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**Metabolic responses to multiple stressors (oxygen,
temperature and pollutants) in benthic marine
invertebrates**

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Zusammenfassung

Die marine Umwelt und besonders die Küstenregionen werden durch hohe Produktivität und Biodiversität charakterisiert und liefern zudem verschiedenste Ressourcen und Ökosystemservices für die Menschheit. Diese Habitate sind jedoch auch starken Variationen an Umweltbedingungen ausgesetzt wie beispielsweise Fluktuation in Temperatur, Sauerstoff oder Trockenstress. Hierdurch ergibt sich die Notwendigkeit der Etablierung von Schutzmechanismen oder der Adaption. Zusätzlich beeinflussen anthropogene Einflüsse, wie beispielsweise Schadstoffe, regelmäßig Küstenhabitate. Verschmutzung durch UV-Filter, welche meist aus Körperpflegeprodukten stammt, ist ein aufkommendes Problem in diesen Ökosystemen. Sowohl physikalische als auch chemische Stressoren beeinflussen die Physiologie des Zielorganismus indem sie beispielsweise oxidativen Stress, Veränderungen der Bioenergetik oder metabolische Störungen verursachen. Das Wissen über kombinierte Stressoren, welche in Küstenhabitaten oft zusammen auftreten, ist limitiert. In dieser Arbeit untersuchen wir die metabolischen Effekte von multiplen Stressoren mit Fokus auf Kombinationen von periodischer Hypoxie und Temperatur, und den UV-Filtern nano-Zinkoxid (nZnO) und Avobenzon auf die Pazifische Auster *Crassostrea gigas* und den Wattwurm *Arenicola marina*.

Die Austern wurden zehn Zyklen periodische Hypoxie (jeweils 12h eingetaucht bzw. aufgetaucht) bei konstanter (15°C oder 30°C) oder wechselnder Temperatur (15°C eingetaucht; 30°C aufgetaucht) ausgesetzt. Unsere Ergebnisse zeigen Stoffwechselveränderungen in allen Expositionen. Unter konstant erhöhter Temperatur (30°C) wurde zudem ein hoher Gewebeschaden sowie erhöhte Mortalität nachgewiesen. Wechselnde Temperatur führte hingegen zum Aufbrauchen der Energiereserven sowie bioenergetischen Anpassungen als Effekt der Stoffwechseländerung. Periodische Hypoxie in Kombination mit wechselnder Temperatur, wie im Intertidal zu finden, können daher überstanden werden, benötigen jedoch energetisch fordernde Stoffwechselanpassungen.

Um die Effekte der UV-Filter zu testen, exponierten wir *A. marina* über einen Zeitraum von 2 bzw. 3 Wochen mit dem anorganischen UV-Filter nZnO (0,1 mg/kg und 1 mg/kg bzw. 3 mg/kg Sediment), dem organischen UV-Filter Avobenzon (50 µg/l Seewasser) und deren Kombination (3mg/kg nZnO und 50µg/l Avobenzon). Unsere Ergebnisse konnten sowohl oxidativen Schaden als auch Stoffwechselanpassungen, wie beispielsweise in den freien Aminosäuren, ausgelöst durch beide UV-Filter, nachweisen. In der kombinierten Exposition wurde der Stress hauptsächlich durch nZnO getrieben.

Zusätzlich konnten wir sowohl bi-phasische Effekte in Bezug auf die nZnO-Dosis nachweisen als auch die Rolle der Hämolymphe in der Stressantwort hervorheben.

Die Studie bietet wichtige Einblicke in die interaktiven Effekte von kombinierten Stressoren und zeigt, dass die Kombination mildernde, verstärkende oder biphasische Effekte in marinen benthischen Organismen auslösen kann. Unsere Resultate verdeutlichen die Limitierung der Extrapolation von Erkenntnissen aus Einzelexpositionen auf Szenarien, die kombinierte Stressoren wie die in Küstensystemen enthalten. Zudem bietet unsere Forschung neue Einblicke in die Ökotoxikologie des geringer untersuchten UV-Filters Avobenzon und bestätigt die Widerstandsfähigkeit von zwei wichtigen Ökosystemingenieuren gegen die getesteten Stressoren.

Summary

Marine environments and especially coastal regions are characterized by high productivity and biodiversity and provide various resources and ecosystem services for humans. However, coastal habitats are also submitted to high variations in environmental conditions such as fluctuation in temperature, oxygen or desiccation, thus creating the necessity for protection and adaptation. Furthermore, anthropogenic stressors such as pollutants frequently affect coastal habitats. Pollution by UV filters, which often derive from personal care products, is an emerging problem in coastal ecosystems. Both physical and chemical stressors can have impacts on the physiology of the targeted organisms such as oxidative stress, bioenergetic shifts and metabolic disruption. However, knowledge about the combined effects of multiple stressors that often co-occur in coastal habitats remains limited. In this work, we investigate the metabolic effects of multiple stressors focusing on different combinations of intermittent hypoxia, temperature and UV filters (nano zinc oxide (nZnO) and avobenzone) in the Pacific oyster *Crassostrea gigas* and the lugworm *Arenicola marina*.

The oysters were submitted to ten cycles of intermittent hypoxia (12h immersion/12h emersion) at constant (15°C or 30°C) or fluctuating temperature (15°C immersion, 30°C emersion). Our results revealed metabolic shifts in all exposures. These shifts were accompanied by high mortality and tissue damage under constant elevated (30°C) temperature whereas fluctuating temperature caused energetic depletion in addition to the metabolic shifts. Thus, intermittent hypoxia in combination with fluctuating temperature, mimicking natural tidal conditions, can be sustained but requires energetically costly metabolic rearrangements.

To test the effects of the UV filters, *A. marina* was exposed for two or three weeks to different concentrations of an inorganic UV filter nZnO (0.1, 1 and 3 mg Zn per kg sediment), an organic UV filter avobenzone (50 µg/l seawater) or their combination (3 mg/kg nZnO + 50 µg/l avobenzone). Our results found signs of oxidative damage and metabolic shifts such as changes in free amino acids caused by both UV filters, but effects were mostly driven by nZnO in the combined exposure. Further, we could highlight the stress response of the body fluids and found evidence for biphasic effects related to the nZnO dosage.

This study provides important insights in the interactive effects of combined stressors and shows that stressor combination may have mitigating, enhancing or biphasic effects in marine benthic organisms. These findings underscore the limitation of extrapolating data from single stressor studies to scenarios involving combined stressors, which are

prevalent in coastal habitats. Furthermore, our research offers new insights into the ecotoxicology of the little-studied organic UV filter avobenzone and confirms the resilience of two important ecosystem engineers against the tested stressors.

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– Dedicated to those who are already gone. –

List of abbreviations

Abbreviation	Meaning
AMP	Adenosine mono phosphate
ATP	Adenosine triphosphate
DO	Dissolved oxygen
ETS	Electron transport system
GSH	Glutathione
NPs	Nanoparticles
nZnO	Nano zinc oxide
PCPs	Personal care products
PLS-DA	Partial least squares discriminant analysis
Q ₁₀	Temperature coefficient
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TiO ₂	Titanium dioxide
ZnO	Zinc oxide

Part I Summary of the doctoral study

1. Coastal benthic ecosystems as a dynamic habitat posing physiological challenges to benthic organisms

Worldwide approximately 356,000 km of coastline connect terrestrial with marine life (Central Intelligence Agency). Taking the coastline paradox into account, which describes the increasing coastline length with increasing resolution, this number is even higher (Mandelbrot, 1967; Richardson, 1961). Further, up to 40 % of the world's population lives within 100 kilometers of the coastline, depending on the coast and sea for living space, energy and food resources, transportation, recreation, and cultural heritage (Frid and Evans, 2023; Mouneyrac et al., 2014). Although coastal areas are well accessible and important for humankind, comprehensive standardized research and monitoring is limited to selected areas (Frid and Evans, 2023). Apart from ecosystem services for humans, coastal areas are important for marine life. Shallow marine waters are often characterized by a high primary production, providing the basis for heterotrophic life (Feuerpfeil et al., 2004; Kröncke and Bergfeld, 2003; Ott et al., 2023; Snoeijs-Leijonmalm et al., 2017). Further, such habitats are the spawning and nursing ground for many different pelagic and benthic species, relying on the protection and food supply of shallow coastal habitats (Ott et al., 2023).

However, living in coastal regions also poses many physiological challenges, with benthic organisms being particularly affected (Vernberg and Vernberg, 1972; Willmer et al., 2005). Shallow coastal areas are characterized by highly variable physical and biological parameters. Changing currents and tidal immersion-emersion cycles lead to drastic changes in temperature and oxygen availability (Snoeijs-Leijonmalm et al., 2017; Willmer et al., 2005). Further, wave actions can cause high sediment dynamics by suspending matter, which might expose burrowing sessile benthic organisms and decrease their chance of survival, and interfere with oxygen uptake and filter feeding of marine organisms (Carniello et al., 2014; Emerson and Grant, 1991; Powilleit et al., 2009; Rios-Yunes et al., 2023). Stronger wave currents in shallow regions, which can reach well above 5 m/s, even have the potential to relocate benthic invertebrates (Emerson and Grant, 1991; Stewart, 2008). The change in salinity caused by freshwater inflow from the land, river outflows, or precipitation can also cause stress in many marine organisms (Herlemann et al., 2011).

In addition to these physical factors, the biological factors in the coastal regions also show strong fluctuations. Depending on the location and season, the food availability and quality can be limited or enhanced due factors such as upwelling events or the limitation of resources, which regulate phytoplankton populations (Snoeijs-Leijonmalm

et al., 2017). Consequently, this can affect higher trophic levels such as the nutrition of filter feeding bivalves (Ott et al., 2023; Phillips, 2005; Puccinelli, 2015). In addition, anthropogenic activities contribute to fluctuations in marine ecosystems. For example, selective fishing of predator species disrupts the top down regulation of the food chain, thereby increasing the prey population which leads to cascading effects in the ecosystem (Madin et al., 2016). One of the largest anthropogenic impacts on the marine environment is chemical pollution. Shallow coastal areas act as a sink for anthropogenic pollutants that accumulate in the water, sediment and biota and can have deleterious effects such as decline of populations of marine organisms or restructuring of the entire ecosystem (Beiras, 2018; Hanna et al., 2013; Vikas and Dwarakish, 2015). Thus, marine ecosystems are impacted by different factors in various levels and intensities, driving the necessity for organisms to adapt to these conditions and maintain sustainable populations. The lack of successful adaptation, particularly to rapid environmental changes such as climate change or chemical pollutants, can lead to population decline and loss of biodiversity (Beiras, 2018; Willmer et al., 2005).

2. The intertidal zone as an example of extreme abiotic environment

The intertidal zone is a habitat characterized by high fluctuations of abiotic conditions (Bernhardt et al., 2020; Vernberg and Vernberg, 1972). This habitat is defined as the area between high and low water and is also called eulittoral zone (Begon et al., 2017; Dictionary Geotechnical Engineering, 2014). The extent of this zone varies depending on the amplitude of the tide as well as the benthic slope (Begon et al., 2017). The seafloor of the intertidal habitat emerges once (diurnal; 24 h period) or twice (semi-diurnal; 12 h period) a day (Desplanque and Mossman, 2004; Stewart, 2008). Hence, depending on the tidal regime and the vertical position on the shore relative to the low water mark, sessile benthic animals can emerge for many hours each day, which exposes them to different threats. Although aquatic predators are present during submersion, emersions introduces and enhances the risk of predation by birds and other terrestrial animals as well as aquatic specialist predators foraging during low tide (Cadée, 1990; Carlton and Hodder, 2003; Meire, 1993; Thiel, 1997). In addition, low tide cuts off the food supply for many suspension feeders, thus limiting the time for nutrition uptake (B.L. Bayne, 2017a; Rijken, 1979; Vernberg and Vernberg, 1972). Another factor to consider is the risk of desiccation during air exposure in the intertidal zone (Vernberg and Vernberg, 1972). Most aquatic animals are adapted to constant surrounding water with dry survival ranging from a few minutes to a few hours for organisms that are not specialized to endure emersion (Bingham et al., 2011; Cook et al., 2015; Davis, 2002). However, many marine intertidal invertebrates can survive air exposure and tolerate a certain degree of desiccation (Brown, 1960; Foster, 1971; Vernberg and Vernberg, 1972). Prolonged desiccation can cause intracellular damage such as protein misfolding, impaired gene expression or disrupted membrane structure (Somero and Yancey, 1997). The water loss can disrupt the osmotic balance of the cells in the organisms, leading to decreased efficiency of metabolic processes and in extreme cases, can result in cell death (B.L. Bayne, 2017b; Clegg et al., 1982; Somero and Yancey, 1997).

Osmotic stress can also be caused by freshwater influx in the intertidal habitats. During low tide, epibenthic organisms are exposed to freshwater from precipitation, whereby sites in river mouths and estuaries are additionally influenced by freshwater outflow (Willmer et al., 2005). Hypoosmotic stress caused by lower salinity in the surrounding medium leads to inflow of water into the cell, thereby disrupting intracellular concentrations of vital molecules (Somero and Yancey, 1997). Further, the cell (or cell organelles) begin to swell and risk ruptures followed by cell death (B.L. Bayne, 2017b; Storey, 2004). Regulatory mechanisms that prevent cell rupture involve regulation of intra- and extracellular osmolarity and are energetically costly, leading to sublethal

effects like the impairment of burrowing capacity of *Mya arenaria* (Haider et al., 2019), decreased filtration rate of *M. edulis* (Riisgård et al., 2013), shifts in hemolymph microbiome and gill morphology of *Sinonovacula constricta* (Yang et al., 2022) or reduced anaerobic capacity of polychaetes (Schöttler et al., 1990).



Figure 1: Detail of a dense *C. gigas* reef with small tide pool in the German Wadden Sea at Sylt. The reef is associated with other organisms such as the invertebrates *Littorina* sp., *Mytilus edulis* or the algae *Uvula* sp. and *Fucus* sp. (own Work).

Hyperosmotic stress (higher salinity in the surrounding medium) can be caused by increased evaporation in summer in the intertidal pools that are disconnected from the sea during low tide (van der Ouderaa et al., 2021). In mudflats these pools are often formed by bivalves (Figure 1) (van der Ouderaa et al., 2021), whereas on rocky shores the intertidal rock pools are formed by physical processes (Healy et al., 2005; Snoeijs-Leijonmalm et al., 2017). Although these tide pools are a welcome refuge during low tide for many animals, evaporation (especially in the summer months) leads to an increase in the water salinity and causes hyperosmotic stress and water loss, similar to that occurring during desiccation (Snoeijs-Leijonmalm et al., 2017; Somero and Yancey, 1997; Storey, 2004; Willmer et al., 2005). Further, this microenvironment present additional threats such as changes in the pH, temperature and dissolved oxygen (DO) concentrations (Goss-Custard et al., 1979; Richards, 2011). Changes in pH can affect the efficiency of the animals respiration or affect calcareous shells (Vernberg and Vernberg, 1972; Waldbusser et al., 2011). Further, the water temperature inside the tide pool can drastically change (by over 10°C in a course of few hours) while isolated from the open sea (Goss-Custard et al., 1979; Huggett and Griffiths, 1986; Klugh, 1924; Willmer et al., 2005). There are also large fluctuations in the oxygen concentrations where DO reaches up to 400% of air saturation due to photosynthesis during daytime and drops to almost

0% at night due to respiration, leading to severely hypoxic conditions (Goss-Custard et al., 1979; Richards, 2011). Temperature and oxygen fluctuations are not limited to tide pools, but also represent a major limiting factor in the periodically air-exposed benthic intertidal habitats during the tidal fluctuations (Vernberg and Vernberg, 1972).

The Wadden Sea, which is part of the North Sea, is an ecosystem of global importance characterized by strong environmental fluctuations (Becker, 1996; Essink et al., 2005; Kröncke and Bergfeld, 2003; Reise et al., 2010; Ricklefs and Vanselow, 2012). The Wadden Sea mudflats spread over 4,700 km² from the Netherlands via Germany to the Danish coasts (Essink et al., 2005; Reise et al., 2010). The Wadden Sea acts as a large biological filter as it is colonized by filter and suspension feeders. Further, densely settled bioturbators enhance biogeochemical processes at the water–sediment interface by reworking of sediments, and the Wadden Seas vegetation collects re-suspended particles (Reise et al., 2010; Retraubun et al., 1996). The Wadden Sea is relatively species poor compared to other coastal ecosystems, but constantly faces the introduction of new taxa (Reise et al., 2005). In this ecosystem, they encounter strongly variable physical factors such as wave energy, temperature and/or oxygen fluctuation which are caused by the semi-diurnal tidal cycle where the seafloor emerges twice a day (Becker, 1996; Essink et al., 2005; Kröncke and Bergfeld, 2003; Reise et al., 2010).

In the Wadden Sea, oxygen fluctuations that cause hypoxic conditions are mostly driven by the absence of water during low tide which can last up to 12 h. However, decomposition of organic material, for example after algal blooms, might lead to the oxygen deficiency in the bottom water also during high tide (Essink et al., 2005). Further, in the summer, temperature fluctuations of up to 25 °C can be observed for the surface benthic sediments. In the winter the temperature shows less pronounced fluctuation, but water-temperature peaks around 0°C and exposure to air temperatures well below -10°C are common (Bundesamt für Schifffahrt und Hydrographie, 2024; Büttger et al., 2011; Deutscher Wetterdienst, 2024; Ricklefs and Vanselow, 2012; van Aken, 2008).

Although the conditions in the Wadden Sea involve variation in multiple abiotic environmental factors, temperature and oxygen availability are among the most important stressors that affect the intertidal organisms (Boutilier and St-Pierre, 2000; Essink et al., 2005; Herreid, 1980; van Aken, 2008; Willmer et al., 2005). The individual effects of both stressors have been studied extensively in various intertidal organisms leading to the discoveries of effective molecular, biochemical, physiological and behavioral adaptations to these stressors (Burnett and Stickle, 2001; Pörtner, 2005; Powell and Garcia, 2000). However, the combined effects of temperature and oxygen

fluctuations, as commonly occurring in the intertidal zone, remain poorly understood (Collins et al., 2023; Pörtner, 2005).

3. Coastal regions as a sink for pollutants

Besides natural physical factors, anthropogenic pollutants emerged as a major stressor in many marine environments (Beiras, 2018; Jambeck et al., 2015). Marine pollution can be defined as “the introduction by man of substances [and energy] into the marine environment resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of seawater and reduction of amenities” (Beiras, 2018; GESAMP, 1969).

Pollutants are introduced into the marine environment through multiple sources including waste disposal, accidents on ships and oil rigs, terrestrial run-off or inefficient wastewater treatment plants (Beiras, 2018; Fisch et al., 2017; Hillenbrand et al., 2005; Li et al., 2007; Ramos et al., 2016; Rousseau et al., 2009). Land-based pollution usually is captured and accumulated by rivers which ultimately enter the marine environment via estuaries and river mouths (Gregory, 1996; Vikas and Dwarakish, 2015). Thus, thousands of tons of different pollutants are discharged into the ocean every day (Vikas and Dwarakish, 2015). These include plastics, trace metals, pesticides, oil, pharmaceuticals, personal care products and nanoparticles (NPs) (Vikas and Dwarakish, 2015). The coastal ecosystems, particularly the marine sediments, act as a sink for pollutants (Fisch et al., 2017; Green et al., 2016; Hanna et al., 2013; Mouneyrac et al., 2014; Snoeijs-Leijonmalm et al., 2017). Further, pollutants buried in the sediment can become again available for the epibenthic and pelagic systems due to bioturbation (Granberg et al., 2008). The pollutants accumulate in marine organisms and can become biomagnified, increasing in concentrations along the trophic chain and may ultimately affect humans (Alberghini et al., 2022; Beiras, 2018; Bhuyan, 2022; Eriksson and Burton, 2003; Kröncke and Bergfeld, 2003; Sánchez-Quiles and Tovar-Sánchez, 2015; Strid et al., 2013; Tsui et al., 2015). The impacts of pollutants on the organisms vary from species to species and depend on the pollutant type, ranging from minor sublethal effects, which the organisms can tolerate without a significant loss in fitness, up to major disruptions of a whole ecosystem (Beiras, 2018; Magni et al., 2004; Siung-Chang, 1997).

3.1. Personal care products: a source of UV filter pollution

Personal care products (PCPs) have many advantages for humans, but due to the increased use and production during the last years, PCPs have drastically accumulated in the environment, raising concerns about their risks and impacts on marine life (Beiras, 2018; Beretta et al., 2014; Fisch et al., 2017; L'Oréal, 2018; Prichard and Granek, 2016; Rodil et al., 2012; Wu et al., 2010). A PCP-type that recently came into focus of the public

and scientific community are sunscreens. Sunscreens contain a variety of ingredients such as sensory enhancers, film formers, emulsifiers, emollients, thickeners and UV filters (Osterwalder et al., 2014). UV filters make up the highest fraction of the sunscreen formula (Osterwalder et al., 2014). To cover a wide UV spectrum, organic and mineral filters are often used in combination (Caloni et al., 2021; National Academies of Sciences Engineering and Medicine, 2022). Organic UV filters mostly protect in the UV-B range (with some exceptions like octinoxate that also absorbs UV-A radiation), whereas mineral UV filters block UV-A as well as UV-B rays (National Academies of Sciences Engineering and Medicine, 2022; Reynertson, 2021; Shaath, 2010). Mineral UV filters are often applied as nanoparticles and reflect as well as absorb the UV radiation (Caloni et al., 2021; Cole et al., 2016; Guo and Mortimer, 2022; Osterwalder et al., 2014). In contrast, the UV protection of organic UV filters relies exclusively on the UV absorption (Huang et al., 2021; Ramos et al., 2015; Serpone et al., 2007).

