the form of organic matter (*Mouneyrac* et al., *2010*).

Among the multiple biomarkers measured in our study, TAC, MGO content, and ETS activity were more sensitive to contaminated sediments. In particular, MGO and ETS levels were strongly associated with multiple contaminants in sediments, suggesting potential causal relationships. Similar results were obtained in previous toxicity tests with ragworms (*Pham et al., 2023, 2024*). These findings suggest that the testing effort could be reduced by focusing on a small set of sensitive biomarkers in this species (*Falfushynska* et al., *2024*).

Our current testing approach with ragworms also has several limitations. First, ragworms used for toxicity testing must be collected from relatively uncontaminated areas, which are becoming rarer due to widespread global contamination (*Williams* et al., *2022*). This challenge could be overcome by breeding and rearing worms under laboratory conditions or indoor systems (*Nesto* et al., *2018*). Secondly, we focused only on a pre-selected set of biomarkers, and the small mass of ragworms necessitates a large sample size to perform all traditional biomarker assays. Future studies could use omics approaches to evaluate a broader range of biomarkers in smaller sample sizes (*Simpson* et al., *2016*).

4.3. Effects of oxygen on test endpoints

Worms under intermittent oxygenation had lower ETS activity and ATP levels. These results are expected because the lack of oxygen as a terminal electron acceptor slows down the mitochondrial electron transport chain, which in turn suppresses ATP production by chemiosmosis (*Sokolova* et al., *2019*). Since ETS activity decreased but the total energy available remained stable, intermittent oxygenation led to an increase in CEA (*Pham* et al., *2023*). The metabolic depression under intermittent oxygenation probably also led to the observed decreases in ADP and total adenylates, which helped maintain the AEC within its narrow range (*Fuente* et al., *2014*; *Sokolova, 2013*).

We found the reduced TAC in worms under intermittent oxygenation, indicating a depleted amount of antioxidants. This could be explained by the excessive production of ROS during both deoxygenation and reoxygenation (*Dröse* et al., *2016*). We also observed a decrease in CES activity during intermittent oxygenation, which could be due to enzyme damage by ROS or low enzyme production under low ATP conditions.

Notably, intermittent oxygenation led to higher worm survival and mass. We postulate that a lower metabolic rate at reduced oxygen levels probably prolonged the lifespan as well as preserved the standing stock of energy reserves. In this regard, continuous oxygenation, often used in laboratory settings, might be considered a stressor for ragworms.

It is a common assumption that defense against contaminants is energetically costly and therefore cannot work at full capacity under limited energy conditions, such as deoxygenation (*Sokolova, 2013*). This is the basis for the concerns about the interaction between contaminants and oxygen. In our study, we found interaction effects only on protein content and consequently on total energy available, while all other endpoints showed additive effects. The lack of interaction could be attributed to the relatively weak effects of intermittent oxygenation, as indicated by small effect sizes with little toxicological significance. This could be explained by the tolerance of ragworms to oxygen fluctuations, including prolonged hypoxia as suggested by previous studies (*Santos* et al., *2016*; *Vismann, 1990*). Our result implies the robustness of the biomarker-based toxicity test using ragworms, as reported in other studies (*Pham et al., 2023, 2024*), in which the deviations of controlled conditions such as temperature and oxygen do not influence the conclusion about sediment toxicity (*Simpson* et al., *2016*).

4.4. Ecological and regulatory implications

The high levels of heavy metals and organic chemicals in sediments from the upper region of the Elbe estuary and their observed toxic effects on ragworms indicate ecological risks to estuarine ecosystems. These contaminants may not only affect local benthic communities but may also affect estuarine organisms in the coastal region, especially if disturbance events such as dredging or flooding cause the transport of contaminated suspended particles downstream (*Crawford* et al., *2022*; *Roberts, 2012*). Therefore, careful management of dredging, disposal, and flood control practices is important to limit adverse ecological effects.

An early proposal for sediment pollution assessment is the Sediment Quality Triad (SQT), which integrates three pillars: sediment chemical analysis, sediment toxicity testing, and benthic community surveys (*Chapman, 1990*). While the first and third pillars have been incorporated into European regulatory frameworks, the adoption of sediment toxicity testing has been slower (*Ausili* et al., *2022*; *Leppanen* et al., *2024*; *Tarazona* et al., *2014*). This delay can be attributed to the stakeholder skepticism about the utility of current toxicity tests, including concerns about the ecological relevance of test conditions, test species, and endpoints such as biomarkers (*Hagger* et al., *2006*; *Heise* et al., *2020*). These concerns could be mitigated by conducting toxicity tests on contaminated sediments under more relevant climatic scenarios, with more ecologically important species, and using multiple biomarkers to provide a more reliable assessment (Pham et al., 2024; Pham and Sokolova, 2023), as exemplified by our study.