Apart from sunscreens, UV filters are also components of other cosmetics, inks, food additives and packages (Beegam et al., 2016; Beiras, 2018; Sirelkhatim et al., 2015). This wide usage leads to multiple potential sources for environmental enrichment. Even modern wastewater treatment plants are not able to completely remove all types of UV filters derived from sunscreens (Li et al., 2007). Apart from introduction via the wastewater, direct UV filter input into aquatic environments has been linked to recreational activities (Brausch and Rand, 2011; Caloni et al., 2021; Cuccaro et al., 2022a; Sánchez-Quiles and Tovar-Sánchez, 2015), resulting in seasonal and daily fluctuation in UV filter concentrations in the water (Bratkovics et al., 2015; Labille et al., 2020; Picot-Groz et al., 2018). Therefore, UV filters of different types have already been detected in various marine areas (Bratkovics et al., 2015; Bratkovics and Sapozhnikova, 2011; Downs et al., 2022b, 2022a; Gottschalk et al., 2015, 2013; Poiger et al., 2004; Ramos et al., 2015; Sun et al., 2016; Tsui et al., 2019). The UV filters have different environmental fates such as degradation, reactive oxygen species (ROS) production or (bio-)accumulation in the sediment or organisms (Cuccaro et al., 2022a; Huang et al., 2021; Sánchez-Quiles and Tovar-Sánchez, 2015).

3.2. Nano zinc oxide (nZnO) and avobenzone in the environment

Zinc oxide (ZnO) nanoparticles are the mineral UV filters commonly used in sunscreens and other PCPs due to their wide protective UV spectrum (Caloni et al., 2021; Cole et al., 2016; Guo and Mortimer, 2022; Osterwalder et al., 2014). Besides their UV protection, the NPs are antimicrobial, have a large and porous surface, show physical and chemical

resistance like thermal stability or compatibility with organic solvents, have high thermal and electrical conductivity and neutral pH (Al Jabri et al., 2022; Davis et al., 2010; Fatehah et al., 2014; López de Discastillo et al., 2021; Sirelkhatim et al., 2015). Further, the characteristics of the NPs can be changed or enhanced by different coatings (Davis et al., 2010). Due to these desirable properties, ZnO NPs are used in many products such as plastics, rubber, pigments, paints, asphalts, batteries, packaging and food processing (Al Jabri et al., 2022; Minetto et al., 2014). The annual production of nano ZnO (nZnO) has been estimated to up to 33,400 tons (Research and Markets (2012) as cited in Bondarenko et al.(2013)). Second only to nano-titanium dioxide, nZnO is one of the most abundant NPs accumulating in sediments at a rate of up to 56 µg/kg/year and estimated persistent concentrations between 6 µg/kg and 20 mg/kg for highly polluted areas (Gottschalk et al., 2015, 2013, 2009; Sun et al., 2016).

The high concentrations and accumulation rates of nZnO are concerning as bioaccumulation and biomagnification of these pollutants have been reported along the food chain (Martin et al., 2022; Wang et al., 2017; Yuan et al., 2022). Due to the overlap of the size range of nZnO (1–100 nm) with the size of the particles retained by marine filter feeders, the filter-feeding organisms like bivalves are susceptible to suspended nZnO (Biswas and Sarkar, 2019; Falfushynska et al., 2019; Gnatyshyna et al., 2017; Wu et al., 2020). In addition, other aquatic organisms including annelids, echinoderms, crustaceans and fish are also negatively affected by nZnO (Buffet et al., 2012; Minetto et al., 2016). In bivalves, oxidative stress, DNA damage and modulated immune response was reported as a result of nZnO exposure (Biswas and Sarkar, 2019; Falfushynska et al., 2019; Gnatyshyna et al., 2017; Wu et al., 2020). In polychaetes, nZnO stimulated the antioxidant system and affected the nervous system (Buffet et al., 2012; Cuccaro et al., 2022b). In contrast, some studies found resistance of certain species of fish and crustaceans against nZnO exposure which emphasizes the species-specific toxicity mechanisms and outcomes of nanoparticles in different organism (Callaghan and MacCormack, 2017; Colas and Le Faucheur, 2024; Wong et al., 2010)

Despite the high environmental load and proven toxicity to marine organisms, the mechanisms behind the nZnO toxicity are not completely understood. Studies suggest that physical interaction of the NPs on cellular level and/or the release of Zn ions could contribute to the toxic effects (Ma et al., 2013; Mouneyrac et al., 2014; Reed et al., 2012; Wong et al., 2010). Current research on the ecotoxicology of nZnO mainly focuses on the water-suspended NPs, neglecting concentrations in the sediment available for endobenthic organisms.

Organic UV filters have recently come into public focus due to their potential impact on coral reefs and other charismatic marine organisms (Chatzigianni et al., 2022). Examples of highly ecotoxic organic UV filters are oxybenzone and octocrylene (Haereticus Environmental Laboratory, 2023). Both have been found in many habitats and show high environmental retention time (Downs et al., 2022a; Schneider and Lim, 2019). Further, they have been linked to coral bleaching due to their photo toxicity and could also be detected in fish tissues, suggesting biomagnification along in the food chain (Downs et al., 2016; Schneider and Lim, 2019; Stien et al., 2019; Tsui et al., 2017). Apart from corals, other habitat-building taxa, such as bivalves, are susceptible to octocrylene accumulation and to physiological damage at environmentally relevant concentrations (Falfushynska et al., 2021; Fisch et al., 2017). Further, this UV filter can degrade to benzophenone, a chemical that is often used as a photoinitiator in industrial UV-curing applications, and has been proven hazardous due to its carcinogenic and DNA damaging effects (Davidson et al., 1995; Downs et al., 2021; Rhodes et al., 2007; Zhang et al., 2017). Findings like these have raised general concerns about organic UV filters and have led to marketing terms for sunscreens such as “reef safe”, claiming to be non-toxic for aquatic habitats or especially corals. However, due to the lack of regulation, the definition of “reef safe” is not consistent for different UV filters and brands (Miller et al., 2021; National Academies of Sciences Engineering and Medicine, 2022).

An UV filter frequently used and considered “(coral) reef safe” by different independent certifications is avobenzene (butyl methoxydibenzoylmethane, BMDM or BMDBM) (Haereticus Environmental Laboratory, 2023). Due to its common use, avobenzene has been detected in many habitats with the concentrations ranging from 1 to 2431 ng/L for various aquatic ecosystems, and reaching 28 µg/g in highly polluted beach sediments (Bratkovics et al., 2015; Bratkovics and Sapozhnikova, 2011; Downs et al., 2022a, 2022b; Poiger et al., 2004; Ramos et al., 2015; Tsui et al., 2019). Further, seasonal and daily fluctuations of organic UV filter concentrations have been reported for recreational areas, which can potentially lead to even higher peak concentrations of avobenzene (Bratkovics et al., 2015; Labille et al., 2020; Picot-Groz et al., 2018).

Although considered “reef safe”, the environmental safety of avobenzene could not be irrefutably confirmed (Clergeaud et al., 2023; Danovaro et al., 2008; Fel et al., 2019; Miller et al., 2021; Mitchelmore et al., 2019). Studies have shown possible impact of the products of avobenzene degradation on the photosynthesis of the coral symbionts (Clergeaud et al., 2023). On the other hand, coral mortality has only been observed at high concentrations that are unrealistic from the environmental point of view (Fel et al., 2019). Danovaro et al. (2008) demonstrated that avobenzene does not cause coral

bleaching, despite some coral symbiont release. In addition to corals, other invertebrates such as mollusks, crustaceans and fish have also been included in the avobenzone toxicity assessments. Previous studies have shown the impact of avobenzone on bioenergetics, oxidative stress, metabolism as well as on the nervous and hormonal system of different aquatic organisms (Bordalo et al., 2022; Boyd et al., 2021; Ka and Ji, 2022; Liu et al., 2022; Park et al., 2017). However, our understanding of the ecotoxic effects of avobenzone remains limited, as existing research has only examined a few taxonomic groups.

The understanding of ecophysiological mechanisms related to pollution by UV filters is on the rise (Cuccaro et al., 2022a). However, much of the research has been concentrated on the effects of the waterborne UV filters (Carve et al., 2021). Moreover, recent studies have frequently utilized high concentrations and short exposure times that are not environmentally relevant (Carve et al., 2021). This narrow focus overlooks the fact that UV filters also impact other parts of habitats, such as the benthos, and some of them lead to chronic exposure due to their persistence. Consequently, there remains a significant gap in our knowledge of UV filter toxicity, particularly in relation to environmentally realistic scenarios (Gottschalk et al., 2015, 2013; Sun et al., 2016). Adding to the complexity is the common practice of focusing solely on the effects of individual UV filters, disregarding the reality that these filters are often used together and released into the environment in combination.

4. Physiological challenges of environmental stressors

4.1. Metabolic and redox regulation under environmental stress

Life is depending on constant energy supply (Berg et al., 2018). This energy is used to produce the building blocks for life and maintaining their integrity (Berg et al., 2018). These processes base on the assimilation of environmental substances into organismal matter by anabolic processes and dissimilation of this matter by catabolic processes (Berg et al., 2018; Schaefer, 2012). Catabolism is responsible for the energy gain to maintain the vital functions of the organism including the metabolic machinery (Schaefer, 2012; Willmer et al., 2005). Further, the acquired energy can be transformed into new biomass or waste products (Willmer et al., 2005). The sum of anabolic and catabolic processes is defined as metabolism (Schaefer, 2012). Usually, both are strictly separated to maintain energy balance (Berg et al., 2018; Vernberg and Vernberg, 1972; Willmer et al., 2005).

External stressors such as oxygen supply, temperature, water availability, osmotic stress or pollutants can disrupt the metabolic balance or individual metabolic processes (Sokolova and Lannig, 2008; Storey, 2004; Willmer et al., 2005). Increased energy demand or lack of available energy are possible consequences, and even a small shift caused by external factors can have major impacts on the energy balance (Willmer et al., 2005). However, apart from the energetic approach, environmental factors can also directly interact with the metabolism via various ways (Berg et al., 2018; Storey, 2004; Willmer et al., 2005). Consequences such as a lack of necessary cellular building blocks, proteins and other molecules, changes in enzymes activity as well as the accumulation of end products or intermediates are the results of these imbalances and interactions (Sokolova, 2021; Storey and Storey, 1988; Vernberg and Vernberg, 1972; Willmer et al., 2005). Thus, countermeasures for metabolic disruptions have evolved. They include mechanisms such as quantitative changes in enzymes or functional macromolecules, qualitative changes like regulation of enzyme activities as well as changes on transcriptional and translational level to adjust to environmental factors (Sokolova, 2021, 2013; Storey, 2004). However, consequences and countermeasures are specific for each stressor and target. Thus, selected environmental factors will be discussed in subsequent subchapters.

Metabolic by-products such as reactive oxygen, nitrogen or carbonyl species contribute to cell signaling but can also lead to cellular damage and disrupt metabolism (Ahmad, 1995; Iovine et al., 2008; Knaus, 2020; Sies et al., 2017). Aerobic metabolism, which requires oxygen consumption, is a common source for reactive oxygen species (ROS)

generation (Ahmad, 1995). Around 4% (and possibly more in invertebrates) of consumed oxygen produces ROS (Hermes-Lima, 2004). Reactive intermediates are produced during different stages of aerobic respiration (Ahmad, 1995). The exact mechanisms are complex, but in general, electrons might leak along the electron transport system and react with molecular oxygen (O_2) to form superoxide anions (Ahmad, 1995; Granger and Kvietys, 2015a). These anions can further react to other ROS like the hydroxyl radical as well as non-radicals such as hydrogen peroxide or ozone (Ahmad, 1995; Hermes-Lima, 2004). In addition, ROS can form other reactive molecules such as reactive nitrogen species, reactive sulfur species or reactive carbonyl species that also shift the redox balance (Sies et al., 2017). The result of excess ROS is oxidative stress which is defined as “a disturbance in the prooxidant-antioxidant balance in favor of the former” (Sies, (1985) as cited in Sies et al. (2017)). Oxidative stress can be triggered by a variety of environmental factors such as hypoxia, temperature or pollutants and is a common marker for ecophysiological stress (Abele et al., 2011; Ahmad, 1995; Willmer et al., 2005).

Due to their high reactivity, ROS can affect various biological molecules including lipids, proteins and DNA (Ahmad, 1995; Dalle-Donne et al., 2003). Polyunsaturated lipids are especially susceptible to ROS modification due to their double bonds (Valavanidis et al., 2006; Young and McEneny, 2001). ROS modification can result in a lipid hydroperoxide which may further react to produce other derivatives (Young and McEneny, 2001). This lipid peroxidation can lead to a chain reaction that once started, can affect other lipids or molecules such as proteins (Suzuki et al., 2010; Valavanidis et al., 2006). ROS damage of proteins is characterized by an introduction of a carbonyl-group (Suzuki et al., 2010; Valavanidis et al., 2006). Primary protein carbonylation often affects lysine, arginine, proline and threonine residues where ketones or aldehydes are formed (Suzuki et al., 2010). In contrast, secondary protein carbonylation involves the introduction of an aldehyde group derived from lipid peroxidation (Suzuki et al., 2010). Further, ROS damage on the DNA can impair transcription, translation and replication and might promote mutations (Ahmad, 1995).

There are many mechanisms to cope with ROS generation to keep a stable redox balance (Ahmad, 1995; Sies et al., 2017). ROS production can be mitigated by slowing down metabolism or activating alternative mitochondrial enzymes like alternative oxidase (B. L. Bayne, 2017). However, such mechanisms come at the cost of lower metabolism and biomass production. In the cells, the ROS are scavenged by the antioxidant system (Ahmad, 1995; B. L. Bayne, 2017; Sies et al., 2017). This system consists of enzymatic and non-enzymatic antioxidants, some of which are general ROS scavengers and some

are specific to certain ROS types (Ahmad, 1995). Common antioxidant enzymes are superoxide dismutase (SOD) and catalase (Ahmad, 1995; B. L. Bayne, 2017; Valavanidis et al., 2006). SOD catalyzes the reaction of superoxide to hydrogen peroxide which is further neutralized by catalase (Ahmad, 1995). In contrast, a common non-enzymatic antioxidant, glutathione (GSH), scavenges different ROS by accepting electrons and becoming oxidized (Ahmad, 1995). The oxidized GSH then can be recycled to its reduced form by glutathione reductase (Ahmad, 1995). However, although effective, the antioxidant system comes with high metabolic costs, making available energy for the system a limiting factor (Abele et al., 2011; B. L. Bayne, 2017).

4.2. Physiological effects of hypoxia

Hypoxia is a commonly occurring environmental factor (Vernberg and Vernberg, 1972; Willmer et al., 2005). This state is defined by an undersupply of oxygen of the organism or environmental system (de Groot and Littauer, 1989; Snoeijs-Leijonmalm et al., 2017; Willmer et al., 2005). In marine context, hypoxia is defined as the dissolved oxygen concentration in the range of 0-2 mL O₂/l (Snoeijs-Leijonmalm et al., 2017). The response to hypoxia is complex and causes various changes to fundamental metabolic pathways (Willmer et al., 2005)

The energy demand of an organism depends on different factors such as basal metabolism, growth, reproduction, storage and activity, which require a precise balance to maintain high organismal fitness (Sokolova et al., 2012). Most metazoans rely on aerobic adenosine triphosphate (ATP) generation – the universal cellular energy carrier – to cover this energy demand (Berg et al., 2018; Willmer et al., 2005). However, aerobic animals can also use anaerobic pathways to supplement aerobic energy metabolism to maintain their energy balance (Sokolova et al., 2012; Willmer et al., 2005). A steady oxygen supply is crucial for aerobic metabolism and the anaerobic fermentation becomes particularly important if the oxygen flow is not sufficient to support aerobic ATP generation (B. L. Bayne, 2017; Storey, 2004; Willmer et al., 2005). The transition between aerobic and anaerobic metabolism is controlled by hypoxia inducible factors (Hochachka and Lutz, 2001; Semenza and Wang, 1992).

Reliance on anaerobiosis during hypoxia is connected to disadvantages for an organism. Many anaerobic end products such as lactate, opines, succinate, acetate, propionate, alanine and ethanol are acidic and their accumulation can disrupt the acid-base homeostasis, inhibiting metabolism and ultimately leading to cell death and tissue necrosis that is for example caused by radical formation (Abele et al., 2011; de Zwaan

and Wijsman, 1976; Müller et al., 2012; Özben, 1998; Sokolova et al., 2012; Willmer et al., 2005). Further, carbohydrates are the main fuel for anaerobic metabolism and thus, availability of the glycogen stores limits the anaerobic survival of animals (B. L. Bayne, 2017; de Zwaan and Wijsman, 1976; Müller et al., 2012). However, to a limited extent, fatty acids and amino acids can also be involved in energy gain after conversion to utilizable molecules such as glucose or oxaloacetate (B. L. Bayne, 2017; de Zwaan and Wijsman, 1976; Müller et al., 2012). The anaerobic ATP yield (2-6 mole ATP per mole glucose, depending on the fermentation type) is 6-16 times lower than corresponding ATP yield from aerobic metabolism (B. L. Bayne, 2017; Sokolova et al., 2012). Therefore, while anaerobic ATP production is essential for hypoxic survival, it comes at a cost of low efficiency, high rates of glycogen depletion and potential disruption of cellular homeostasis due to metabolic acidosis and is therefore time limited.

The extent of efficient anaerobic metabolism varies between taxa and conditions (B. L. Bayne, 2017; Willmer et al., 2005). The hypoxic tolerance is modest in most vertebrates (with some exceptions like diving mammals or organisms inhabiting underground burrows) and they mostly upregulate anaerobic pathways during high physical activity to support high ATP demand of the muscles (Cook et al., 2015; Willmer et al., 2005). On the other hand, many invertebrates show much higher hypoxic resistance with efficient and sustained anaerobic metabolism (Müller et al., 2012; Sokolova et al., 2012). Facultative anaerobes such as sessile intertidal organisms experience oxygen fluctuations on daily basis (see chapter 2) and are well adapted for the transition to anaerobic metabolism (B. L. Bayne, 2017; Willmer et al., 2005).

There are different strategies to enhance anaerobic capacity. The acid-base homeostasis can be buffered to counter acidification (Abele et al., 2011; Sokolova, 2018; Sokolova et al., 2012). Further, organisms that periodically encounter hypoxic periods, maintain high carbohydrate storages to fuel anaerobic ATP production (B. L. Bayne, 2017). Specialized invertebrate species such as intertidal bivalves increase this ATP gain through adapted glycolysis which is more efficient than the conventional lactate pathway (B. L. Bayne, 2017; Sokolova et al., 2012; Willmer et al., 2005). Another key mechanism is the efficient aerobic recovery once oxygen is reintroduced into the system. The body fluids and tissues are reoxygenated and aerobic metabolism is upregulated (B. L. Bayne, 2017; Ellington, 1983). Accumulated end products are excreted, reconverted to anaerobic substrates or oxidized by aerobic processes (B. L. Bayne, 2017; Ellington, 1983). At the end, ATP pools are restored and transition to aerobic conditions is complete (B. L. Bayne, 2017; Ellington, 1983). Hereby, aerobic recovery is highly active and leads to the so

called oxygen debt where additional oxygen (compared to normal aerobic metabolism) is consumed to cover the energy demand during aerobic transition (Ellington, 1983).

However, a burst in ROS production can be the result once sufficient oxygen is reintroduced and reoxygenation of the tissues starts (Kalogeris et al., 2014). The ROS burst is a challenging factor in environments experiencing fluctuating oxygen conditions such as the intertidal zone, and can quickly disrupt the prooxidant-antioxidant balance (Abele et al., 2011; Kalogeris et al., 2014; Sokolova, 2018). Excess of ROS generated by the ROS burst during reoxygenation can cause far-reaching damages in the cell (Kalogeris et al., 2014; Sokolova, 2018). The electron transport system (ETS) is a major contributor to post hypoxic ROS generation (Granger and Kvietys, 2015b). ETS intermediates are reduced during hypoxic phases which leads to decreased availability of electron-accepting intermediates or substrates (Granger and Kvietys, 2015b). Further, mitochondrial supercomplexes can be dislocated or disassembled during anaerobiosis (Granger and Kvietys, 2015b). Once oxygen is reintroduced into the system, the mitochondrial ETS is reactivated or reassembled at an insufficient rate, thus increasing the electron leak due to faulty electron transfer which results in the ROS burst (Granger and Kvietys, 2015b). Further, various enzymes such as xanthine oxidase may be relevant contributors to the ROS burst (Ahmad, 1995; Kalogeris et al., 2014). Hypoxanthine accumulates during hypoxia and is oxidized to xanthine by xanthine oxidase after reoxygenation, thereby generating ROS (Kalogeris et al., 2014). Thus, ROS formation during reoxygenation is an important stressor, especially under fluctuating oxygen regime (Hochachka et al., 1993; Sokolova et al., 2019; Solaini et al., 2010). However, animals that often encounter hypoxia have evolved efficient mechanisms to cope with post hypoxic ROS generation, including high baseline levels and rapid upregulation of antioxidants, which are associated with increased energy costs (Abele et al., 2011).

Decrease of the energy demand during hypoxic episodes is another strategy to survive low oxygen conditions. Facultative anaerobes like intertidal invertebrates and some hypoxia-tolerant species of fish and reptiles have evolved the metabolic strategy called metabolic rate suppression that allows them to extend their survival in the absence of oxygen (Abele et al., 2011; Hochachka et al., 1993; Hochachka and Lutz, 2001; Snoeijs-Leijonmalm et al., 2017; Sokolova et al., 2012; Steffen et al., 2021; Storey and Storey, 2005). Further, metabolic rate suppression has also evolved under conditions where access to energy is severely limited including hibernation and food or water limitation (Storey and Storey, 2004). During hypoxia, facultative anaerobes can suppress their metabolic activity to a small fraction of the normal basal metabolism (Sokolova et al., 2012; Willmer et al., 2005). Suppression affects both, ATP production and ATP

consumption of energy demanding processes such as protein turnover, ion transport and nitrogen excretion to keep a stable ratio between ATP and its energy depleted precursor adenosine diphosphate (B. L. Bayne, 2017; Hochachka and Lutz, 2001; Sokolova et al., 2012).

4.3. Physiological responses to temperature

Temperature is a major environmental factor affecting physiology, ecology and evolution of organisms (Vernberg and Vernberg, 1972; Willmer et al., 2005). Thermal extremes experienced by animals range from 50°C in the desert or -60°C in polar regions (Willmer et al., 2005). However, most animals live under conditions far away from these extreme temperatures and have a relatively narrow thermal range (Sunday et al., 2012; Willmer et al., 2005). While temperature is important for all life, poikilotherms (organisms with variable body temperature) are particularly susceptible due to the direct effects of ambient temperature on their body temperature (Sokolova, 2019). This includes most ectotherms that rely on external heat supply for maintaining the organismal heat balance (Begon et al., 2017; Sokolova, 2019). Most aquatic animals, which includes intertidal invertebrates such as bivalves, are ectothermic poikilotherms that often experience temperature fluctuations accompanied by changes in metabolic rates (Begon et al., 2017; Pilditch and Grant, 1999; Vernberg and Vernberg, 1972).

Low temperatures are limiting to life due to their rate-lowering effect on all physiological and biochemical reactions and possible freezing of cellular water. The freezing point of the surrounding water is of relevance for aquatic organisms (Vernberg and Vernberg, 1972; Willmer et al., 2005). Further, the freezing point of the body fluids is of major importance, as formation of intracellular ice is incompatible with survival (Storey and Storey, 1988; Vernberg and Vernberg, 1972; Willmer et al., 2005). Freezing can lead to the formation of ice crystals which physically damage the cells and tissues (Storey and Storey, 1988; Willmer et al., 2005). In addition, freezing cuts off the oxygen supply, leading to symptoms somewhat similar to hypoxia (Storey and Storey, 1988). Some (semi-)terrestrial animals such as frogs and insects have evolved protective mechanisms against longer freezing including the production of high concentrations of organic osmolytes that act as cryoprotectants and the control of ice formations within the body (Storey and Storey, 1988; Willmer et al., 2005). Some intertidal organisms, that are periodically exposed to freezing temperatures, such as bivalves, gastropods and barnacles also established resistance against freezing by tolerating cellular ice formation and maintaining a high cellular osmolarity (Kennedy et al., 2020; Storey and Storey,

2013, 1988). However, low temperatures above freezing can be a challenge as well: they might disrupt the metabolism as different linked pathways are unevenly affected by temperature changes (Storey and Storey, 1988; Willmer et al., 2005).

(Bio)chemical kinetics are directly linked to the temperature (Latscha and Kazmaier, 2016; Willmer et al., 2005). Thus, with increasing or decreasing temperature, the biochemical reaction rates increase or decrease, respectively (Storey and Storey, 1988; Willmer et al., 2005). Commonly, the thermal coefficient Q_{10} is used to describe the change in reaction kinetics over a 10°C change (Willmer et al., 2005). Q_{10} values are normally between 1 and 4 for most physiological reactions but values like 13.8 for the hemoglobin coagulation can be found (Latscha and Kazmaier, 2016; Willmer et al., 2005). However, beyond the optimal temperature range the Q_{10} rule cannot be applied and the apparent Q_{10} becomes negative reflecting enzyme denaturation and loss of activity (Willmer et al., 2005). Hence, the animals' metabolism is strongly affected by temperature changes (Willmer et al., 2005).

The disruption of metabolic activity at temperatures above the optimum temperature range is attributed to different mechanisms, depending on the degree of warming. Animals can actively downregulate their metabolism in response to increasing temperatures to protect against negative effects of over-enhanced metabolism as for example shown for intertidal snails (Marshall and McQuaid, 2011) (see metabolic rate suppression in chapter 4.2). However, different metabolic pathways are differently affected by temperature changes, thus the balance between linked pathways can become compromised (Willmer et al., 2005). Increase in the pathway rate due to warming may deplete the pool of intermediates or substrates may accumulate if downstream pathways are less temperature-sensitive than the upstream ones (Willmer et al., 2005). This may result in diminished energy supply or disturbing/inhibiting effects of the accumulated intermediates (Willmer et al., 2005). In addition, higher metabolic rates cause higher energy demand which might not be covered by the animals' reserves or compromised ATP yield due to co-occurring factors such as hypoxia (B. L. Bayne, 2017; Marshall and McQuaid, 2011; Sokolova et al., 2012; Willmer et al., 2005). These effects are commonly found in ectotherms from different environments but the temperature-induced metabolic misbalance is especially threatening for animals that live in habitats with small fluctuations in temperature such as Antarctic fish (Willmer et al., 2005). Furthermore, physical-chemical effects of high temperatures such as the denaturation of biomolecules or the change in membrane structure becomes a threat (Willmer et al., 2005). Denaturation leads to loss of function whereas impaired membranes impact the transport systems, which, if not repaired or compensated, leads to drastic cellular

damage (Willmer et al., 2005). Therefore, animals that are adapted to temperature fluctuation maintain high concentrations of assisting molecules such as heat shock proteins and compatible osmolytes, which support protein folding and stabilize membrane fluidity (Asea and Kaur, 2018; Feder and Hofmann, 1999; Yancey, 2005).

4.4. Physiological responses to pollutants

Pollutants can be found in aquatic habitats worldwide, where they come from various sources such as industrial discharge, agricultural and urban runoff, and wastewater treatment plants (Beiras, 2018; Jambeck et al., 2015; Willmer et al., 2005). The pollutants can enter the organism due to ingestion, ion transport or passively via transepithelial diffusion (Beiras, 2018). Further, pollution particles can enter the cell via phagocytosis (Stoika, 2022). In addition, the pollutants might form metabolites due to degradation or biotransformation which possess a different and sometimes higher toxic potential than the parent compound (Beiras, 2018; Downs et al., 2021). The physiological consequences of pollutant exposure depend on the pollutant and the exposed organism (Ahmad, 1995; Beiras, 2018; Stoika, 2022).

Pollutants in particulate form can cause mechanical damage to the cells and tissues (Kumar and Shekhar, 2022; Yung et al., 2013). They block the intestines or respiratory organs such as the gills (Berry et al., 2021; Kumar and Shekhar, 2022). Further, particulate pollutants may cause ruptures in tissues or cells (Kumar and Shekhar, 2022). Particle attachment to external structures such as the exoskeleton can cause impairment of mobility and other physical effects (Baun et al., 2008). Non-particulate pollutants such as oil also have physical-mechanical effects on organisms by impairing their mobility, respiration and other crucial functions (Beiras, 2018; Goulletquer et al., 2014).

In addition to physical-mechanical effects, pollutants can have various chemical impacts on exposed organisms (Beiras, 2018; Rainbow, 2007). Commonly, exposure to pollutants has been associated with oxidative stress (Ahmad, 1995). Pollutants inhibit the respiratory system and can lead to increased ROS production inducing oxidative stress (Bossy-Wetzel et al., 2004; Li et al., 2012; Stoika, 2022). ROS production by photocatalytic processes is an additional source of pollution-generated ROS (Sirelkhatim et al., 2015).

Apart from impacts on the respiratory system, other metabolic pathways can also be modulated by pollutants (Vernberg and Vernberg, 1972). Some trace metals, such as

zinc, which might be released from nZnO due to dissolution, have important roles in enzymes, and dedicated uptake mechanisms for these metals exist in the cells (Asea and Kaur, 2018; Newman and McIntosh, 2020; Rainbow, 2002). However, if uptake of metals exceeds excretion and/or binding to intracellular ligands, toxic effects are the result (Rainbow, 2007). Some toxic metals (like Cd) might mimic essential metals (e.g. Zn), replacing the essential metals from the catalytic sites of the enzymes and metal-binding sites in transcription factors, thereby impacting various metabolic pathways (Bury et al., 2003; Rainbow, 2007). Further, high concentrations of metals could interfere with signaling or destabilize the membrane (Asea and Kaur, 2018; Plattner and Verkhatsky, 2016). Organic pollutants also have various physiological and molecular targets such as the hormone homeostasis, cell signaling or enzymes (Maipas and Nicolopoulou-Stamati, 2015; Mearns et al., 2013; Mezzelani and Regoli, 2022).

Detoxification of pollutants is of high importance to prevent cellular damage (Amiard et al., 2006; Beiras, 2018). The animal cells possess various repair and protection mechanisms some of which (such as heat shock proteins) are involved in the protective response against multiple stressors including chemical pollutants, temperature or radiation, while other are specific for detoxification of metals and organic xenobiotics (Amiard et al., 2006; Asea and Kaur, 2018; Beiras, 2018; Willmer et al., 2005). Many animals can actively excrete excess of metals, while others (notably some invertebrates) accumulate high levels of metals raising the need for their effective detoxification (Rainbow, 2007, 2002). The important detoxification mechanism of metals involves glutathione and metallothioneins which bind the metals to their thiol groups, followed by storage or excretion of the bound metals (Amiard et al., 2006; Rainbow, 2007; Willmer et al., 2005). In addition, pollutants (including organic contaminants) can be detoxified by biotransformation or conjugation with molecules such as glutathione, which are then metabolized or excreted (Beiras, 2018; Rodrigo and Costa, 2017).

Pollutants often occur in combinations in the environment (Beiras, 2018). Synergistic or antagonistic mechanisms between compounds might affect the toxicity and detoxification of the pollutant mixture (Beiras, 2018; Pirotta et al., 2022; Sokolova and Lannig, 2008). Due to these non-linear interactions, the effects of exposure to an individual stressor cannot always be extrapolated to combined exposures (Pirotta et al., 2022). Recently, the investigation of combined stressors has come into the focus of research as an environmentally relevant exposure, opening new branches on the evaluation of xenobiotics such as toxicometabolomics (Araújo et al., 2021; Beiras, 2018; Norkko et al., 2005; Pirotta et al., 2022; Schnackenberg et al., 2012).

5. Invertebrates as model organisms

Model organisms are the key to targeted research (Ankeny and Leonelli, 1998). The work with such organisms compiles and combines knowledge which can, among other aspects, reduce technical obstacles to investigations (Müller and Grossniklaus, 2010). Model organisms are commonly used in biomedical fields to study aging, development, diseases, and to develop and test pharmaceutical compounds (Bodnar, 2009; Munkácsy and Pickering, 2021; Ségalat, 2007; Wilson-Sanders, 2011) as well as for assessing the mechanisms of responses to environmental factors like hypoxia, eutrophication or exposure to prooxidants (Ahmad, 1995; Camargo et al., 2005; Herreid, 1980). Traditionally, vertebrate models have been used as model organisms but due to an increase in public concerns about the animal rights, invertebrates have become the model of choice for many studies (Müller and Grossniklaus, 2010; Wilson-Sanders, 2011). Thus, since the mid-1940s, the number of studies on invertebrates has increased by orders of magnitude (Wilson-Sanders, 2011). There are many advantages of using invertebrates as model species. Many invertebrate taxa are relatively easy to maintain, multiple cellular functions are well understood, conserved and transferrable to other groups, including humans, so that invertebrates can serve as simplified models for a variety of research fields (Ankeny and Leonelli, 1998; Müller and Grossniklaus, 2010; Munkácsy and Pickering, 2021; Wilson-Sanders, 2011). Further, invertebrates make up around 95% of the known animal species, representing 31 of the animal phyla (Eisenhauer and Hines, 2021). They play an important role in the ecosystems, provide crucial ecosystem services and they can be found in major parts of the ecosphere including extreme habitats (Eisenhauer et al., 2023; Foottit and Adler, 2017; Poisot et al., 2013; Reynoldson and Metcalfe-Smith, 1992; Seibold et al., 2018). Consequently, the broad spectrum of species and inhabited ecosystems including various environmental factors highlights the importance of invertebrates as research subjects. In this work, I have used two invertebrate species as models for my research: the Pacific oyster *Crassostrea gigas* and the lugworm *Arenicola marina*. These species are common in many coastal habitats worldwide where they provide important ecosystem services like filtration, bioturbation and formation of biogenic reefs (Hartmann-Schröder, 1996; MolluscaBase, 2023; Read and Fauchald, 2018; Reise et al., 2023).

5.1. *Crassostrea gigas*: an invasive reef builder

The Pacific oyster *Crassostrea (Magallana) gigas* (Thunberg, 1793) is a bivalve species from the family Ostreidae distributed in intertidal habitats worldwide. Due to ongoing

discussion on the new assignment to the genus *Magallana*, the traditionally accepted species name, *Crassostrea gigas*, will be used in the present work (Bayne et al., 2019). The native range of the Pacific oyster encompasses the coastal habitats of the Pacific Ocean in Japan, the Yellow Sea and the Sea of Okhotsk. However, due to expanding aquaculture, *C. gigas* has been introduced into coastal ecosystems and established wild populations worldwide (Molnar et al., 2008; Rilov and Crooks, 2009). The introduced range of this species includes North and South American Pacific, European and African Atlantic, southern Australia, New Zealand, the Mediterranean Sea, Fiji, Samoa, New Caledonia, Micronesia as well as the Hawaiian islands (Molnar et al., 2008). Newly invaded areas are often characterized by existing mussel beds, which provide suitable ground for the settlement of oysters. Once settled, the oysters transform this mussel bed to mixed beds (Reise et al., 2023, 2017). In this context, oysters act as ecosystem engineers by forming a reef that provides settling grounds and shelter for other organisms (B.L. Bayne, 2017c; Reise et al., 2023; Troost, 2010). Further, the created reef can influence the food availability to other organisms by modifying water turbulence or providing hard substrate for algae (Reise et al., 2023; Troost, 2010).

Over 60% of extinction events are due to the invasion of alien species, meaning that, besides favorable ecosystem services, invasions by *C. gigas* also entail risks (Dolmer et al., 2014; IPBES, 2023). The transformation of existing mussel beds can drastically affect the populations of other important ecosystem engineers such as *Mytilus edulis*. Besides active displacement of native bivalves, *C. gigas* may alter the parasite-host interaction of the mussels (Goedknecht et al., 2020). *C. gigas* also alters the pathogen composition, leading to additional potential risks for neighboring organisms (Dolmer et al., 2014; Friedman et al., 2005). In addition, the decline of native mussel populations by invasive species can affect the populations of birds that feed on shellfish, posing risks for multiple trophic levels (Dolmer et al., 2014; Wolff and Reise, 2002).

The introduction of *C. gigas* along the North Sea coasts and especially in the Wadden Sea is well documented (Reise et al., 2023). Since the 1960s, the oysters have been introduced into the waters of Texel (Netherlands) and the Oosterschelde estuary as well as Sylt (Germany) for aquaculture purposes, from where they spread across the German Wadden Sea and formed dense reefs (Figure 1) (Drinkwaard, 1998; Reise et al., 2023, 2005). The initial concerns about the introduction of *C. gigas* for aquaculture purposes were overruled by the considerations that larvae and spat escaping from the oyster cages could not survive the winters in the Wadden Sea (Reise et al., 2017). However, it became apparent that the winters in the North Sea did not reach the critical threshold to deplete initial settlements, but only delayed the spread (Dolmer et al., 2014; Reise et

al., 2017). Further, the oyster benefits from climate warming which improves conditions for reproduction and development of Pacific oysters in the Wadden Sea (Diederich et al., 2005; Reise et al., 2023; Thomas et al., 2016).

C. gigas is a eurythermal species with a wide thermal tolerance range found in habitats with the sea surface temperature ranging between -1.9°C and 28.6°C (Carrasco and Barón, 2010). The oysters can tolerate temperatures between -4°C and 30°C without major damages under the laboratory conditions of short-term exposures (Bougrier et al., 1995; Bruhns et. al., unpublished data; Le Gall and Raillard, 1988). Further, temperature extremes at -22°C and 35°C can be endured, but they cause severe physiological effects, often leading to increased mortality rates (Rajagopal et al., 2005; Strand et al., 2011). In addition, the oysters can enhance their thermal tolerance by pre-conditioning to the heat or cold stress (B.L. Bayne, 2017d; Rajagopal et al., 2005; Strand et al., 2011). The heat tolerance of oysters is most likely attributed to a well-developed and diversified heat shock protein system, which provides a better thermal protection compared to mussels commonly found in the same habitats (Clegg et al., 1998; De Marco et al., 2023; Lim et al., 2016; Rajagopal et al., 2005; Reise et al., 2023). In contrast, the tolerance to freezing may be achieved by accumulation of metallothioneins as it has been already shown for other freeze-tolerant invertebrates like a gastropod *Littorina littorea* (English and Storey, 2003; Strand et al., 2011).

The summer mortality of *C. gigas* has been frequently documented in the Wadden Sea (Samain, 2011). The exact causes of this mortality are not yet completely understood but studies suggest that a complex interaction of environmental factors builds up the mortality risk which is triggered by increasing temperatures (Samain, 2011). The phytoplankton blooms in the spring increase the food availability for the oysters which leads to increased growth rates and reproduction activity. Growth and reproduction are energetically costly which is enhanced by accelerated metabolism due to increasing temperatures. As a result, the oysters deplete their energy reserves which cannot be compensated during high tide due to an unbalanced oxygen regime caused by algal biomass depletion. Hence, the oysters' homeostasis gets disturbed, making it susceptible to pathogens and environmental stress such as heat shock (Goulletquer et al., 1998; Samain, 2011).

C. gigas is commonly found in the intertidal zone, where they frequently experience emersion during low tides (Reise et al., 2017). Like other sessile bivalves, the oyster responds to this emersion by closing its shell, leading to internal hypoxic conditions (Platvoet and Pinkster, 1995; Vernberg and Vernberg, 1972; Willmer et al., 2005).

However, under typical environmental conditions, oysters endure this phase without sustaining damage (B. L. Bayne, 2017; Ivanina and Sokolova, 2016). Moreover, survival of prolonged (20 days) hypoxia has been reported in oysters, albeit accompanied by a pronounced hypoxic stress response (Le Moullac et al., 2007; Sussarellu et al., 2010).

A key survival strategy of oysters under hypoxic conditions is metabolic rate suppression (B.L. Bayne, 2017d; Hochachka, 1988, 1986; Moullac et al., 2007). This state involves reducing the metabolic rate below the normal resting level, with the remaining cellular energy demand met by ATP output from fermentative processes (Storey and Storey, 1990, 2004) (see chapter 4.2). Additionally, metabolic rate suppression in oysters has been observed with other stressors such as starvation or low/freezing temperatures (García-Esquivel et al., 2002; Gorr, 2017; Storey and Storey, 1990). Overall, oysters demonstrate remarkable robustness against a broad range of abiotic stressors (Zhang et al., 2012). However, the precise factors contributing to this resilience are not yet fully understood, though recent studies suggest that microbiome dynamics and amplification of the genes involved in stress protection (the so called stressome expansion) may play a role (Bang et al., 2018; Lokmer and Wegner, 2015; Purchiaroni et al., 2013; Zhang et al., 2012). The high environmental tolerance exhibited by the Pacific oyster contributes significantly to its successful establishment worldwide and highlights potential adjacent risks of this species' further expansion. Therefore, further research on the physiological mechanisms underlying this phenomenon is necessary to evaluate the oysters' invasive potential.