4.5. Conclusions

Sediments from the Elbe and Oder estuaries were contaminated with various heavy metals and organic chemicals. Based on survival and multiple biomarkers, we identified several sediment samples as toxic to ragworms. The most problematic sediments were from the upper region of the Elbe estuary, which contained high levels of contaminants. The effects of sediments on ragworms were generally not modified by oxygen regimes, suggesting the robustness of the sediment toxicity test. Our study demonstrates the utility of biomarker-based toxicity testing for estuarine sediment pollution assessment and its potential for incorporation into regulatory frameworks.

CRediT authorship contribution statement

Duy Nghia Pham: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Safia El Toum:** Writing – review & editing, Investigation. **Raphaëlle Martineau:** Writing – review & editing, Investigation. **Susanne Heise:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation. **Inna M. Sokolova:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.120451.

Data availability

Research data are publicly available on Zenodo at https://doi.org/10.5281/zenodo.14037268.

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Sediment contamination in two German estuaries: A biomarker-based toxicity test with the ragworm *Hediste diversicolor* under intermittent oxygenation

Supplementary material

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Introduction

Table S1. Sediment sampling sites in the Elbe and Oder estuaries. Six sites (E1 to E6) were selected in two regions of the Elbe estuary and five sites (O1 to O5) were selected in two regions of the Oder estuary. Ragworms *Hediste diversicolor* and control sediment were collected from a site (W) in the Warnow estuary.

Sediment	Region	Latitude	Longitude
E1	Transitional Elbe	53.829720	8.886049
E2	Transitional Elbe	53.838480	9.310470
E3	Transitional Elbe	53.680654	9.489343
E4	Limnic Elbe	53.530810	9.806947
E5	Limnic Elbe	53.470472	10.001008
E6	Limnic Elbe	53.408223	10.252025
O1	Peenestrom	54.016483	13.846550
O2	Peenestrom	53.884833	13.865450
O3	Szczecin Lagoon	53.803250	14.022483
O4	Szczecin Lagoon	53.785567	14.077633
O5	Szczecin Lagoon	53.767983	14.129067
W	Warnow	54.172800	12.141400

Materials and methods

Sediment characterization

PAHs consisted of 16 compounds: Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenz[a,h]anthracene, and Benzo[g,h,i]perylene. CLBs consisted of Pentachlorobenzene and Hexachlorobenzene. PCBs consisted of seven congeners: PCB 28, PCB 52, PCB 101, PCB 118, PCB 153, PCB 138, and PCB 180. HCH consisted of five isomers: alpha-HCH, beta-HCH, gamma-HCH, delta-HCH, and epsilon-HCH. DDX consisted of six compounds: o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT, and p,p'-DDT.

Dry matter content and grain size distribution were measured according to DIN ISO 11465:1996-12 and DIN EN ISO 17892-4:2017-04, respectively. Heavy metals were analyzed according to DIN EN 16171:2017-01, except for mercury, which was measured according to DIN ISO 16772:2005-06. PAHs and PCBs were analyzed according to DIN ISO 18287:2006-05 and DIN EN 17322:2021-03, respectively. HCH, CLBs, and DDX were measured according to DIN ISO 10382:2003-05. Measurement uncertainties were calculated according to DIN ISO 11352:2013-03. In this study, all 95% confidence intervals (expanded uncertainties) were less than ±20% of the measured values.