5.2. *Arenicola marina*: important marine ecosystem engineer and indicator species

The lugworm *Arenicola marina* (Linnaeus, 1758) from the family Arenicolidae (Annelida: Sedentaria) is an ecologically important species in coastal habitats worldwide (Tyler-Walters, 2016; WoRMS, 2024). This species has circumpolar distribution in the Northern hemisphere including the Atlantic Ocean and adjacent seas, the Baltic Sea, the Pacific and Arctic Ocean as well as the Mediterranean Sea and adjacent seas (Hartmann-Schröder, 1996; WoRMS, 2024). It is found in the intertidal as well as subtidal zones down to 20 m water depth (Hartmann-Schröder, 1996). Microhabitat preferences of *A. marina* depend on sediment characteristics and animal age. Generally, lugworms prefer sandy habitats, and younger individuals are more abundant closer to the shore line, while larger individuals occur deeper down the shore (Hartmann-Schröder, 1996; Longbottom, 1970; Sommer, 2005). The lugworm establishes microhabitats for other

organisms such as nematodes and often establish zones of high population density, which may be further associated with elevated abundance of other organisms such as *Macoma baltica* or *Corophium* sp. (Hartmann-Schröder, 1996; Wetzel et al., 1995). These zones are characterized by sand casts on the sediment surface that indicate the exit of the U shaped burrow of the worm (Hartmann-Schröder, 1996; Sommer, 2005). Within its burrow, the polychaete dwells at the bottom in the feeding pocket which can be up to 1 m deep (Sommer, 2005). It ingests organic rich sediment supplied via the feeding funnel at the burrow entrance (Fauchald and Jumars, 1979; Hartmann-Schröder, 1996). Due to the constant transport of surface sediment and pumping muscle contractions, the lugworm supplies the burrow and surrounding sediment with fresh, oxygen rich water (Longbottom, 1970; Rijken, 1979; Sommer, 2005). Further, this type of feeding leads to the reworking of the sediment with rates of up to 7 cm per year which can be enhanced to 35 cm per year when the *Arenicola*-zone is associated with other bioturbators (Cadée, 1990, 1976). These abilities underline its relevance as an important ecosystem engineer (Volkenborn, 2005).

Coastal marine sediments are a sink for pollutants, exposing *A. marina* to accumulated pollutants. The lugworm is a size selecting feeder with a preferred grain size between 125-500 μm but larger particles can also be ingested (Andresen and Kristensen, 2002; Cadée, 1976; Gebhardt and Forster, 2018). This size selection might occur to ensure optimal ingestion and digestion (Andresen and Kristensen, 2002). As a side effect, the size selectivity of feeding might protect the polychaete from ingestion of pollutants above the preferred particle size as shown for larger microplastics (Gebhardt and Forster, 2018). However, sediment-adsorbed pollutants and small particulate matter like nanoparticles can be ingested and represent a threat (Attademo et al., 2023; Besseling et al., 2013; Gebhardt and Forster, 2018; Van Cauwenberghe et al., 2015; Wright et al., 2013). Engineered NPs, which are smaller than 100 nm, pose a hazard despite not being in the preferred size range for feeding. NPs can form larger aggregates or attach to organic matter, thereby becoming available to *A. marina* through its feeding activity (Baker et al., 2014; Biswas and Sarkar, 2019; Lei et al., 2016). Further, nanoparticles might be taken up by phytoplankton which, after sedimentation, can become part of the diet of the worm (Fauchald and Jumars, 1979; Sánchez-Quiles and Tovar-Sánchez, 2015; Wright et al., 2013). Similarly, dissolved pollutants can be accumulated in organic materials or adsorb onto the sediment particles which might be then ingested by the polychaete (Baker et al., 2014; Cuccaro et al., 2022a; Tsui et al., 2015).

Many species of polychaetes are robust against pollution and may be used as indicator species for polluted habitats (Beiras, 2018; Davies et al., 1984; Dean, 2008; Tomassetti and Porrello, 2005). These opportunistic polychaete species might possess advantages for survival or recolonization when compared to other species decimated by massive impacts like oil spills or high organic loads from aquaculture (Peterson, 2001; Tsutsumi, 1987). Despite their robustness, polychaetes exhibit sublethal health impacts due to cellular stress induced by pollutants. Like other aquatic invertebrates, exposure to metal contaminants causes oxidative stress, resulting in the formation of reactive oxygen species. Consequently, the organism activates its antioxidant system to mitigate these effects. (Falfushynska et al., 2015; Pires et al., 2017; Sun and Zhou, 2008; Trevisan et al., 2014; Won et al., 2012). Moreover, polychaetes exhibit a robust capability for detoxifying trace metals, as demonstrated by the upregulation of heat shock proteins and metallothioneins (Feder and Hofmann, 1999; García-Alonso et al., 2011). However, this detoxification process leads to the bioaccumulation of pollutants, thereby posing potential threats to the food chain (Dean, 2008; García-Alonso et al., 2011; Martin et al., 2022; Monserrat et al., 2003; Sokolova and Lannig, 2008; Wang et al., 2017). Like metals, organic pollutants also affect the antioxidant system of the worms (Nusetti et al., 2005; Sun and Zhou, 2008). Consequently, these studies suggest that reactive oxygen species formation is a primary outcome following exposure to pollutants (Monserrat et al., 2003; Sokolova et al., 2012). While impaired respiration after metal exposure was reported (Everaarts, 1986; Everaarts et al., 1979; Everaarts and Reichert, 1988), other metabolic shifts caused by pollutant exposures have not been well studied.

Although ecotoxicology of polychaetes are extensively studied (Reish and Gerlinger, 1997), research predominantly focuses on population-level dynamics or the uptake of pollutants rather than physiological responses. Furthermore, there remains a notable gap in investigating the ecotoxicological effects of many important classes of pollutants like PCPs and nanoparticles, in polychaetes. Particularly, studies examining the combined effects of multiple substances are scarce, highlighting the need for more detailed investigations to enhance the utility of polychaetes as indicator organisms (Dean, 2008). While more than 48 polychaete species are commonly used as pollution indicators, research often concentrates on a limited selection such as *Alitta succinea*, *Hediste diversicolor* or *Capitella capitata* (Dean, 2008; Reish and Gerlinger, 1997; Tsutsumi, 1987). However, other species like *A. marina* also hold potential as indicator species (Morales-Caselles et al., 2009, 2008; Ramos-Gómez et al., 2011a).

Exposure of *A. marina* to metal and organic pollution results in various physiological impacts such as impairment of the lugworms' respiratory system, induction of oxidative

stress responses and DNA damage (Everaarts, 1986; Everaarts et al., 1979; Everaarts and Reichert, 1988; Morales-Caselles et al., 2009; Ramos-Gómez et al., 2011a, 2011b). Moreover, microplastics have been found to induce metabolic shifts (Green et al., 2016). As a scenario with high ecological impact, a simulated pollution by an oil spill, caused mortality after long term exposure, but also demonstrated survival of *A. marina* at lower oil concentrations (Morales-Caselles et al., 2008). This survival may be attributed to the efficient ability of the organism to detoxify organic pollutants as well as other toxicants such as hydrogen sulfide (Abele et al., 2011; Lee et al., 2012; Ramos-Gómez et al., 2011b; Sommer, 2005). However, despite effective detoxification systems, pollution levels exceeding the organisms' tolerance limits may have negative impacts on the ecosystem. The impacts of pollutants on *A. marina* might influence its bioturbating ability, as already demonstrated for different pollutants (Green et al., 2016; Morales-Caselles et al., 2009), thus diminishing its role as an ecosystem engineer.

6. Goals and results of the thesis work

The aim of this study was to determine the metabolic responses to combined stressors in two common marine invertebrates, the Pacific oyster *C. gigas* and the lugworm *A. marina*. We focused on the oxidative stress response as a common proxy for the general cellular stress and explored the metabolic shifts underlying cellular responses to pollutants. The study on *C. gigas* focused on the response to intermittent hypoxia in combination with temperature stress, whereas the studies in *A. marina* focused on the response on the two commonly used UV filters nZnO and avobenzone. The main objectives of this study were:

1. To understand the possible interactive effects of commonly co-occurring abiotic stressors (oxygen and temperature fluctuations) on metabolic responses of oysters.
2. To understand the stress response of single or combined exposure to the UV filters nZnO and avobenzone in infaunal marine organisms using lugworms as a model.

To achieve these objectives, we tested the following hypotheses:

1. Intermittent hypoxia will induce oxidative stress and cause metabolic shifts in *C. gigas*.
2. Elevated temperatures will enhance the metabolic and redox shifts caused by intermittent hypoxia in *C. gigas*.
3. Single pollutant exposures to nZnO or avobenzone will induce oxidative stress and cause metabolic shifts in *A. marina*.
4. Increasing concentrations of nZnO or combined exposure to nZnO and avobenzone will intensify metabolic and redox stress in *A. marina*.

6.1. Effects of intermittent hypoxia combined with temperature on *Crassostrea gigas*

To investigate the interactive physiological effects of intermittent hypoxia and temperature (objective 1), we exposed *C. gigas* for 10 days to intermittent hypoxia (12:12h emersion: immersion) in combination with different temperature regimes: a constant temperature of 15°C (normal) or 30°C (elevated), or a fluctuating (15°C immersion; 30°C emersion) temperature regime (Figure 2). Animals constantly submerged at 15°C served as normoxic controls. We sampled the gills and the digestive gland after the last recovery (immersion) cycle to investigate persistent effects of

periodic air exposure. We measured markers for the bioenergetic status (ETS activity, and tissue levels of glycogen and proteins) as well as oxidative stress markers (lipid peroxidation, protein carbonylation and total antioxidant capacity) using spectrophotometric methods in the gills and the digestive gland tissues. Furthermore, concentrations of metabolites related to energy, nitrogen and amino acid metabolism were analyzed in the gills using liquid-chromatography tandem mass-spectrometry and the metabolomics tool MetaboAnalyst 5.0 (Pang et al., 2021).

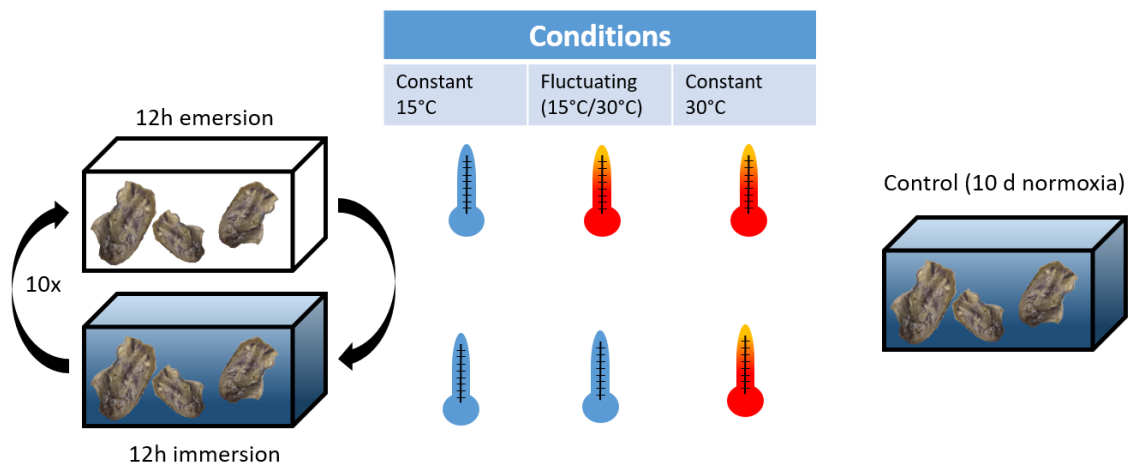


Figure 2: Experimental layout of the manuscript II.1

Our results show metabolic shifts in the oysters triggered by intermittent hypoxic exposure and warming that caused energetically costly metabolic responses and led to increased tissue damage and mortality. Based on a partial least square discriminant analysis (PLS-DA), the metabolic profile of the groups exposed to intermittent hypoxia were (partially) separated from the normoxic controls (Bruhns et al. (2023a), Fig. 3). Further, metabolic profiles of oysters exposed to intermittent hypoxia and fluctuating temperature were well separated from exposures at constant temperatures. Intermittent hypoxia at fluctuating temperature caused 13 pathway shifts – 4 of which are only change under this specific exposure regime – compared to 10 and 5 altered metabolic pathways under the intermittent hypoxia combined with the constant normal (15°C) and elevated (30°C) temperature, respectively (Bruhns et al. (2023a), Fig. 4, Table 3). The distinct metabolic response of oysters exposed to intermittent hypoxia with fluctuating temperature, which includes shifts in energetic pathways such as glyoxylate and dicarboxylate metabolism or Ala, Asp and Glu metabolism, is accompanied by signs of energetic depletion. Specifically, the main energy storage of intertidal bivalves, glycogen (B. L. Bayne, 2017; de Zwaan and Wijsman, 1976), significantly decreased compared to the normoxic control (Bruhns et al. (2023a), Fig. 1). Hence, we suggest that glycogen is used up under hypoxia to fuel the anaerobic metabolism, which is not

compensated during recovery (immersion). This mismatch could be the result of shifts in enzyme kinetics caused by the temperature differences (see chapter 4.3) which stimulates glycogen use (at 30°C emersion) and decreases glycogen synthesis during recovery at comparably low (15°C) temperatures.

It is worth noting that oysters exposed to intermittent hypoxia with fluctuating temperature showed decreased ETS activity (Bruhns et al. (2023a), Fig. 1) which might be an indicator for metabolic rate suppression commonly observed under stressful conditions such as the tidal cycle (see chapter 4.2). One of the important mechanisms regulating metabolic suppression in bivalves involves nitric oxide radicals formed by the conversion of Arg to citrulline (Jacklet, 1997; Strahl and Abele, 2020). Our study showed a 13-fold accumulation of citrulline in the gills of oysters exposed to intermittent hypoxia at fluctuating temperature compared to the normoxic baseline (Bruhns et al. (2023a), Table 4), which may indicate enhanced NO production as a regulatory metabolic signal. The energetic misbalance of this group is also indicated by an decrease in adenosine mono phosphate (AMP; Bruhns et al. (2023a, Table 4)) (Hardie, 2003). Alternatively, changes in AMP can indicate stimulation of the protein synthesis as AMP is a RNA-precursor and a regulating molecule for biosynthetic processes (Bolster et al., 2002; Horman et al., 2002; Jauker et al., 2015; Rider, 2016). The latter hypothesis is indirectly supported by our findings of protein accumulation in the oysters' gills under exposures to intermittent hypoxia with fluctuating temperatures (Bruhns et al. (2023a), Fig. 1) and warrants further investigation . Furthermore, we detected depletion of essential branched chain amino acids and aromatic amino acids (Bruhns et al. (2023a), Table 4), consistent with the notion of elevated protein synthesis (Fitzgerald and Szmant, 1997; Manahan, 1990). Our data does not allow to unequivocally conclude about the effects of intermittent hypoxia and fluctuating temperature on the protein synthesis rates or explain the mechanisms behind the increase in protein content, but the accumulation of heat shock proteins is proposed as a potential cause for this increase (Schlesinger, 1990). These proteins make up to 10% of the total protein content and respond to a variety of stressors (Finka and Goloubinoff, 2013; Karademir and Sari-Kaplan, 2018) (see chapters 4.3 and 4.4). Further, response of heat shock proteins in oysters after temperature and hypoxic stress have already been reported (David et al., 2005; Ivanina et al., 2009; Kawabe and Yokoyama, 2012).

Exposures to intermitted hypoxia at constant temperatures showed deviating responses compared to hypoxia at fluctuating temperatures. Constant elevated temperatures led to tissue damage indicated by increases in lipid peroxidation and methionine sulfoxide levels as well as protein depletion in the oysters' gills (Bruhns et al. (2023a), Fig. 1, Fig.

2). The damage to the cellular proteome found under these conditions was associated with elevated mortality rate (~60%) indicating that warming to 30°C used in our study approaches the upper thermal limit of *C. gigas* (Le Gall and Raillard, 1988). The high mortality rate might have led to survival bias where only the tolerant organisms survived and were available for biochemical analyses. This survivor bias might explain the lack of significant changes in bioenergetic biomarkers (AMP, ETS and glycogen) and limited shifts in metabolic pathways (n=5) in this group (Bruhns et al. (2023a), Fig. 1, Fig. 4, Table 3, Table 4). In contrast, intermittent hypoxia at normal temperature led to shifts in 10 metabolic pathways including bioenergetics pathways such as glyoxylate and dicarboxylate metabolism similar to the oysters exposed to fluctuating temperature (Bruhns et al. (2023a), Fig. 4, Table 3). Unlike the fluctuating temperature regime, intermittent hypoxia combined with low temperature did not change the energy status or caused tissue damage in oyster gills (Bruhns et al. (2023a), Fig. 1, Fig. 2).

This study underscores the resilience of the Pacific oyster to thermal stress and intermittent hypoxia but also shows that the combination of hypoxia and constant heat stress overwhelms the protective mechanisms resulting in elevated mortality and oxidative damage. In contrast, environmentally relevant conditions like combination of intermittent hypoxia with fluctuating temperature (see chapter 2), induced pronounced metabolic rearrangement accompanied by energetic depletion. Thus, our initial hypotheses can be partially accepted: intermittent hypoxia leads to metabolic rearrangement which is enhanced in combination with fluctuating temperature. However, sole exposure to intermittent hypoxia does not induce oxidative stress in *C. gigas* unless combined with elevated temperature. Our results add to the understanding of the mechanisms contributing to the oysters' success in invading new habitats and contribute to the knowledge of physiological background of the summer mortality. Detailed results and discussions are described in the publication II.1 (Bruhns et al., 2023a).

6.2. Effects of UV filters and their combination on the lugworm *Arenicola marina*

To investigate the stress response of single or combined exposure to the UV filters nZnO and avobenzone (objective 2), we conducted two studies on the lugworm *A. marina*. The animals were exposed for two weeks to nZnO (0.1 mg or 1 mg Zn per kg sediment) (manuscript II.2; Figure 3). The second study included exposures for three weeks to nZnO (3 mg Zn per kg sediment), 50 µg/l waterborne avobenzone or their combination

(manuscript II.3; Figure 3). We sampled the body wall and body fluids and investigated concentrations of metabolites related to energy, nitrogen and amino acid metabolism using liquid-chromatography tandem mass-spectrometry and the metabolomics tool MetaboAnalyst (version 5.0 and 6.0) (Pang et al., 2021). Further, we investigated oxidative damage (lipid peroxidation, protein carbonyls, methionine sulfoxide content) and the antioxidant system (superoxide dismutase, catalase and the total antioxidant capacity) in the body wall by using spectrophotometric methods.

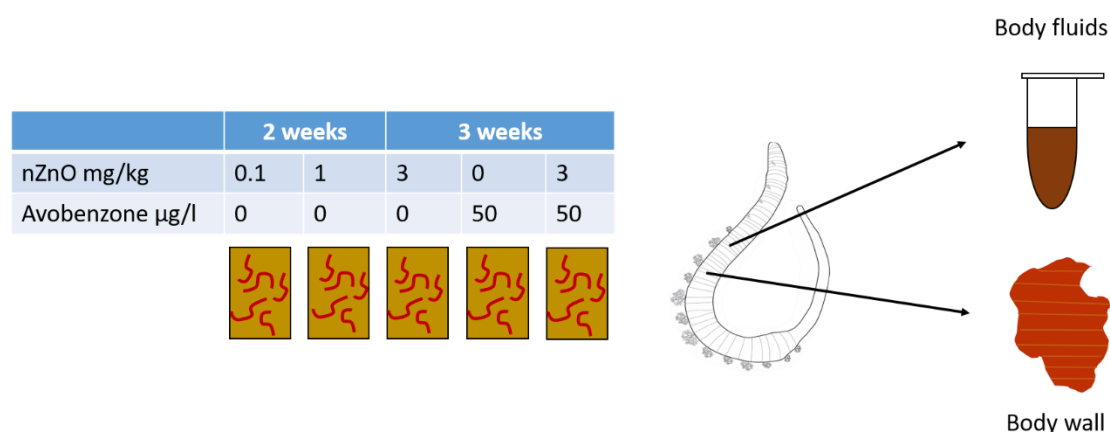


Figure 3: Experimental layout of the manuscripts II.2 (2 weeks) and II.3 (3 weeks).

Our results summarized in the manuscript II.2 demonstrate oxidative tissue damage caused by nZnO exposure as well as metabolic shifts, which, however, differ between body wall and body fluids. nZnO exposure at the chosen concentrations (0.1 and 1 mg/kg sediment) did not cause oxidative protein damage (Bruhns et al. (2023b), Fig. 1), which is consistent with earlier studies (Falfushynska et al., 2019; Wu et al., 2022). However, we could detect an increase of lipid peroxidation caused by nZnO exposure which was enhanced with increasing UV filter concentration (Bruhns et al. (2023b), Fig. 1). The more sensitive response of lipids might be attributed to the chain reaction mechanism of lipid peroxidation or to the direct contact of the cell membrane with nZnO particles (Ahmad, 1995; Ma et al., 2013; Wei et al., 2022). Based on the PLS-DA analysis (Figure 4 A), we found a partial separation of the body wall metabolomes between the control worms and those exposed to 1 mg nZnO. The body fluids metabolome of all experimental groups showed considerable overlap (Figure 4 B). However, pathway analysis revealed 14 significantly shifted pathways in the body fluids caused by nZnO exposure (Bruhns et al. (2023b), Table 1). Shifts occurred in amino acid metabolism, bioenergetics, antioxidant metabolism, nitrogen metabolism as well as neurotransmitter metabolism. In contrast, the body wall metabolism of nZnO-exposed worms only showed six shifts including amino acid metabolism, bioenergetic branches

and nitrogen metabolism (Bruhns et al. (2023b), Table 1). The impact on the body fluids is additionally highlighted by the correlation analysis which found a cluster of 19 positively correlated free amino acids (Bruhns et al. (2023b), Fig. 10). Further, the bioenergetic metabolites citrate and malate as well as the osmolyte taurine were negatively correlated with most metabolites. In contrast, within the body wall, only nine free amino acids showed a positive correlation in a cluster, while taurine was negatively correlated with most metabolites (Bruhns et al. (2023b), Fig. 10).