Results

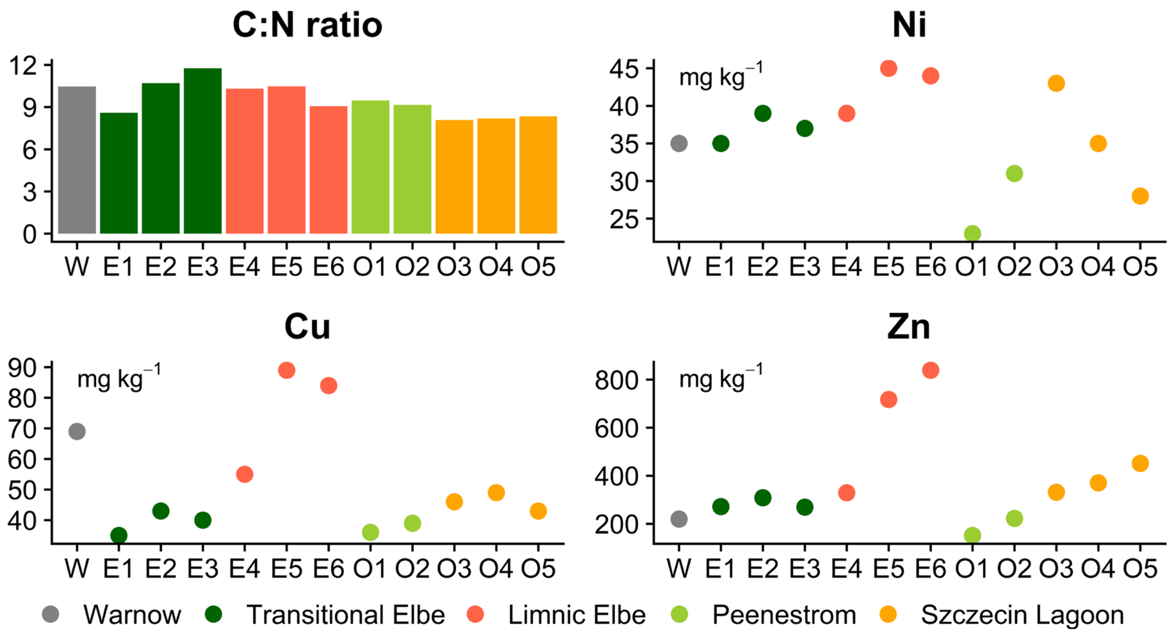

Figure S1. C:N ratio and contents of Ni, Cu, and Zn of sediments collected at 12 sites in the Warnow, Elbe, and Oder estuaries.