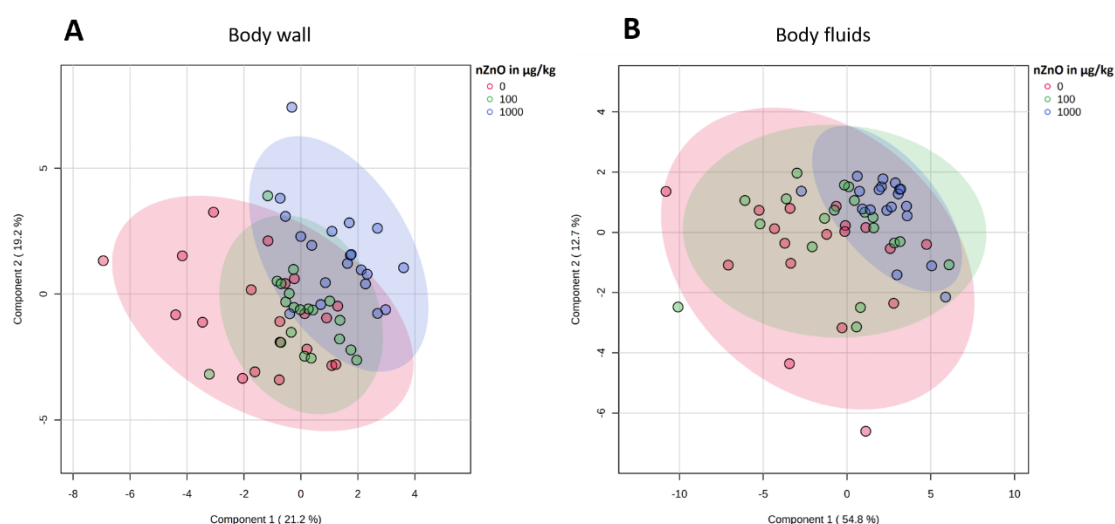


Figure 4: PLS-DA 2D scores plot for the body wall (A) and body fluids (B) metabolome of manuscript II.2 using MetaboAnalyst 6.0 with the auto-scaling setting and relevance of increasing concentrations. The PLS-DA analysis is not part of the original manuscript.

Combined effects of nZnO and the organic UV filter avobenzone on *A. marina* described in the manuscript II.3 differed from those of nZnO alone. We detected avobenzone concentrations of $\sim 2 \mu\text{g/l}$ seawater two days after addition. These levels are considerably lower than the nominal added concentration ($50 \mu\text{g/l}$) but are plausible for highly polluted environments (Downs et al., 2022a; Sánchez-Quiles and Tovar-Sánchez, 2015). The determination of the mechanism behind the avobenzone degradation was outside of the scope of this study, but the results emphasize the importance of considering the ratio between nominal and actual concentrations when investigating organic UV filters that are prone to rapid degradation under environmental conditions (National Academies of Sciences Engineering and Medicine, 2022; Trebše et al., 2016).

The biochemical results of manuscript II.3 are partially consistent with manuscript II.2 as we could detect oxidative stress and found metabolic shifts driven by nZnO exposure. Specifically, lipid peroxidation and methionine sulfoxide accumulated significantly in the body wall during co-exposure to 3 mg/kg nZnO and $50 \mu\text{g/l}$ avobenzone (Bruhns et al. (2024), Fig. 2). The combined exposure further led to a significant decrease in SOD

activity (Bruhns et al. (2024), Fig. 1). In contrast, the total antioxidant capacity was increased in both single-pollutant exposures and catalase activity was enhanced by sole nZnO exposure (Bruhns et al. (2024), Fig. 1). Hence, we suggest, that oxidative stress was induced by nZnO and avobenzone (alone or in combination), but antioxidant upregulation in the single-pollutant exposures was sufficient to prevent oxidative tissue damage (Ahmad, 1995; Lushchak et al., 2001). However, the combination of both UV filters exhausted the antioxidant response causing oxidative tissue damage (Ramos-Gómez et al., 2011b).

PLS-DA analysis of the body wall further revealed that the metabolic profile of lugworms exposed to nZnO (alone or in combination with avobenzone) was partially shifted relative to those exposed to avobenzone only (Bruhns et al. (2024), Fig. 4). The metabolic profile of the control lugworms was partially shifted to the worms exposed to avobenzone (alone or in combination) (Bruhns et al. (2024), Fig. 4). The pathway enrichment analysis revealed significant metabolic shifts in the body wall of lugworms exposed to nZnO alone (Bruhns et al. (2024), Table 2). Lack of response in exposures involving avobenzone suggests a lower toxic potential for this compound, or even potential mitigating effects in combined exposures, as reported in previous studies (Kudłak et al., 2022; Marcin and Aleksander, 2023; Martin et al., 2022; Migdal et al., 2010; Park et al., 2017). Consistent with the findings reported in the manuscript II.2, the metabolic shifts caused by nZnO included bioenergetic pathways such as glyoxylate and dicarboxylate metabolism, tricarboxylic acid cycle and Ala, Asp and Glu metabolism as well as a nitrogen metabolism pathway, Arg biosynthesis (Bruhns et al. (2024), Table 2). Some of the altered pathways such as Ala, Asp and Glu metabolism and Arg biosynthesis are also involved in the amino acid metabolism. It is interesting to note that the correlation analysis of the body fluids found a cluster (19) of positively correlated metabolites whereas in the body wall, a smaller cluster of 12 metabolites was detected (Bruhns et al. (2024), Fig. 3). This pattern is consistent with the findings of manuscript II.2.

Bringing together both studies, nZnO and avobenzone can be seen as UV filters with moderate toxicity to *A. marina*. Both compounds induce oxidative stress that is increased in combined exposures. However, the oxidative stress caused by single pollutant exposures can be partially compensated by the antioxidant system. This compensation might be connected to additional metabolic adjustments as reflected in the observed shifts of bioenergetic pathways. Energy fluxes are often affected by environmental stressors such as pollutants (Britto et al., 2020; Sokolova, 2021; Sokolova and Lannig, 2008). The mechanisms behind the pollutant-induced metabolic

adjustments are complex but shifts of the antioxidant system or anabolic processes such as protein synthesis might contribute to these shifts (Abele et al., 2011; Sokolova and Lannig, 2008). The metabolic shifts observed in amino acid metabolism support this suggestion (Fitzgerald and Szmant, 1997; Lane, 2020). Besides potential metabolic shifts resulting from modulated protein synthesis, alterations in nitrogen metabolism may also be linked to bioenergetic changes, potentially indicating shifts in nitrogen excretion of the polychaete (Liu et al., 2009; O'Malley and Terwilliger, 1975). However, shifts in nitrogen metabolism could alternatively indicate adjustments in stress response signaling, given that nitric oxide serves as a common signaling molecule in invertebrates (Jacklet, 1997). It is worth noting that metabolic shifts are more pronounced in the body fluids compared to the body wall of *A. marina*. This finding emphasizes the importance of the body fluids in the stress response of the lugworm and opens the possibility of repeated non-lethal sampling for ecotoxicological studies involving these animals.

However, some effects of nZnO exposures differ between the two independent experiments. Although the second study used higher nZnO concentration (3 mg/kg) than the first one (0.1 and 1 mg/kg), the metabolic disturbances and oxidative damage caused by nZnO in the lugworms were less pronounced in the second study. Interestingly, our first study found a depletion of most free amino acids whereas in the second study the significant responses of the amino acids were mostly characterized by an increase caused by nZnO exposure (alone or in combination with avobenzone). The reason behind this discrepancy remains unknown but free amino acid depletion can have negative consequences for sustained protein synthesis and osmotic regulation (B. L. Bayne, 2017; Wiesenthal et al., 2019; Yancey, 2005). In addition, the baseline (control) concentrations of most studied metabolites were notably lower in the second study. This shows different physiological baseline status in the experimental batches of animals used for the two experiments that were obtained in different seasons and years. Thus, the different baselines and the different exposure time could have led to acclimation minimizing the toxic effects of nZnO or general suppression of metabolism leading to a biphasic response of selected stress markers (Colas and Le Faucheur, 2024). Conclusively, our initial hypothesis regarding oxidative stress and metabolic shifts caused by single exposures can be accepted. Both nZnO and avobenzone caused oxidative stress but only nZnO led to relevant metabolic shifts. However, our second hypothesis proposing intensified physiological responses caused by increased nZnO concentrations and UV filter combination must be rejected. We could not find clear evidence for a positive dose dependence of the physiological response to nZnO

exposure. Furthermore, while nZnO and avobenzone co-exposure intensified the oxidative stress, it did not cause significant shifts in metabolic pathways.

These studies provide important insights into the ecotoxicological stress response of the important ecosystem engineer *A. marina*. We highlight the role of the body fluids and demonstrate the resilience of the lugworms to the chosen concentrations of the pollutants that are at the higher end of the expected environment levels. Thus, this species might have ecological advantage in highly polluted habitats. Detailed results and discussions are described in the publications II.2 (Bruhns et al., 2023b) and II.3 (Bruhns et al., 2024).

6.3. Conclusions

The use of multiple combined stressors in experimental designs is important because such combined exposures more closely resemble the field conditions in coastal habitats (Breitburg et al., 2019; Mack et al., 2022; Norkko et al., 2005). Furthermore, stressor interaction can result in cumulative effects of multiple stressors that cannot be predicted based on the single stressor effects (Matzelle et al., 2015; Todgham and Stillman, 2013). Our study in *C. gigas* and *A. marina* using combined exposures to different environmental stressors supports this paradigm and shows both additive and non-additive (interactive) effects of single and combined stressors on the metabolism of these species. Further, we show variation in the physiological response among different exposure regimes and different tissues. Thus, we could detect mitigating as well as enhancing effects caused by combined stressors. Further, we found indications for biphasic effects of nZnO dosage and batch-to-batch variability in response to nZnO between lugworms collected at different times. These results highlight that effects from single exposures in principle cannot be extrapolated to combined scenarios. Thus, we emphasize the importance of evaluating multiple stressors in ecophysiological and ecotoxicological studies to better understand both the mechanisms and consequences of abiotic stress under environmentally realistic multi-factor scenarios.

Our study also contributes to better characterization of the ecological tolerance of two important ecosystem engineers under multiple stressor scenarios. We were able to demonstrate the remarkable resilience of *C. gigas* when faced with combinations of intermittent hypoxia and warming during emersion, conditions often encountered during the summer in the Wadden Sea. However, our findings show that the limits of *C. gigas* resilience are exceeded when intermittent hypoxia is coupled with sustained warming, a scenario resembling marine heatwaves in shallow intertidal zones (De Marco

et al., 2023). Our study also demonstrated high tolerance of *A. marina* against two common UV filters at high environmentally relevant concentrations. This tolerance may confer advantages to *A. marina* in terms of survival or establishment in highly polluted areas, potentially positioning it as an indicator species for polluted habitats. Nevertheless, combined exposure to two commonly co-occurring UV filters (nZnO and avobenzone) led to oxidative damage, suggesting chronic sublethal stress and potential compromised health in the exposed animals. Understanding the mechanisms of pollutant tolerance in *A. marina* requires further investigation, but our data provide preliminary evidence for potential involvement of enhanced biosynthesis of proteins (such as heat shock or other cytoprotective proteins) in the stress response of *C. gigas*. The lack of concentration-dependence of metabolic responses to nZnO also raises questions about the potential biphasic effects of this pollutant on metabolism of *A. marina* that warrants further investigation. In addition, the detected degradation of avobenzone prompts the investigation of potential toxic degradation products in future studies (Lebedev et al., 2020; Trebše et al., 2016).

The scope of variables that can be explored experimentally is constrained to ensure precision and maintain control over experiments (Gunderson et al., 2016; Todgham and Stillman, 2013). Thus, instead of complex stressor combinations, studies with focus on few but frequently co-occurring stressors could provide a compromise between environmental realism and feasibility. Hereby, possible antagonistic or synergistic effects could be detected which then warrants further research. Based on our results, *C. gigas* might successfully invade habitats that are characterized by strong fluctuations in oxygen and temperature but marine heatwaves that become more frequent with the climate change, may limit the species' spread and threat existing populations. Further, the low toxicity of avobenzone on *A. marina* might justify using it as a suitable replacement of more toxic organic UV filters in sunscreens as already done in "reef-safe" formulas. Although nZnO was the driving force in the metabolic response of the lugworm, negative effects were limited and thus, continued use appears to pose low environmental risk to this species, considering that the concentrations used in our study were at the higher end of expected environmental concentrations. Nevertheless, due to the NPs persistence, environmental concentrations might drastically increase in the future which warrants continuous monitoring and potential ecotoxicological re-evaluations.

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Part II Manuscripts

II.1 Combined effects of temperature and emersion-immersion cycles on
metabolism and bioenergetics of the Pacific oyster *Crassostrea*
(*Magallana*) *gigas*

Contribution letter

The material of the manuscript titled “**Combined effects of temperature and emersion-immersion cycles on metabolism and bioenergetics of the Pacific oyster *Crassostrea (Magallana) gigas***” was published in 2023 in the journal *Marine Environmental Research*.

My work was the major contribution to this manuscript. I was involved in the experimental design and planning, collection and maintenance of the experimental animals, setup and execution of the experimental exposures, tissue sampling, and the determination of bioenergetic (proteins, electron transport system, glycogen content) and oxidative stress (lipid peroxidation and protein carbonylation) markers. Determination of the total antioxidant capacity was done in collaboration with Sonja Engelhaupt and Nina Feußner where I was involved in the initial adaptation of the methods and quality control. Determination of the metabolites was done in collaboration with Dr. Stefan Timm where I carried out the initial extraction of the metabolites. Further, I conducted the statistical and graphical analysis of the gained data and wrote the first draft of the manuscript. I contributed to the revision of the draft and to the responses to the reviewers during the submission process.

Based on the CRediT taxonomy (Brand et al., 2015), my contributions to this manuscript were as follows:

- Conceptualization
- Methodology
- Validation
- Formal analysis
- Investigation
- Data Curation
- Writing - Original Draft
- Writing - Review & Editing
- Visualization

Signatures of consent

Prof. Dr. Inna Sokolova
(Supervisor)

Torben Bruhns
(PhD candidate)

Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J., 2015. Beyond authorship: attribution, contribution, collaboration, and credit. *Learn. Publ.* 28, 151–155. <https://doi.org/10.1087/20150211>



Combined effects of temperature and emersion-immersion cycles on metabolism and bioenergetics of the Pacific oyster *Crassostrea (Magallana) gigas*

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ABSTRACT

Life on tidal coasts presents physiological major challenges for sessile species. Fluctuations in oxygen and temperature can affect bioenergetics and modulate metabolism and redox balance, but their combined effects are not well understood. We investigated the effects of intermittent hypoxia (12h/12h) in combination with different temperature regimes (normal (15 °C), elevated (30 °C) and fluctuating (15 °C water/30 °C air)) on the Pacific oyster *Crassostrea (Magallana) gigas*. Fluctuating temperature led to energetic costly metabolic rearrangements and accumulation of proteins in oyster tissues. Elevated temperature led to high (60%) mortality and oxidative damage in survivors. Normal temperature had no major negative effects but caused metabolic shifts. Our study shows high plasticity of oyster metabolism in response to oxygen and temperature fluctuations and indicates that metabolic adjustments to oxygen deficiency are strongly modulated by the ambient temperature. Co-exposure to constant elevated temperature and intermittent hypoxia demonstrates the limits of this adaptive metabolic plasticity.

1. Introduction

Living in coastal regions presents major physiological challenges for sessile benthic species, which must contend with fluctuating environmental conditions that can disrupt their metabolic and redox homeostasis (Willmer et al., 2005; Abele et al., 2012; Haider et al., 2020). One significant source of variability is the natural tidal cycle, which exposes intertidal organisms to intermittent submersion and emersion coinciding with the changes in oxygen and food availability, temperature and desiccation stress (Vernberg and Vernberg, 1972). Lacking the ability for escape, intertidal sessile organisms like bivalves, limpets and barnacles evolved protective behaviors that involve shell closures to isolate the body from the stressful environment. While shell closure is effective in preventing desiccation, it also limits oxygen supply, leading to hypoxia within the shell (Vernberg and Vernberg, 1972; Platvoet and Pinkster, 1995; Willmer et al., 2005). Consequently, intertidal animals

have developed strategies to counteract oxygen deprivation, such as metabolic rate suppression, which can reduce aerobic rates by up to 99% (Hochachka et al., 1993; Hochachka and Lutz, 2001; Storey and Storey, 2004; Abele et al., 2012; Janas et al., 2017; Haider et al., 2020; Steffen et al., 2021). Upon re-submersion, the organism experiences reoxygenation, which can induce a burst in reactive oxygen species (ROS) production due to the rapid reintroduction of oxygen (Kalogeris et al., 2014). While intertidal organisms express high levels of antioxidants, compensating for this ROS burst remains challenging (Hermes-Lima et al., 1998; Sokolova et al., 2011; Abele et al., 2012; Bayne, 2017b).

Temperature is an important stressor that varies on the seasonal, daily and tidal scales in the intertidal zone and can directly affect the physiology and metabolism of ectothermic organisms (Vernberg and Vernberg, 1972; Sommer, 2005; Lushchak, 2011). During summer low tides, body temperature of sessile intertidal organisms rapidly increases due to contact with the warm air and substrate and insolation (Sokolova

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et al., 2000; Ricklefs and Vanselow, 2012; Bayne, 2017d; Sokolova, 2019; Janetzki et al., 2021). As a result, organisms in the intertidal zone experience diurnal temperature fluctuations that occasionally exceed their critical temperatures and can affect energy metabolism, ROS generation and ultimately, fitness (Newell and Bayne, 1973; Sommer et al., 1997; Lushchak, 2011; Strand et al., 2011; Ricklefs and Vanselow, 2012; Bayne, 2017d). Under fluctuating temperature conditions, physiological plasticity plays an important role in an organism's adaptation as described in the "beneficial acclimation hypothesis" (Leroi et al., 1994; Kingsolver and Huey, 1998; Wilson and Franklin, 2002; Seebacher et al., 2015). However, physiological plasticity is limited when stronger or unpredictable fluctuating environments do not provide clear signals for directional adaptation (Kingsolver and Huey, 1998; Kawecki, 2000; Wilson and Franklin, 2002). Earlier studies on the effects of stable vs. fluctuating temperature regimes on adaptive physiological responses and thermal tolerance of different ectotherms species yielded controversial results (Ashmore and Janzen, 2003; Dong and Dong, 2006; Pernet et al., 2007; Byrne et al., 2009; van Dooremalen et al., 2011; Paaijmans et al., 2013; Seebacher et al., 2015; Kingsolver et al., 2020; Slein et al., 2023). This variability in physiological and metabolic responses to temperature might reflect evolutionary adaptations of different species to certain thermal regimes, but may also be modulated by other abiotic factors like oxygen availability. While the individual effects of hypoxia (oxygen deficiency) and temperature have been well studied in intertidal organisms, their combined effects remains poorly understood and require further investigation (Pörtner, 2005; Collins et al., 2023).

The aim of this study was to investigate the combined impacts of intermittent hypoxia and elevated or fluctuating temperature on the metabolism and redox balance of the Pacific oyster, *Crassostrea (Magallana) gigas* (Thunberg, 1793), an invasive species commonly found in European intertidal waters. The oyster was introduced worldwide for aquaculture purposes but frequently escaped, establishing dense intertidal reefs (Reise et al., 2005; Ruesink et al., 2005; Reid and Valdés, 2011). Invasive populations of *C. gigas* in the German Wadden Sea inhabit major parts of the intertidal zone, where they emerge twice daily for several hours during a semi-diurnal tide (Reise et al., 2017). As a robust and stress-tolerant organism (Zhang et al., 2012), *C. gigas* is a suitable model to investigate the interaction between hypoxia and temperature stress. Here, we hypothesized that intermittent hypoxia will disrupt the redox balance and metabolic profiles of the oysters, with this disruption being intensified by elevated temperature. To test our hypotheses, we exposed Pacific oysters to ten cycles of intermittent hypoxia, with 12 h of hypoxia (air exposure) followed by 12 h of recovery (submersion) per cycle. This regime was chosen to simulate the maximum emersion experienced by oysters in the upper intertidal habitats (Bundesamt für Schifffahrt und Hydrographie, 2023). Extended (12 h) recovery period was chosen to detect the persistent effects of periodic emersion. Additionally, we subjected the oysters to elevated temperatures (30 °C) considered critical for *C. gigas* (Le Gall and Railard, 1988; Bougrier et al., 1995) and plausible for summer low tide conditions of the German Wadden Sea (Ricklefs and Vanselow, 2012). The temperature was either kept constant at 15 °C or 30 °C throughout the emersion-immersion cycle or fluctuated between the emersion (30 °C) and immersion (15 °C) conditions as would be expected during summer tides. Our results provide insights into the role of metabolic plasticity in responses to intermittent hypoxia and thermal stress in oysters and shows mitigating effects of fluctuating temperatures on the hypoxia-induced metabolic and redox disturbances in this stress-tolerant intertidal species.