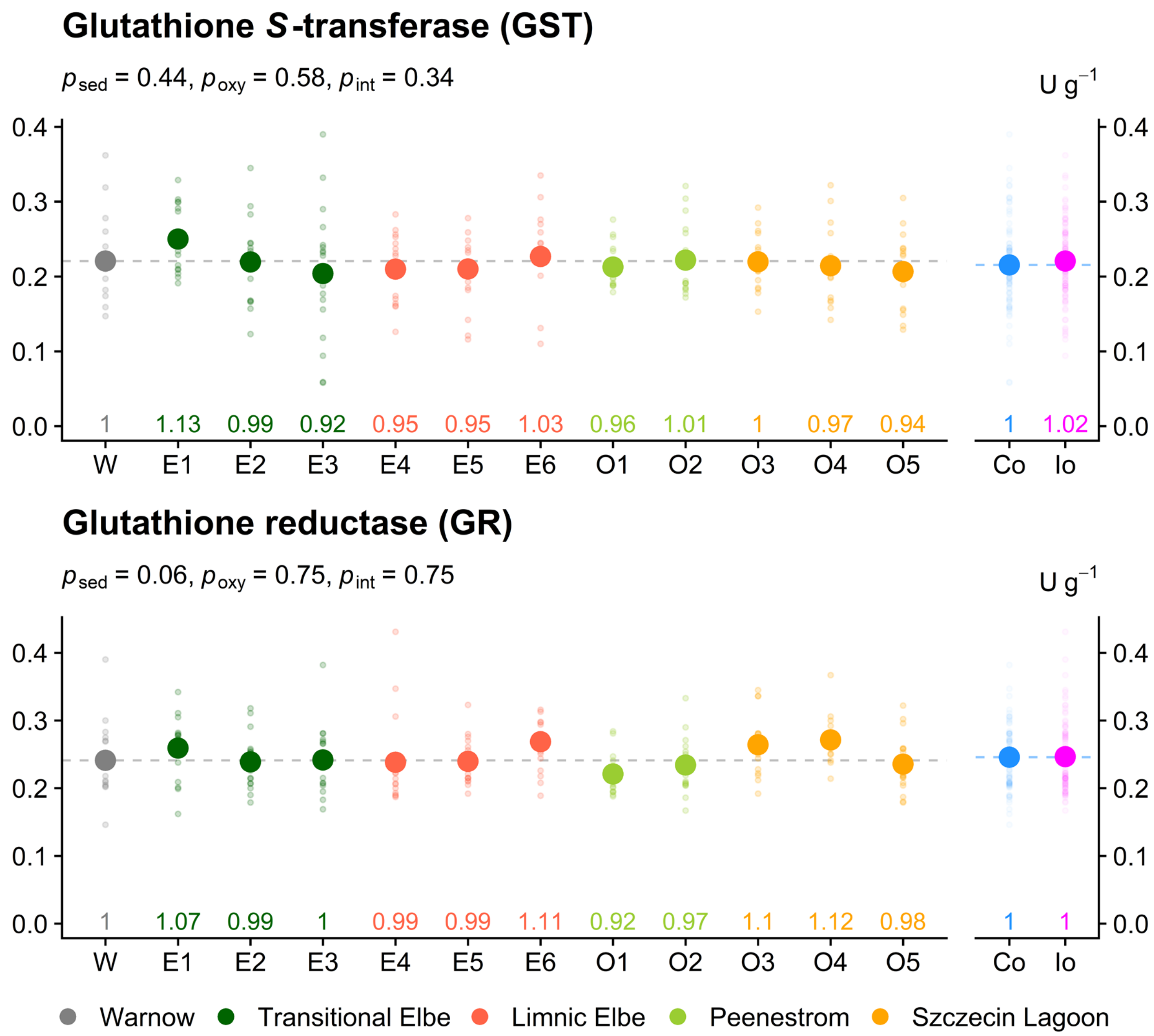

Figure S2. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on glutathione *S*-transferase (GST) and glutathione reductase (GR) activities of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots. Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation *p*-values are given for sediment (sed), oxygen (oxy), and their interaction (int).

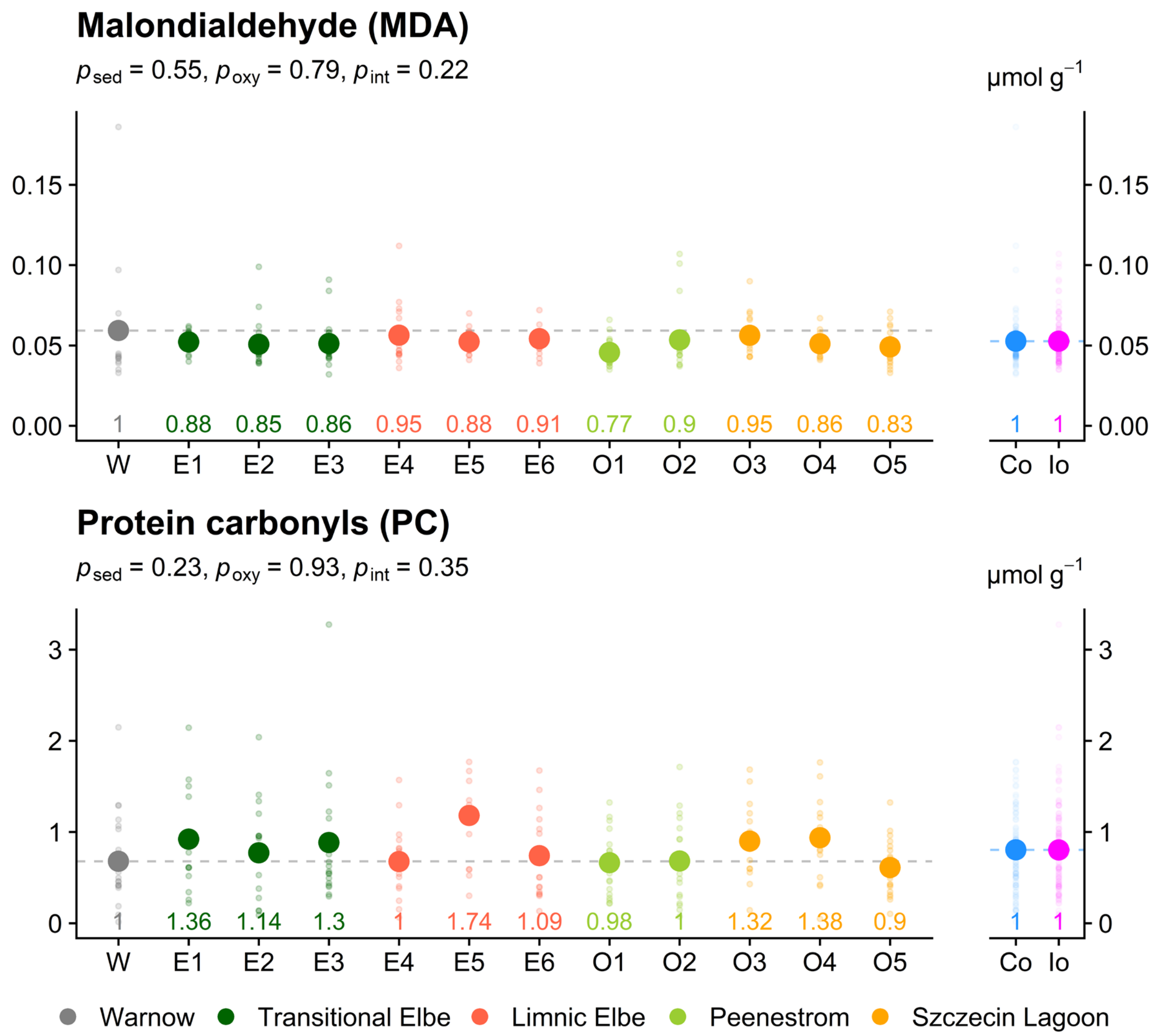

Figure S3. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on malondialdehyde (MDA) and protein carbonyl (PC) levels of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots. Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation *p*-values are given for sediment (sed), oxygen (oxy), and their interaction (int).

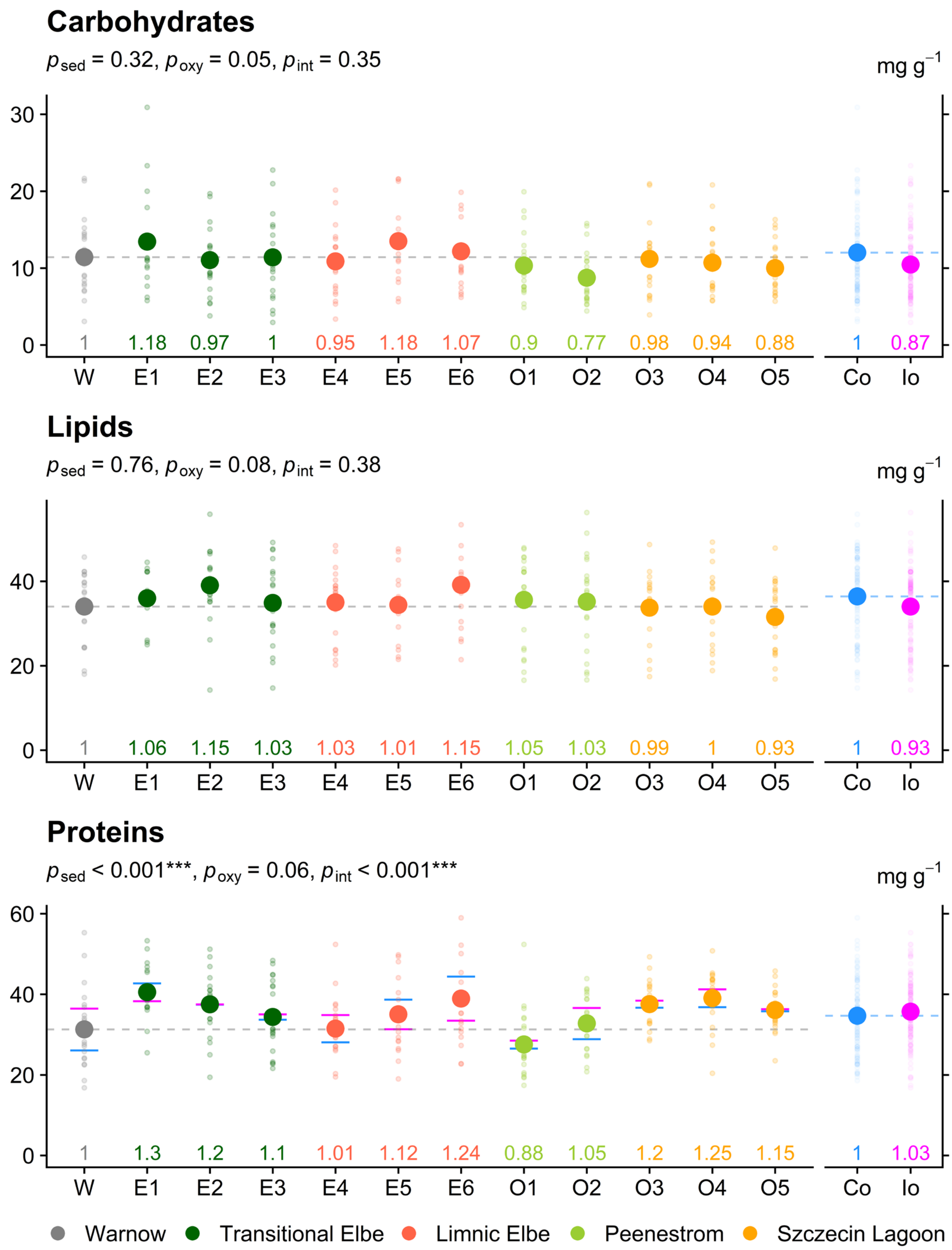

Figure S4. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on carbohydrate (CAR), lipid (LIP), and protein (PRO) contents of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots. Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation *p*-values are given for sediment (sed), oxygen (oxy), and their interaction (int). To illustrate the interactive effect on protein content, two short segments indicating predicted means of oxygen regimes are additionally shown for each sediment sample.