2. Material and methods

2.1. Oyster collection and experimental exposures

Oyster were collected from a mixed intertidal oyster-mussel reef in

the intertidal zone of Königshafen (Oddewatt, List, Sylt, Germany) in June 2020. Oysters were cleaned of epibionts and placed in habituation tanks at 15 °C and salinity 28 (practical salinity units) (Pro-REEF Sea Salt; Tropic Marin). These conditions were similar to the habitat water conditions at the time of collection. Starting on day two, salinity was increased to 33 at a rate of ~1 unit per day. After one week, oysters were transferred into a recirculated maintenance system (700l) with biological filtration, aeration and protein skimmer and kept at 15 °C and salinity 33 for 4–9 weeks until experimental exposures. Oysters were continuously fed with a commercial algal mixture (*Nanochloropsis* sp., *Chlorella* sp. *Phaeodactylum* sp., *Cylindrotheca* sp. and *Nitzschia* sp., provided by Aquacopa or Sustainable Aquatics), supplemented with 30–50% (v:v) of freshly cultured *Rhodomonas* sp. using automatic feeding systems (Reefdoser EVO 4, Aqua Medic). The final concentration of algae in the maintenance tanks was kept at approximately 200 µg/l (chlorophyll a equivalents).

For each experimental treatment group, 19–20 randomly selected oysters (average length 51 mm) were placed into three 5 l tanks (6–7 oysters per tank, salinity 33). Water in the tanks was constantly aerated, and the tanks were kept under the dim light conditions throughout the exposures. For each experimental group, the oysters were exposed to ten cycles of emersion-immersion (12 h: 12 h) under different temperature regimes including either normal (15 °C in water and air), elevated (30 °C in water and air) or fluctuating (15 °C in water and 30 °C in air) temperature. During emersion, the oysters were placed for 12 h in dry plastic containers lined with plastic spacers to allow drainage. Air temperature was controlled by placing the containers into a water bath (15 °C) or temperature-controlled incubator (30 °C). Humidity was not controlled during emersion but was kept high (>80%) by closing the plastic container with a lid. Oysters maintained at 15 °C under normoxia and constant immersion were used as controls. During exposures, oysters were fed every other day with the algal mix (0.3–0.4 ml/tank; Premium Reef Blend, Sustainable Aquatics). Ammonium and nitrite concentrations were monitored and water exchanged if the threshold of 10 mg/l for either compound was exceeded.

Tissue samples were collected from all experimental oysters at the end of the last (10th) immersion cycle. This time point corresponded to 12 h of recovery after hypoxia ensuring that all oysters were sampled under the normoxic conditions. Normoxic controls were collected after 3, 5 and 10 days of exposure. Pilot analyses showed that there was no significant difference in the studied endpoints in the normoxic controls over time ($p > 0.05$; data not shown). Therefore, all control samples were treated as a single experimental group. Gill and digestive gland were dissected on ice, immediately frozen in liquid nitrogen and stored at –80 °C until further analyses.

2.2. Determination of energy reserves and electron transport system (ETS) activity

Gill or digestive gland tissues (15.6 ± 3.1 mg per sample) were placed into 2 ml tubes containing zirconium beads (Ø 1 mm and 2 mm, ~420 mg and ~230 mg, respectively) and ice-cold homogenization buffer (0.1 M Tris-HCl, pH 8.5, 1.5 g/l polyvinylpyrrolidone, 153 µM MgSO₄ and 0.2% Triton X-100 (w/v) in the tissue to buffer ratio of 1:25 (w/v). Tissues were homogenized using FastPrep-24 homogenizer (MP Biomedicals) with five 60 s cycles at 6.5 m/s and 5 min cooling on ice between the cycles. The homogenate was centrifuged for 10 min at 4 °C and 3000×g. A 75 µl aliquot of the supernatant was collected and stored at –80 °C for ETS measurement. The remaining homogenate was lysed using four freeze-thaw cycles (5 min each) alternating between liquid nitrogen and water bath (50–55 °C). The samples were centrifuged for 10 min at 4 °C and 3000×g and stored at –80 °C for carbohydrate and protein analyses.

ETS was measured in an assay buffer containing 91 mM Tris-HCl, pH 8.5, 0.21% Triton X-100 (w/v), 0.34 mM NADH and 51 µM NADPH as described elsewhere (King and Packard, 1975; De Coen and Janssen,

1997). To account for the background activity of non-mitochondrial oxidoreductases, blanks were set with addition of mitochondrial ETS inhibitors (1 M KCN and 0.4 mM rotenone) instead of NADH and NADPH. The reaction was started with 2.7 mM P-iodonitrotetrazolium (INT) and monitored at 490 nm (SpectraMax iD3, Molecular Devices). Using the extinction coefficient of the produced INT formazan $\epsilon = 15900 \text{ M}^{-1} \text{ cm}^{-1}$, the ETS activity was calculated as the background-corrected oxygen consumption rate assuming the molar ratio 2:1 of formazan to oxygen.

Tissue levels of carbohydrates were measured using phenol-sulfuric acid colorimetric method at 492 nm with glucose as a standard (Masuko et al., 2005). Proteins were measured using the Bradford assay at 595 nm (Bradford, 1976) using bovine serum albumin as a standard.

2.3. Oxidative stress markers

Gills or digestive gland tissues ($45.5 \pm 8.0 \text{ mg}$ per sample) were homogenized in phosphate-buffered saline (PBS, 50 mM, pH 7.4) using 1:10 (w/v) tissue to buffer ratio. The homogenate was split into two aliquots and the proteins were precipitated by adding two volumes of 50% trichloroacetic acid (TCA). After 10–15 min of incubation on ice, the samples were centrifuged for 10 min at 4°C and $5000 \times g$. The resulting supernatants were combined. Pellets and supernatant were stored at -80°C .

Lipid peroxidation products were determined in the supernatant as the concentration of the thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). Thiobarbituric acid (21 mM in 0.1 M HCl) was added 1:1 (v/v) to the supernatant and incubated at $90\text{--}100^\circ \text{C}$ for 20 min. Samples were then cooled on ice and absorbance measured at 532 nm. TBARS level was calculated using $\epsilon = 156000 \text{ M}^{-1} \text{ cm}^{-1}$.

Protein carbonyl content was measured in the precipitated protein pellets using 2,4-dinitrophenylhydrazine (DNPH, 10 mM in 2 M HCl) as described before (Levine et al., 1990). The second pellet from each sample was treated with HCl and used as a negative control. The DNPH- or HCl-treated samples were centrifuged for 10 min at 4°C and $10000 \times g$, washed twice with 1 ml 5% TCA and centrifuged for 10 min at 4°C and $10000 \times g$. The pellets were dissolved in 1.5 ml of 8 M urea, centrifuged for 10 min at 4°C and $10000 \times g$ and absorbance measured in the supernatant at 370 nm. The sample absorbance was corrected for the absorbance of the negative controls, and the protein carbonyls concentration calculated using $\epsilon = 22000 \text{ M}^{-1} \text{ cm}^{-1}$.

Total antioxidant capacity (TAOC) was measured in the homogenates of the gill or digestive gland tissues. Tissues ($29.8 \pm 6.0 \text{ mg}$) were homogenized 1:15 (w/v) in 50 mM PBS, pH 7.8, containing 0.1 mM phenylmethylsulfonyl fluoride (Re et al., 1999). Samples were centrifuged for 10 min at 4°C and $5000 \times g$ and the supernatant stored at -80°C . For TAOC measurements, samples were mixed with freshly prepared ABTS⁺ working solution in PBS (the final concentrations: 104 μM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 37 μM K₂S₂O₈) and incubated for 6 min on a shaker at room temperature in the dark. The absorbance was measured at 734 nm and TAOC was calibrated with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as a standard and expressed in μmole Trolox-equivalents per g wet mass.

2.4. Metabolite profiles

Metabolite content was measured in the gill as described elsewhere (Haider et al., 2020). Metabolites were extracted from $25.7 \pm 4.1 \text{ mg}$ of tissue in 1 ml 80 % ethanol with 1 $\mu\text{g/ml}$ 2-(N-morpholino)ethanesulfonic acid (MES) as an internal standard using FastPrep-24 homogenizer (MP Biomedicals). The homogenate was centrifuged for 10 min at 4°C and $13000 \times g$ to remove the debris. The supernatants were freeze-dried under vacuum (Alpha 1–4 LSCplus and RVC 2–25 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH) and stored at -80°C . The samples were then dissolved in ROTISOLV® LC-MS-grade water

(Carl Roth), filtered (0.2 μm , Omnifix®-F, Braun, Germany) and analysed with the high-performance liquid chromatograph-mass spectrometer LCMS-8050 (Shimadzu). The sample separation was conducted as described earlier (Haider et al., 2020). The metabolites were identified and quantified with the built-in LC-MS/MS package for primary metabolites (Vers. 2, Shimadzu, P/N 225–24,862-92) and the LabSolutions software package (Shimadzu) (Selim et al., 2018). Each compound was calibrated using the respective standard substances (Merck) and loading-corrected relative to the internal standard (MES) peak. The peaks of citrate and isocitrate could not be distinguished, and the respective concentrations are combined and reported as (iso)citrate.

2.5. Statistics

Effects of different exposure regimes on the measured biomarkers were tested using one-way ANOVA. Any outliers with values outside the 1.5x interquartile range were excluded from further analyses. Data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). Data showing lack of homogeneity of variances or not normal distribution were analysed using Welch-ANOVA or Kruskal-Wallis test, respectively. Bonferroni, Games-Howell or Dunn-Bonferroni were used as post-hoc tests for ANOVA, Welch-ANOVA or Kruskal-Wallis analysis, respectively. Data was analysed using IBM SPSS Statistics 27.

Metabolite data was processed using MetaboAnalyst 5.0 (Pang et al., 2021). Pathway analyses was conducted using auto scaling, *Drosophila melanogaster* as a reference library, global test as the enrichment method and relative-betweenness centrality for topology analysis. (iso)citrate was treated as citrate. Exposure groups were compared to the normoxic control with pathways considered as significantly affected if the pathway impact was >0 and the false discovery rate (FDR) was <0.05 . Partial least squares-discriminant analysis (PLS-DA) was conducted to reduce the dimensionality of the data set using autoscaling. Metabolites that contributed most to the separation along 1st and 2nd component were identified based on the loadings (cut-off ± 0.2) and were used for further analyses.

3. Results

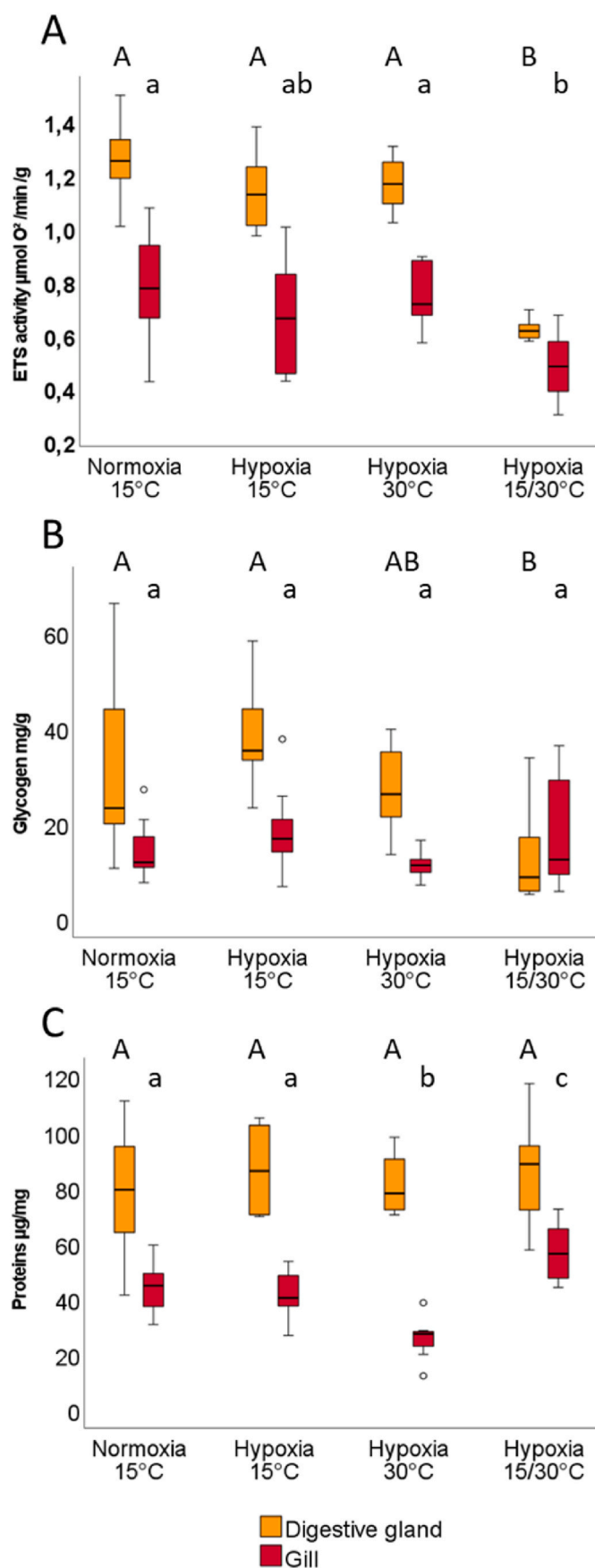
3.1. Mortality

No mortality was observed in the control oysters or those exposed to intermittent hypoxia (emersion-immersion cycles) at 15°C ($N = 20$). In the group exposed to intermittent hypoxia combined with the fluctuating temperature, one oyster died at exposure day 8 (mortality rate of 5%, $N = 19$), whereas intermittent hypoxia combined with the constantly elevated temperature (30°C) led to the mortality rate of 60% which was distributed over the whole course of the exposure ($N = 20$; 1.7 casualties per day).

3.2. Cellular bioenergetic parameters

The oyster gills showed lower ETS activity than the digestive gland (Fig. 1A). The experimental treatment showed significant effects on the ETS activity in oyster tissues (Table 1). This, oysters exposed to intermittent hypoxia combined with fluctuating temperature exhibited a notable decrease in ETS activity, with reductions of 37% and 52% in the gill and digestive gland, respectively, compared to the normoxic control (Fig. 1A). No change of ETS activity was observed in other hypoxia-exposed groups relative to the normoxic controls ($p > 0.05$).

The gills of oysters had generally lower glycogen content compared to the digestive gland (Fig. 1B). Glycogen content in the gills did not change in response to the intermittent hypoxia regardless of the temperature regime (Table 1). However, in the digestive gland, glycogen depletion was observed in oysters exposed to intermittent hypoxia combined with fluctuating temperature relative to the controls and



(caption on next column)

Fig. 1. Bioenergetic markers: electron transport system (ETS, A), carbohydrate content in glycogen equivalents (B) and protein content (C) are shown for digestive gland (yellow, upper case letters) and gills (red, lower case letters) and scaled to tissue fresh mass; columns that do not share the same letter are significantly different ($p < 0.05$); box plots show median, interquartile range (IQR) as well as maximum and minimum; outliers ($>1.5 \times$ IQR) are indicated by circles.

hypoxia-exposed oysters kept at 15 °C (Fig. 1B).

The digestive gland had higher levels of proteins compared to the gill in all exposures (Fig. 1C). Intermittent hypoxia, irrespective of the temperature, showed no significant effect on the protein content of the digestive gland in oysters ($p > 0.05$, Table 1). However, in the gills, intermittent hypoxia combined with 30 °C caused protein depletion, and combined with fluctuating temperature - protein accumulation (Table 1, Fig. 1C).

3.3. Oxidative stress markers

Lipid peroxidation (LPO) levels were higher in the oyster gills compared to the digestive gland in all experimental groups (Fig. 2A). Moderate elevation of LPO levels was observed in the gills of the oysters exposed to hypoxia at 30 °C, but this increase was only significant when compared to the hypoxic group kept at fluctuating temperature (Fig. 2A). In the digestive gland, LPO levels were lower in oysters exposed to intermittent hypoxia at 15 °C compared to the normoxic controls (Table 1, Fig. 2A).

Protein carbonyl content was generally lower in the gills than in the digestive gland of oysters (Fig. 2B). The experimental treatment showed no evidence of effect on protein carbonyl levels in the gills and the digestive gland (Table 1).

Oysters exposed to intermittent hypoxia at 30 °C exhibited an increase in methionine sulfoxide concentration in the gills compared to those of the normoxic controls (Table 4, Fig. 2C). Hypoxia at constant or fluctuating temperature did not cause significant changes in methionine sulfoxide levels in oyster gills (Fig. 2C).

The total antioxidant capacity (TAOC) was lower in the gill compared to the digestive gland (Fig. 4D). There was no evidence for the effect of the experimental treatment on the TAOC in the gill or the digestive gland (Table 1).

3.4. Metabolite profiles in the gill tissue

PLS-DA analysis revealed that two first principal components accounted for 24.7% and 17.2% of the variance in the data, respectively (Fig. 3). All hypoxia-exposed groups were clearly separated from the control group in the scatter plot of the two principal components (Fig. 3). Samples from oysters exposed to intermittent hypoxia at fluctuating temperatures were strongly separated from the controls (along the 1st component axis) as well as from two other hypoxia-exposed groups (along the 2nd component axis) (Fig. 3). Samples from oysters exposed to intermittent hypoxia at constant temperatures (15 °C or 30 °C) showed overlapping positions, suggesting similarity in their metabolite profiles.

Based on the loadings of the PLS-DA analysis, 21 metabolites were identified as contributing to the separation of the model (loadings >0.2 or <-0.2 ; Table 2). Alanine, citrulline, tryptophan, tyrosine, glutamate, isoleucine, leucine, phenylalanine, succinate, and valine were associated with the 1st component (the fluctuating temperature axis), while aconitate, aspartate, glutamine, lysine, AMP, asparagine, (iso)citrate, malate, methionine sulfoxide, and threonine were associated with the 2nd component (hypoxia and constant temperature axis). Taurine contributed to the separation along both components.

Pathway enrichment analysis revealed alterations in 13, 10, and 5 out of 20 analysed metabolic pathways (with pathway impact >0) in the gills of oysters exposed to intermittent hypoxia under fluctuating

Table 1

Analysis of the effects of the experimental treatments on the bioenergetic and oxidative stress markers in the gills and the digestive gland tissues of *C. gigas*. The treatment effects were tested by ANOVA (a), Kruskal-Wallis test (k) or Welch ANOVA (w). Significant effects are highlighted in bold.

Trait	Gill	DG
ETS activity	F(3,39) = 5.700 p = 0.002^a	F(3,35) = 50.384 p < 0.001^a
Glycogen	F(3,17.2) = 2.757 p = 0.074^a	$\chi^2 = 15.619$ p = 0.001^k
Proteins	F(3,38) = 18.082 p < 0.001^a	F(3,39) = 0.486 p = 0.694^a
Lipid peroxidation products	F(3,13.5) = 6.819 p = 0.005^a	F(3,10.5) = 3.687 p = 0.048^w
Protein carbonyls	F(3,10.4) = 1.746 p = 0.218^a	Chi2 = 2.695 p = 0.441^k
Total antioxidant capacity	F(2,25) = 0.337 p = 0.717^a	F(2,6.6) = 0.729 p = 0.518^w

temperature conditions, at 15 °C, and at 30 °C, respectively (Table 3, Supplementary Table). Metabolic pathways such as alanine, aspartate, and glutamate metabolism, arginine biosynthesis, tryptophan metabolism, and tyrosine metabolism were affected in all exposure groups (Table 3, Fig. 4 A, B, C). Glycine, serine, and threonine metabolism were altered by intermittent hypoxia at 15 °C, while the TCA cycle changed in response to intermittent hypoxia exposure at 30 °C. In the gills of oysters exposed to intermittent hypoxia under the fluctuating temperature regime, alterations in phenylalanine, tyrosine, and tryptophan biosynthesis, arginine and proline metabolism, and butanoate metabolism were detected (Fig. 4 C).