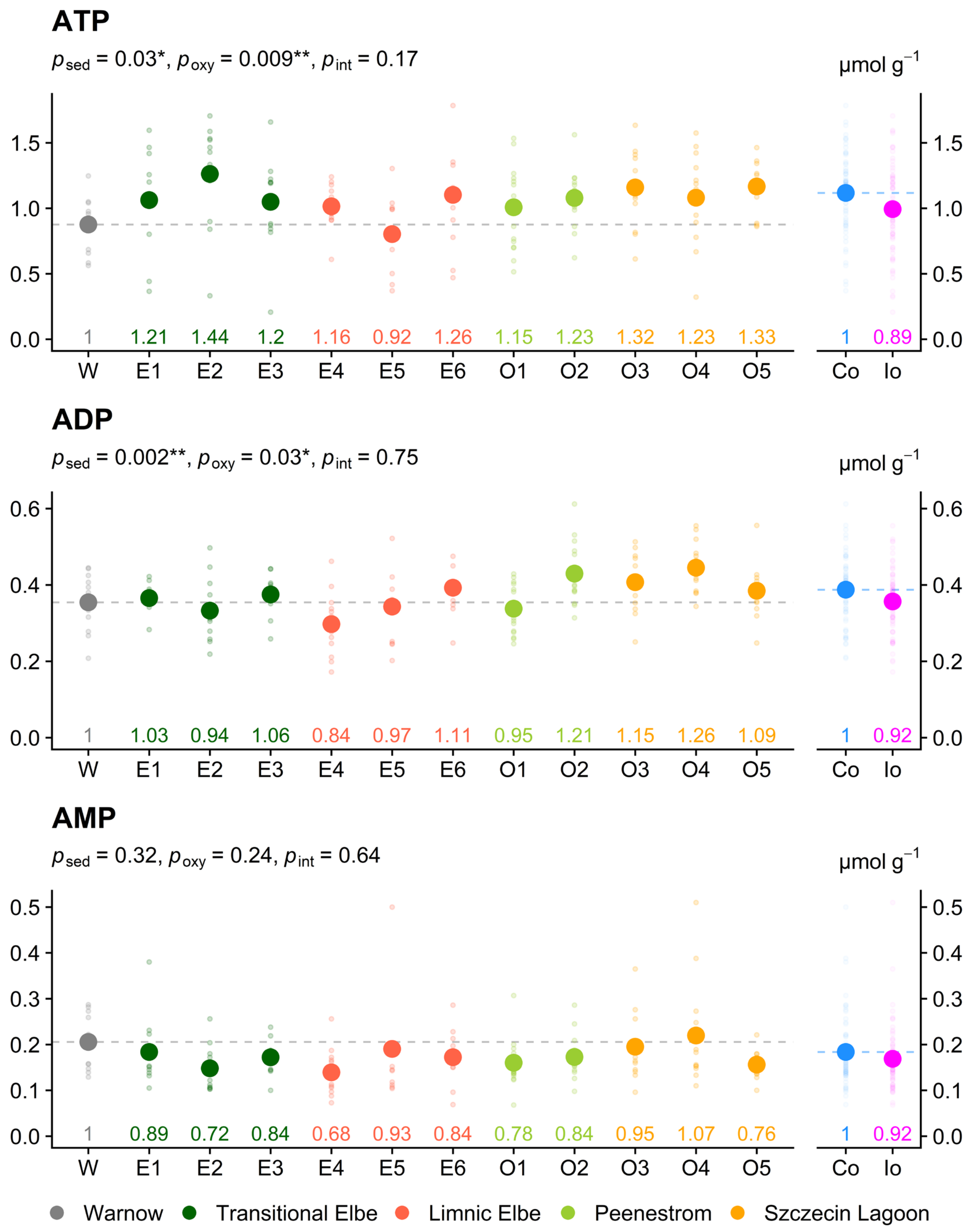

Figure S5. Effects of the Elbe and Oder estuary sediments (E1-E6 and O1-O5) and intermittent oxygenation (Io) on ATP, ADP, and AMP contents of ragworms, with the Warnow estuary sediment (W) and continuous oxygenation (Co) as controls. Individual observations are shown as small dots and predicted means are shown as large dots.

Dashed lines indicate the control means. Response ratios (RR) are given for 12 sediment samples and two oxygen regimes. Permutation *p*-values are given for sediment (sed), oxygen (oxy), and their interaction (int).

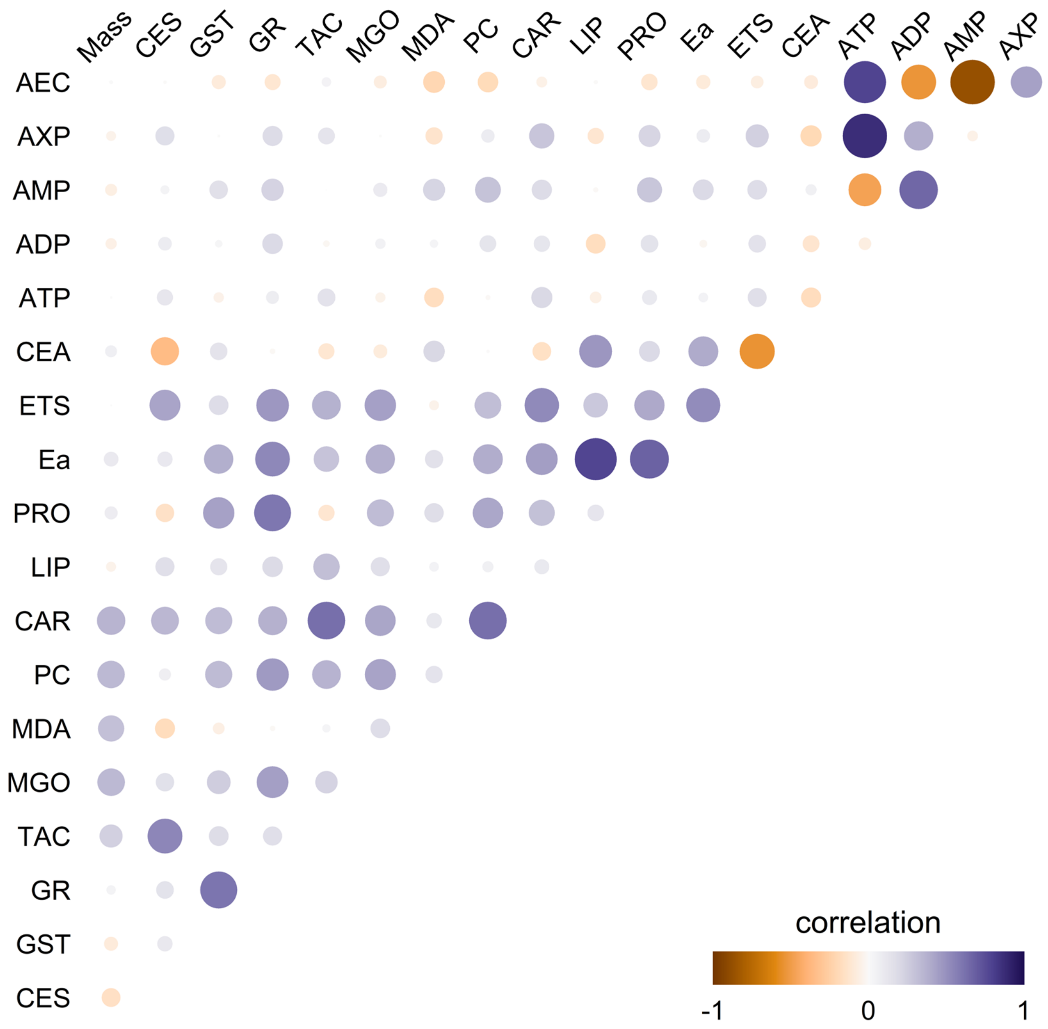

Figure S6. Correlations between test endpoints in post-exposure ragworms. The size and color of the circles represent the magnitude of Pearson's coefficient.

Table S2. Permutation tests for differences in mass and biomarker responses between pre-exposure worms and post-exposure control worms.