3.5. TCA and urea cycle metabolites

The combined exposure of oysters to intermittent hypoxia and heat at 30 °C resulted in an upregulation of aconitate and (iso)citrate, and a decrease in succinate levels in the gills (Table 4). Additionally, carnitine concentrations were elevated in the gills of oysters exposed to intermittent hypoxia at 30 °C (Table 4). However, there were no significant changes in the concentrations of TCA intermediates and carnitine in other experimental treatment groups compared to the control, except for a decrease in succinate levels observed in all experimental treatments (Table 4). Citrulline content in the gills significantly increased (by 12.8-fold) under the fluctuating temperature regime compared to control (Table 4).

3.6. Levels of free amino acids

All exposures led to a significant decrease in tryptophan and tyrosine (Table 4). Moreover, exposure of oysters to hypoxia at 15 °C resulted in a significant increase in the concentrations of taurine, glutamine, glycine, asparagine, and lysine content in the gill tissues (Table 4). Combined exposure to intermittent hypoxia and 30 °C led to an increase in the content of aspartate and asparagine, but a depletion of alanine and proline, in the oyster gills (Table 4). Furthermore, intermittent hypoxia at fluctuating temperature resulted in a significant decrease in the gill content of taurine, alanine, phenylalanine and proline (Table 4).

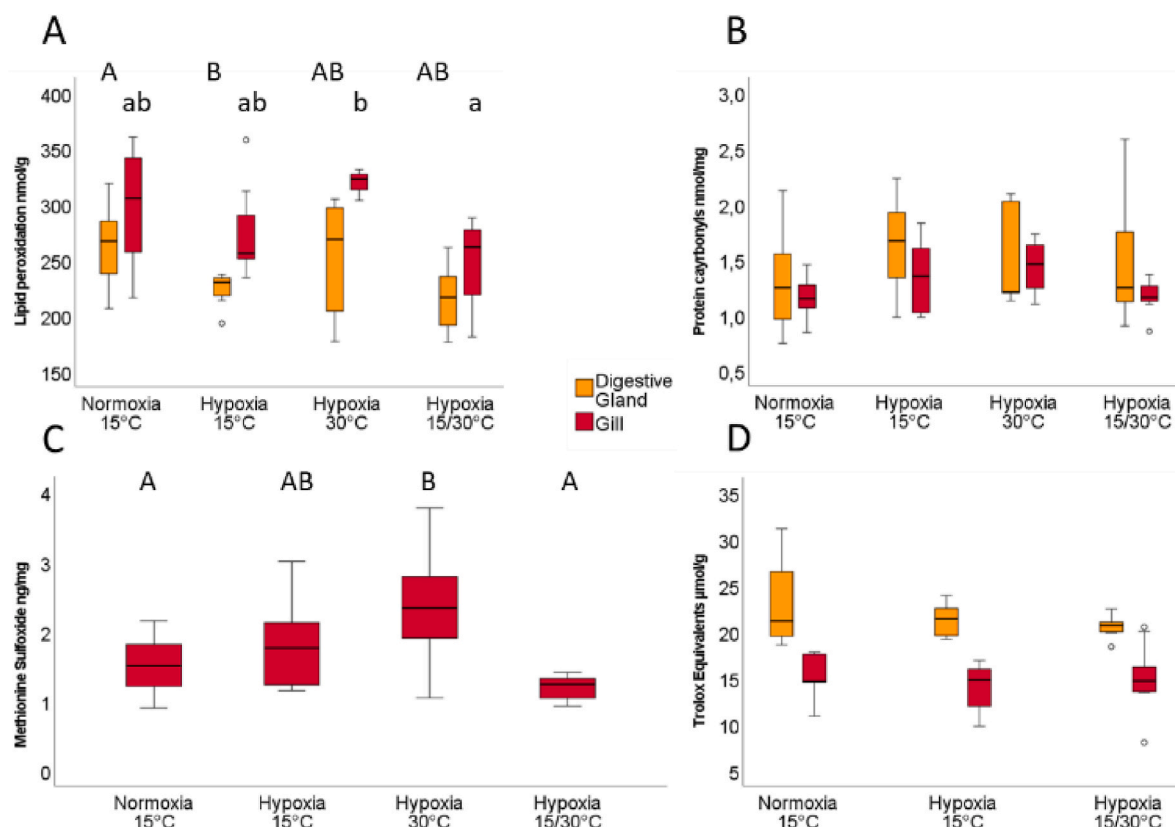


Fig. 2. Oxidative stress markers: lipid peroxidation in thiobarbituric acid-reactive substances equivalents (A), protein carbonyls (B), methionine sulfoxide (C) and total antioxidant capacity in trolox equivalents (D) are shown for digestive gland (yellow, upper case letters) and gills (red, lower case letters) and scaled to tissue fresh mass; columns that do not share the same letter are significantly different ($p < 0.05$); box plots show median, interquartile range (IQR) as well as maximum and minimum; outliers ($>1.5 \times$ IQR) are indicated by circles.

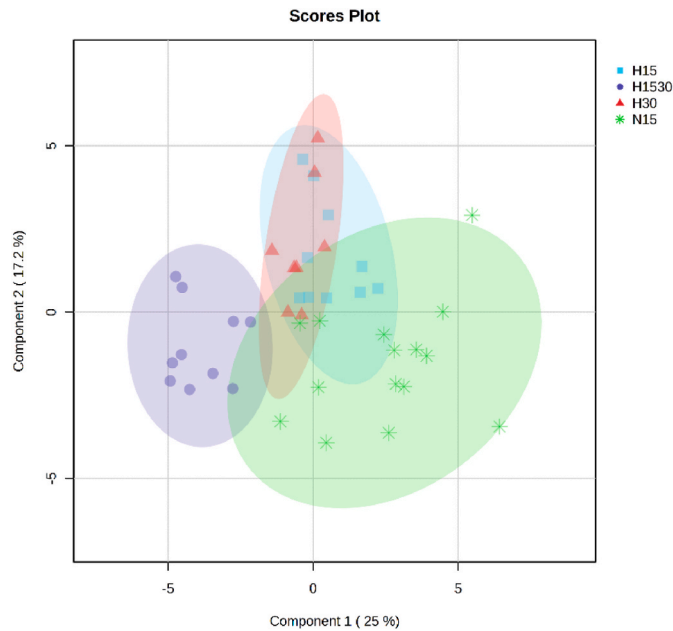


Fig. 3. PLS-DA scores plot: results for the first two components based on LC-MS data of the gill samples. Hypoxia exposure combined with fluctuating temperature (H1530) is separated along the second component from hypoxia exposure with constant normal (H15) or elevated (H30) temperature. Normoxic control (N15) and the hypoxia exposures are separated along the first component.

4. Discussion

4.1. Temperature regime modulates bioenergetic effects of intermittent hypoxia

Oysters can tolerate short hypoxic events without adverse effects on their energy metabolism (Ivanina and Sokolova, 2016; Bayne, 2017b; Haider et al., 2020). Hypoxic survival in oysters and other hypoxia-tolerant facultative anaerobes is commonly associated with metabolic rate suppression (Hochachka, 1986, 1988), which is enhanced in hypometabolic states caused by low temperatures (Gorr, 2017). In our present study, exposure of *C. gigas* to intermittent hypoxia at a low temperature (15 °C) had little impact on tissue bioenergetic parameters. Specifically, there was no change in the electron transport system (ETS) activity, which serves as an index of cellular energy demand, and glycogen was preserved in both gill and digestive gland

tissues. In addition, the level of AMP in the gill tissue remained stable, indicating that the energy status of the cell was not severely compromised (Hardie, 2003). A similar pattern was observed in oysters exposed to intermittent hypoxia at a constant elevated temperature (30 °C), where ETS activity, glycogen reserves, and AMP levels remained stable.

The lack of negative shifts in tissue energy status at 15 °C may be attributed to the combination of cold-induced hypometabolism and the metabolic rate suppression during hypoxic periods, which are typical for intertidal bivalves like oysters (Storey and Storey, 1990; Storey and Churchill, 1995; Samain, 2011; Bayne, 2017d). However, at a constant elevated temperature (30 °C), higher energy expenditure is expected during both hypoxic and recovery phases, which could potentially lead to energy deficiency, particularly since feeding is restricted to the recovery periods. This deficiency may be supplied by the breakdown of glycogen, which serves as the primary energy fuel for anaerobic metabolism during emersion (de Zwaan and Wijsman, 1976; Bayne, 2017c). Despite the energetically challenging conditions of heat combined with intermittent hypoxia, the lack of glycogen loss indicates a low rate of glycogen breakdown during emersion in hypoxia-adapted organisms like oysters (Gäde et al., 1975; Ivanina et al., 2016; Bayne, 2017b; Janas et al., 2017) and/or a rapid glycogen resynthesis during the recovery

Table 2

PLS-DA loadings of selected metabolites (cutoff ± 0.2). Loadings exceeding the cutoff are presented bold.

	C1	C2
Aconitate	0.0387	0.2986
Alanine	0.2649	0.0957
Aspartate	0.0937	0.3305
Citrulline	-0.2613	-0.0460
Glutamine	-0.0914	0.2415
Lysine	-0.0948	0.2394
Taurine	0.2161	0.2550
Tryptophan	0.3039	-0.0667
Tyrosine	0.2987	0.01500
AMP	0.1770	0.2011
Asparagine	-0.0356	0.3414
(Iso)citrate	0.0843	0.3362
Glutamate	0.2670	0.1765
Isoleucine	0.3248	0.0041
Leucine	0.3179	0.0048
Malate	-0.0098	0.2232
Methionine sulfoxide	0.0373	0.2807
Phenylalanine	0.3196	0.0577
Succinate	0.2466	-0.1634
Threonine	0.0483	0.2757
Valine	0.3260	-0.0197

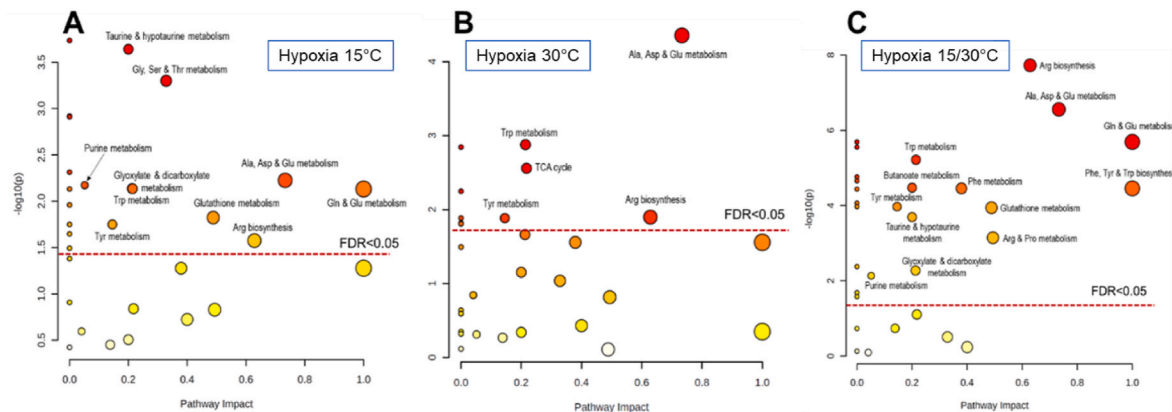


Fig. 4. Pathway enrichment: analyses based on LC-MS data of gill samples. Hypoxia exposure at constant normal (15 °C, A), elevated (30 °C, B) or fluctuating (15/30 °C, C) temperature are compared to normoxic control at 15 °C. Y axis: $\log_{10}(p)$, X axis: pathway impact. Horizontal red dashed line indicates the false discovery rate (FDR) defined as the threshold p value (0.05) after adjustment for multiple comparisons. Symbol size corresponds to the pathway impact, colour shading (yellow to red) reflects the relative significance (with red showing the highest significance).

Table 3

Summary of the significantly enriched pathways in different experimental treatment groups relative to the normoxic controls. “+” indicates significant differences for the respective treatment relative to the control (FDR<0.05). “-” indicates that the certain pathway was not significantly altered in the respective treatment group relative to the control (FDR>0.05). Only pathways with the impact >0 were considered.

	Hypoxia 15 °C	Hypoxia 30 °C	Hypoxia 15/ 30 °C
Alanine, aspartate and glutamate metabolism	+	+	+
Arginine biosynthesis	+	+	+
Tryptophan metabolism	+	+	+
Tyrosine metabolism	+	+	+
D-Glutamine and D-glutamate metabolism	+	-	+
Glutathione metabolism	+	-	+
Glyoxylate and dicarboxylate metabolism	+	-	+
Purine metabolism	+	-	+
Taurine and hypotaurine metabolism	+	-	+
Phenylalanine metabolism	-	-	+
Phenylalanine, tyrosine and tryptophan biosynthesis	-	-	+
Arginine and proline metabolism	-	-	+
Butanoate metabolism	-	-	+
Glycine, serine and threonine metabolism	+	-	-
Citrate cycle (TCA cycle)	-	+	-
Total # of altered pathways	10	5	13

phase (Ouillon et al., 2022). It is worth noting that due to the high mortality (~60%) in co-exposures to constant elevated temperature and hypoxia, the less tolerant individuals (and those that presumably show the largest disruptions of metabolic and energetic bias) were likely those that died first leading to the survivor bias to the more stress-tolerant individuals in our data set. Overall, our findings suggest that oysters are capable of maintaining their energy homeostasis when exposed to intermittent hypoxia at a constant temperature of 15 °C, and a certain fraction of the population is also capable of doing so when co-exposed to intermittent hypoxia at a constant temperature of 30 °C.

In contrast to the unchanged energy status during exposure to intermittent hypoxia at constant temperatures, we detected negative energetic shifts in oysters co-exposed to hypoxia and fluctuating temperature. Aerobic capacity (ETS) and glycogen levels decreased significantly in this group. Glycogen depletion in the digestive gland is especially meaningful, as it is a key storage site for this carbohydrate (de Zwaan and Wijsman, 1976; Bayne, 2017c). These findings indicate higher energy demand during hypoxic episodes in the oysters exposed to the combination of hypoxia and fluctuating temperature relative to other hypoxia-exposed groups. The glycogen used up during hypoxia does not appear to be resynthesized during recovery (possibly due to the low temperature in the normoxic phase) leading to a mismatch between the glycogen utilization and synthesis and a decline in the overall glycogen content of the digestive gland. Further, the AMP decrease in the gills might signal misbalance of the cellular energy status (Hardie, 2003). Alternatively, this decrease may be a result of enhanced synthesis rates of nucleic acids, since AMP is a precursor of RNA (Jauker et al., 2015). Upregulated RNA synthesis would align with the observed protein accumulation in the gills of this exposure group (Bolster et al., 2002; Horman et al., 2002; Chan et al., 2004; Rider, 2016). Taken together, these findings show high energy costs of survival in the fluctuating environments for the oysters, and indicate that upregulation of protein synthesis might contribute to this costs as shown in other organisms in extreme fluctuating environments (Blewett et al., 2022).

4.2. Effects on oxidative stress and protein levels

The impact of intermittent hypoxia on the protein content of oysters tissues varied depending on the tissue and temperature conditions. Protein content of the digestive gland remaining unchanged across all treatments, indicating a balance between protein synthesis and degradation. However, the gill protein content varied in different hypoxia-temperature treatments. Exposure to intermittent hypoxia combined with a constant elevated temperature (30 °C) resulted in gill protein loss accompanied by elevated levels of methionine sulfoxide (MetO), an oxidized form of the amino acid methionine. Both markers indicate proteome damage. Methionine is particularly susceptible to oxidation by reactive oxygen and nitrogen species (Maisonneuve et al., 2009; Lee and Gladyshev, 2011), and thus might be more sensitive marker of oxidative damage than protein carbonyl levels that did not change in any of our experimental treatments. The protein loss and oxidative damage observed in the gill tissue under the combined hypoxia and heat stress may have negative consequences for the function of this vital organ and, ultimately, the survival and performance of the organism (Friedman et al., 1991, 2005; Gouletquer et al., 1998; Bouchet et al., 2007; Samain, 2011). It is worth noting that the observed effects of hypoxia-heat combination may underestimate the physiological stress experienced by oysters under these conditions, as the high mortality observed in this treatment likely resulted in a survivor bias, with only the most resilient individuals being included in the analyses (Ioannidis, 2005).

Unexpectedly, the increase in protein accumulation in the gill of oysters exposed to intermittent hypoxia and fluctuating temperatures indicate that protein synthesis outstrips the degradation. The mechanisms behind this increase are not fully understood. Dehydration and water loss as a reason for protein accumulation (McCarthy et al., 2013) can be excluded since we did not see a corresponding increase in the concentrations of other biomolecules. Instead, it is more likely that the elevated protein content in the gills is due to the de novo synthesis of protective proteins, such as heat shock proteins (HSPs). HSPs accumulate in response to various stressors, including elevated temperatures and hypoxia, and can account for up to 10% of the total protein mass (Finka and Goloubinoff, 2013; Karademir and Sari-Kaplan, 2018). In oysters, a strong upregulation of HSPs has been reported after exposure to hypoxia or temperature stress, suggesting that HSP accumulation may explain the elevated protein content in the gills (David et al., 2005; Ivanina et al., 2009; Kawabe and Yokoyama, 2012). Interestingly, no protein accumulation was observed in the digestive gland, which may reflect its higher hypoxia tolerance and delayed stress response compared to the gills (Oehler et al., 2000; David et al., 2005; Willmer et al., 2005; Kawabe and Yokoyama, 2012).

There was no evidence for the oxidative damage to the membrane lipids or change in total antioxidant capacity induced by the exposures to intermittent hypoxia regardless of the temperature (constant 30 °C not tested due to the lack of samples). Taken together, our data indicate that oxidative stress is not a major contributor to the physiological stress response of *C. gigas* under the studied exposure conditions, consistent with generally high stress tolerance of this species (Samain, 2011; Zhang et al., 2012; Bayne, 2017a).

4.3. Hypoxia-induced shifts in oyster metabolome: amino acid metabolism

Gluconeogenesis was strongly modulated by intermittent hypoxia in oysters as demonstrated by shifts in the Ala, Asp and Glu metabolism. Specifically, certain temperature combinations resulted in increased levels of key amino acids such as Asn, Asp, and Glu, while Gln and Ala showed a decrease. Given that we collected samples after 12 h of normoxic recovery, stimulation of gluconeogenesis might assist in replenishing of glycogen stores used during hypoxia like shown in another hypoxia-tolerant bivalve, the soft-shell clam *Mya arenaria* (Picard et al., 2014; Ouillon et al., 2022). However, glycogen resynthesis was

Table 4

Effects of the experimental treatments on the concentrations of metabolites in the gills of *C. gigas*. Tests: A -ANOVA, KW - Kruskal-Wallis test, W - Welch ANOVA. Experimental groups: N - normoxia, H15 - cyclic hypoxia at 15 °C, H30 - cyclic hypoxia at 30 °C, H15/30 - cyclic hypoxia with immersion at 15 °C and emersion at 30 °C. Different letters in "Post hoc" column indicates significant differences between the treatment groups listed in the order: N – H15 – H30 – H15/30. Methionine sulfoxide data are also presented in Fig. 2. "n" specifies number of replicates.