	F	*p*
Mass	0.88	0.32
CES	0.00	0.97

	F	*p*
GST	0.26	0.62
GR	0.52	0.52
TAC	0.06	0.81
MGO	2.41	0.10
MDA	0.59	0.55
PC	1.13	0.31
CAR	0.42	0.53
LIP	0.14	0.72
PRO	2.51	0.15
Ea	1.77	0.18
ETS	0.43	0.53
CEA	0.00	1.00
ATP	0.82	0.38
ADP	1.80	0.21
AMP	0.09	0.77
AXP	2.33	0.13
AEC	0.05	0.82

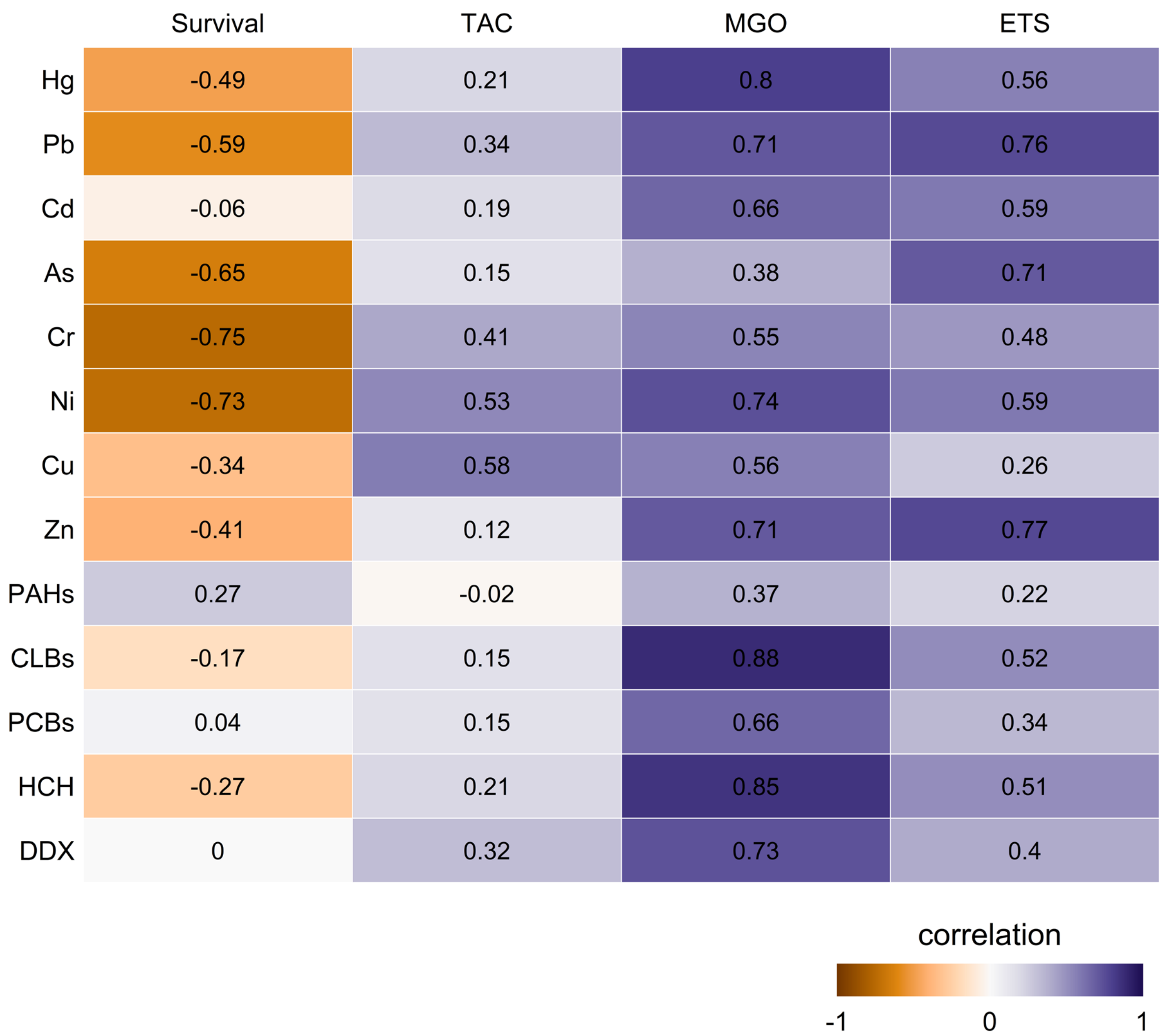

Figure S7. Correlations between significant test endpoints and contaminant levels of sediments. Color represents the magnitude of the given Spearman's coefficient.

Declaration of authorship

I hereby declare that I am solely responsible for the content of my dissertation and that I have used only the sources or references cited in the dissertation.

Rostock, 2025-04-06

Duy Nghia PHAM

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