Metabolite	Test	Statistic	Average, ng/mg $\pm$ standard error				Post hoc
			N	H15	H30	H15/30	
Aconitate	A	F(3,38) = 4.8 p = 0.006	26.41 $\pm$ 3.1 (n = 15)	32.11 $\pm$ 2.6 (n = 9)	47.34 $\pm$ 7.7 (n = 8)	28.11 $\pm$ 2.8 (n = 10)	A-AB-B-A
Alanine	A	F(3,37) = 30.9 p < 0.001	138.99 $\pm$ 10.5 (n = 15)	157.11 $\pm$ 7.1 (n = 10)	75.55 $\pm$ 5.8 (n = 7)	43.78 $\pm$ 7.4 (n = 9)	A-A-B-B
AMP	A	F(3,35) = 5.1 p = 0.005	222.37 $\pm$ 9.6 (n = 12)	207.14 $\pm$ 14.8 (n = 10)	214.79 $\pm$ 11.9 (n = 8)	164.78 $\pm$ 7.3 (n = 9)	AB-ABC-B-C
Arginine	A	F(3,37) = 0.5 p = 0.652	42.53 $\pm$ 3.0 (n = 15)	39.21 $\pm$ 3.6 (n = 9)	44.48 $\pm$ 4.8 (n = 8)	45.27 $\pm$ 1.9 (n = 9)	Not tested
Asparagine	KW	Chi2 = 17.5 P < 0.001	5.13 $\pm$ 0.43 (n = 14)	7.97 $\pm$ 0.86 (n = 10)	9.90 $\pm$ 1.26 (n = 8)	5.85 $\pm$ 0.42 (n = 9)	A-B-B-AB
Aspartate	A	F(3,38) = 14.1 p < 0.001	505.13 $\pm$ 36.7 (n = 15)	615.24 $\pm$ 25.0 (n = 10)	744.48 $\pm$ 55.9 (n = 8)	387.97 $\pm$ 22.5 (n = 9)	AC-AB-B-C
Carnitine	KW	Chi2 = 19.5 P < 0.001	46.44 $\pm$ 3.2 (n = 14)	43.46 $\pm$ 2.0 (n = 8)	85.49 $\pm$ 4.2 (n = 8)	47.43 $\pm$ 2.6 (n = 9)	A-A-B-A
(Iso)citrate	A	F(3,37) = 6.1 p = 0.002	337.11 $\pm$ 48.9 (n = 15)	504.76 $\pm$ 47.3 (n = 9)	546.33 $\pm$ 61.2 (n = 7)	266.73 $\pm$ 40.8 (n = 10)	AC-AB-B-C
Citrulline	KW	Chi2 = 28.3 P < 0.001	0.20 $\pm$ 0.009 (n = 14)	0.27 $\pm$ 0.019 (n = 8)	0.29 $\pm$ 0.015 (n = 8)	2.62 $\pm$ 0.113 (n = 9)	A-A-A-B
Cystine	KW	Chi2 = 9.7 P = 0.021	0.30 $\pm$ 0.040 (n = 15)	0.41 $\pm$ 0.067 (n = 10)	0.28 $\pm$ 0.087 (n = 8)	0.15 $\pm$ 0.030 (n = 9)	AB-A-AB-B
GABA	A	F(3,37) = 1.1 p = 0.379	18.45 $\pm$ 3.9 (n = 14)	26.54 $\pm$ 7.1 (n = 9)	13.45 $\pm$ 4.4 (n = 8)	16.30 $\pm$ 5.0 (n = 10)	Not tested
Glutamate	W	F(3,19.5) = 31.1 p < 0.001	266.95 $\pm$ 17.7 (n = 15)	265.90 $\pm$ 9.9 (n = 9)	258.59 $\pm$ 15.3 (n = 8)	151.34 $\pm$ 8.5 (n = 10)	A-A-A-B
Glutamine	KW	Chi2 = 11.1 P = 0.011	16.82 $\pm$ 1.5 (n = 15)	31.94 $\pm$ 4.5 (n = 10)	20.06 $\pm$ 2.2 (n = 8)	26.42 $\pm$ 4.4 (n = 10)	A-B-AB-AB
Glycine	A	F(3,39) = 6.1 p = 0.002	102.32 $\pm$ 6.9 (n = 15)	145.87 $\pm$ 7.5 (n = 10)	109.93 $\pm$ 8.9 (n = 8)	119.37 $\pm$ 8.1 (n = 10)	A-B-A-AB
GSH	A	F(3,38) = 0.5 p = 0.71	59.2 $\pm$ 5.3 (n = 15)	66.32 $\pm$ 6.7 (n = 10)	68.05 $\pm$ 7.8 (n = 8)	65.85 $\pm$ 4.8 (n = 9)	Not tested
Histidine	A	F(3,34) = 1.6 p = 0.219	5.94 $\pm$ 0.36 (n = 14)	6.59 $\pm$ 0.32 (n = 8)	5.63 $\pm$ 0.40 (n = 8)	5.45 $\pm$ 0.32 (n = 8)	Not tested
Isoleucine	W	F(3,20.8) = 15.5 p < 0.001	10.03 $\pm$ 0.04 (n = 15)	7.86 $\pm$ 0.52 (n = 10)	7.83 $\pm$ 0.54 (n = 8)	4.53 $\pm$ 0.44 (n = 10)	A-A-A-B
Lactate	A	F(3,36) = 1.1 p = 0.358	33.2 $\pm$ 2.4 (n = 13)	30.89 $\pm$ 2.2 (n = 9)	33.15 $\pm$ 3.2 (n = 8)	38.39 $\pm$ 3.8 (n = 10)	Not tested
Leucine	KW	Chi2 = 18 P < 0.001	11.64 $\pm$ 0.93 (n = 15)	8.92 $\pm$ 0.45 (n = 10)	8.54 $\pm$ 0.61 (n = 8)	5.85 $\pm$ 0.64 (n = 10)	A-AB-AB-B
Lysine	W	F(3,17.8) = 4.2 p = 0.021	16.29 $\pm$ 1.5 (n = 15)	31.33 $\pm$ 4.5 (n = 10)	19.31 $\pm$ 2.2 (n = 8)	26.29 $\pm$ 4.4 (n = 10)	A-B-AB-AB
Malate	A	F(3,37) = 5.9 p = 0.002	3980 $\pm$ 436 (n = 14)	5726 $\pm$ 4716 (n = 10)	6270 $\pm$ 412 (n = 8)	3700 $\pm$ 666 (n = 9)	A-AB-B-A
Methionine	KW	Chi2 = 5 P = 0.173	2.14 $\pm$ 0.20 (n = 15)	2.01 $\pm$ 0.24 (n = 10)	2.47 $\pm$ 0.16 (n = 8)	1.84 $\pm$ 0.20 (n = 10)	Not tested
Methionine Sulfoxide	A	F(3,38) = 7.6 p < 0.001	1.51 $\pm$ 0.11 (n = 15)	1.79 $\pm$ 0.19 (n = 10)	2.35 $\pm$ 0.29 (n = 8)	1.20 $\pm$ 0.06 (n = 9)	A-AB-B-A
OH-Proline	A	F(3,36) = 2.5 p = 0.078	2.82 $\pm$ 0.27 (n = 14)	3.91 $\pm$ 0.53 (n = 9)	4.03 $\pm$ 0.27 (n = 8)	4.27 $\pm$ 0.70 (n = 9)	Not tested
Ornithine	A	F(3,36) = 3.1 p = 0.04	6.62 $\pm$ 0.44 (n = 14)	5.25 $\pm$ 0.45 (n = 9)	6.78 $\pm$ 0.28 (n = 7)	5.62 $\pm$ 0.33 (n = 10)	Not tested
Phenylalanine	W	F(3,21.1) = 17.3 p < 0.001	11.04 $\pm$ 0.81 (n = 15)	9.72 $\pm$ 0.51 (n = 10)	8.97 $\pm$ 0.46 (n = 8)	5.79 $\pm$ 0.43 (n = 10)	A-A-A-B
Proline	KW	Chi2 = 25.4 P < 0.001	77.75 $\pm$ 13.3 (n = 15)	122.75 $\pm$ 9.2 (n = 9)	17.32 $\pm$ 2.4 (n = 7)	27.03 $\pm$ 7.1 (n = 8)	A-A-B-B
Serine	A	F(3,38) = 6.7 p = 0.001	13.73 $\pm$ 0.9 (n = 15)	14.97 $\pm$ 0.8 (n = 9)	18.15 $\pm$ 1.8 (n = 8)	21.53 $\pm$ 2.0 (n = 14)	A-A-AB-B
Succinate	W	F(3,15.5) = 13.8 p < 0.001	61.13 $\pm$ 11.69 (n = 14)	37.07 $\pm$ 1.54 (n = 10)	8.24 $\pm$ 1.83 (n = 8)	2.58 $\pm$ 0.33 (n = 9)	A-B-BC-C
Taurine	A	F(3,33) = 57.4 p < 0.001	9059 $\pm$ 270 (n = 12)	11796 $\pm$ 119 (n = 7)	8339 $\pm$ 410 (n = 8)	6143 $\pm$ 262 (n = 10)	A-B-A-C
Threonine	A	F(3,37) = 3.2 p = 0.035	10.38 $\pm$ 0.6 (n = 14)	14.61 $\pm$ 1.2 (n = 10)	13.76 $\pm$ 1.9 (n = 7)	11.09 $\pm$ 1.4 (n = 10)	Not tested
Tryptophan	W	F(3,19.2) = 20 p < 0.001	1.61 $\pm$ 0.139 (n = 15)	1.03 $\pm$ 0.117 (n = 10)	0.87 $\pm$ 0.067 (n = 8)	0.59 $\pm$ 0.038 (n = 10)	A-B-B-C
Tyrosine	W	F(3,21.3) = 9.1 p < 0.001	10.17 $\pm$ 0.85 (n = 15)	7.27 $\pm$ 0.52 (n = 10)	6.86 $\pm$ 0.43 (n = 8)	4.83 $\pm$ 0.56 (n = 10)	A-B-B-C
Valine	W	F(3,18.7) = 13 p < 0.001	10.67 $\pm$ 0.74 (n = 15)	8.29 $\pm$ 0.40 (n = 10)	7.92 $\pm$ 0.12 (n = 6)	5.22 $\pm$ 0.52 (n = 10)	A-B-B-C

insufficient to fully compensate for the breakdown in the digestive gland of the oysters exposed to hypoxia-heat combination. Apart from that, our findings agree with the earlier studies that report enhanced carbohydrate metabolism during hypoxia in human endothelial cells (Oehler et al., 2000), polychaetes (Kamp and Juretschke, 1989) and bivalves (Isani et al., 1989; Greenway and Storey, 1999).

Arginine biosynthesis was modulated in all exposure groups demonstrated by shifts of the key amino acids Asp, Gln, Glu and citrulline. A modulation of urea cycle is unlikely as urea contributes only a small fraction of the total nitrogen excretion in oysters (Bayne, 2017b). Therefore, alteration of Arg biosynthesis under hypoxic conditions might reflect other functions of the pathway intermediates such as the role synthesis of phosphagens (phospho-L-arginine) or signaling functions. Interestingly, we detected a massive increase (12.8 times) of citrulline in oysters exposed to hypoxia and fluctuating temperature compared to the normoxic controls. In *C. gigas*, the conversion of Arg to citrulline via nitric oxide synthase forms nitric oxide radicals (NO $\cdot$) which acts as an important signaling molecule for metabolic regulation, immune response, and inflammation in different animals (Jacklet, 1997; Scholz and Truman, 2000; Hermes-Lima, 2004; Storey, 2004; Lambert et al., 2007; Swamy et al., 2010). Nitric oxide is involved in metabolic

suppression and cytoprotection during hypoxia in bivalves (Strahl and Abele, 2020) and vertebrates (Fu et al., 2013; Heidorn et al., 2018), respectively. Thus, the high citrulline accumulation observed in our study might reflect elevated NO production (Johansson and Carlberg, 1995) to support metabolic arrest during the hypoxic phase and provide cellular protection during the recovery. The lack of citrulline accumulation in the oysters exposed to hypoxia under constant temperature regime indicates that unlike the fluctuating temperature and hypoxia combinations that mimic summer tidal conditions, less realistic scenarios of intermittent hypoxia at constant temperature might not provide all the necessary cues to trigger this physiological response in oysters.

Gill tissue levels of essential branched-chain amino acids (leucine, isoleucine and valine; BCAAs) and aromatic amino acids (phenylalanine, tyrosine, tryptophan; AAAs) decreased in all hypoxia exposures in the oysters (Manahan, 1990; Fitzgerald and Szmant, 1997). BCAAs are critical for cellular metabolism, growth, and stress signaling, as well as stimulating protein synthesis and inhibiting proteolysis, which aids protein deposition (De Bandt and Cynober, 2006; Holeček, 2018; Nambara et al., 1998). Aromatic amino acids (AAAs) play a crucial role in protein synthesis (Han et al., 2019; Li et al., 2021, 2023) and serve as

precursors for signaling molecules including hormones and neurotransmitters (Kutchko and Siltberg-Liberles, 2013). Our data do not allow determining whether the observed decline in BCAAs and AAAs are due to their impaired uptake from the food, increased incorporation into the proteins or elevated catabolism for energy during hypoxia (Willmer et al., 2005; Haider et al., 2019). Regardless of the underlying mechanisms, the decrease in the tissue levels of these important amino acids might have negative consequences for protein synthesis, and endocrine and neural function of oysters (Kutchko and Siltberg-Liberles, 2013; Adeva-Andany et al., 2017).

4.4. Temperature modulates TCA cycle in combined exposures

PLS-DA analysis showed significant shifts in the TCA cycle metabolite profiles along the 2nd component that could be assigned to temperature effects. Further, pathway enrichment analysis identified the TCA cycle as a pathway significantly altered in the oysters co-exposed to intermittent hypoxia with constant elevated temperature. Here, three of the measured key metabolites of this pathway (aconitate, (iso)citrate and malate) increased in the oyster gills. These findings indicate activation of TCA cycle during temperature stress, possibly to support the elevated energy demand or biosynthetic needs of the cells. Furthermore, TCA cycle metabolites are important for retrograde signaling between the mitochondria and other cell components (Martínez-Reyes and Chandel, 2020) and regulation of the immune response (reviewed in Choi et al., 2021). Accumulation of the TCA metabolites might therefore signal the heat-induced mitochondrial damage (Choi et al., 2021) and stimulate immune response and other cellular protective mechanisms as already reported for other invertebrates (Wang et al., 2008; Matozzo and Marin, 2011; Catalán et al., 2012; Dang et al., 2012; Applebaum et al., 2014; Bayne, 2017d). Further investigations are needed to determine whether TCA-related signaling plays a role in this immune activation. Notably, exposure to fluctuating temperature did not lead to accumulation of TCA cycle intermediates in hypoxia-exposed oysters indicating milder mitochondrial stress in this environmentally relevant exposure scenario.

The only studied TCA metabolite that consistently responded to intermittent hypoxia rather than temperature stress, was succinate, which was consistently depleted in all hypoxia-exposed groups compared to the normoxic baseline. Succinate is a common anaerobic end product that accumulates during hypoxia in marine bivalves (Schiedek, 1997a, 1997b; Storey, 2004; Bayne, 2017b; Haider et al., 2020) and is rapidly depleted during recovery (Ouillon et al., 2021, 2022). This depletion often demonstrates overshoot with succinate levels decreasing below the normoxic baseline (Ouillon et al., 2021, 2022), similar to the pattern found in our present study. This might reflect overactivation of respiration (the so called oxygen debt) commonly observed after hypoxia and interpreted as the metabolic costs of restoring cellular and systemic homeostasis disrupted by hypoxia (Herreid, 1980; Ellington, 1983). This hypothesis is supported by the enhanced mitochondrial ability to oxidize succinate during post-hypoxic recovery in *C. gigas* (Adzighli et al., 2022) and might be an adaptive mechanism that allows this hypoxia-tolerant species to use accumulated anaerobic end-product (succinate) as a mitochondrial fuel to rapidly restore ATP levels.

4.5. Taurine metabolism is modified by temperature and hypoxia

Loadings of taurine were above the threshold for the two first PLS-DA components, indicating combined effects of hypoxia and temperature on this metabolite. Pathway enrichment analysis also identified taurine and hypotaurine metabolism as a significantly modulated pathway in hypoxia exposures combined with normal or fluctuating temperature involving an increase or decrease in taurine content, respectively. In bivalves including oysters, taurine acts as an important compatible osmolyte (Yancey, 2005). Furthermore, taurine enhances mitochondrial

performance and mitigates the mitochondrial ROS production in bivalves and other organisms (Hansen et al., 2006; Jong et al., 2012; Bin et al., 2017; Sokolov and Sokolova, 2019). In cross-species comparisons of bivalves, high taurine levels were associated with higher tolerance to hypoxia-reoxygenation stress (Haider et al., 2020). Depletion of taurine might therefore partially explain the lower mitochondrial performance (shown by suppressed ETS activity levels) in oysters exposed to intermittent hypoxia combined with fluctuating temperature observed in our present study. No decrease in ETS activity was found in the other two experimental groups that maintained normal or elevated taurine concentrations. Overall, our present study and earlier published research indicate that *C. gigas* effectively maintain taurine homeostasis with only mild variation under most stress scenarios (Hummel et al., 1996; Kube et al., 2007; Haider et al., 2020). This ability might contribute to exceptionally high stress tolerance of *C. gigas* (Zhang et al., 2012; Bayne, 2017a) and requires further investigation.

5. Conclusion and outlook

Our present study demonstrated that intermittent hypoxia combined with fluctuating temperature caused metabolic shifts in *C. gigas*, which was accompanied by depletion of energy reserves. Studies show that 30 °C is the upper thermal limit of *C. gigas* (Le Gall and Raillard, 1988; Bougrier et al., 1995), albeit submerged North Sea oysters can survive this temperature for at least three days without mortality (Bruhns, unpublished data). However, the combination of this high temperature with cyclic hypoxia exceeded the oysters' stress tolerance leading to tissue damage and high mortality. In contrast, exposure to cyclic hypoxia at 15 °C caused metabolic shifts in different pathways in the Pacific oyster but no negative changes in bioenergetics or oxidative stress. This study highlights the importance of investigating combined stressors affecting metabolic modulation caused by different combinations of stress intensities or modes. In general, we showed that *C. gigas* is quite robust against natural stress conditions but gains this robustness from energetically costly adaptations. Further studies are needed to test the putative mechanisms of phenotypic plasticity and their role in stress adaptation in oysters, like the role of HSPs synthesis in the protein accumulation in the gills, the implications of the altered BCAAs and AAAs metabolism in endocrine signaling and proteome maintenance, and the role of NO as a metabolic regulator during fluctuating temperature and oxygen regimes.

Authors' statement

Conceptualization – IMS, TB
 Methodology – TB, ST, IMS
 Validation – TB, ST
 Formal analysis – TB
 Investigation – TB, ST, NF, SE
 Resources – IMS, ST
 Data Curation – TB, ST
 Writing - Original Draft - TB
 Writing - Review & Editing – TB, ST, NF, SE, ML, MW, IMS
 Visualization – TB, IMS
 Supervision – IMS, ML, MW
 Project administration – IMS, ML, MW
 Funding acquisition – IMS, ML, MW

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2023.106231>.

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II.2 Metabolomics-based assessment of nanoparticles (nZnO) toxicity in an infaunal marine annelid, the lugworm *Arenicola marina* (Annelida: Sedentaria)

Contribution letter

The material of the manuscript titled “**Metabolomics-based assessment of nanoparticles (nZnO) toxicity in an infaunal marine annelid, the lugworm *Arenicola marina* (Annelida: Sedentaria)**” was published in 2023 in the journal *Science of The Total Environment*.

My work was a major contribution to this manuscript. I was involved in the experimental design and planning, maintenance of the experimental animals, setup and execution of the experimental exposures, tissue sampling and the determination of oxidative stress (lipid peroxidation and protein carbonylation) markers. Determination of the metabolites was done in collaboration with Dr. Stefan Timm where I carried out the initial extraction of the metabolites. The statistical and graphical analysis of the gained data as well as the writing of the first draft was done in collaboration with Prof. Dr. Inna Sokolova. I focused on the analysis of the oxidative stress markers as well as selected metabolites. Further, I wrote the material and methods section and further edited the complete draft. I contributed to the revision of the draft and to the responses to the reviewers during the submission process.

Based on the CRediT taxonomy (Brand et al., 2015), my contributions to this manuscript were as follows:

- Conceptualization
- Methodology
- Validation
- Formal analysis
- Investigation
- Data Curation
- Writing - Original Draft
- Writing - Review & Editing
- Visualization

Signatures of consent

Prof. Dr. Inna Sokolova

(Supervisor)

Torben Bruhns

(PhD candidate)

Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J., 2015. Beyond authorship: attribution, contribution, collaboration, and credit. Learn. Publ. 28, 151–155. <https://doi.org/10.1087/20150211>



Metabolomics-based assessment of nanoparticles (nZnO) toxicity in an infaunal marine annelid, the lugworm *Arenicola marina* (Annelida: Sedentaria)

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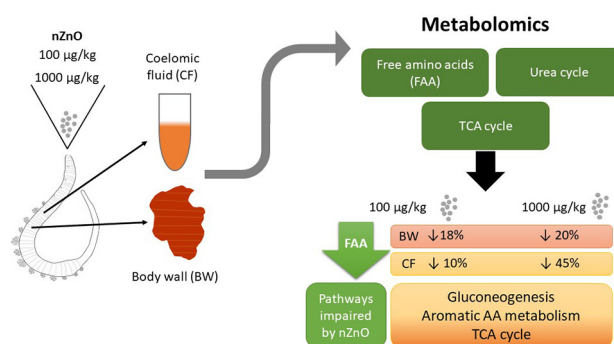
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HIGHLIGHTS

- The lugworms *A. marina* were exposed to 100 and 1000 $\mu\text{g kg}^{-1}$ nZnO in sediment.
- nZnO exposure led to oxidative stress and metabolic disruption.
- The affected pathways involve gluconeogenesis, metabolism of aromatic amino acids and Krebs cycle.
- Amino acid levels in the coelomic fluid decreased concentration-dependently with nZnO.
- Depletion of amino acids pool by nZnO might impair osmoregulation and protein synthesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Nanopollutants such as nZnO gain importance as contaminants of emerging concern due to their high production volume and potential toxicity. Coastal sediments serve as sinks for nanoparticles but the impacts and the toxicity mechanisms of nZnO in sediment-dwelling organisms are not well understood. We used metabolomics to assess the effects of nZnO-contaminated sediments on a benthic ecosystem engineer, an infaunal polychaete *Arenicola marina*. The worms were exposed to unpolluted (control) sediment or to the sediment spiked with 100 or 1000 $\mu\text{g Zn kg}^{-1}$ of nZnO. Oxidative lesions (lipid peroxidation and protein carbonyls) were measured in the body wall as traditional biomarkers of nanopollutant toxicity. Metabolite profiles (including amino acids, tricarboxylic acid (TCA) cycle and urea cycle intermediates) were determined in the body wall and the coelomic fluid. Exposure to nZnO altered metabolism of the lugworms via suppression of the metabolism of gluconeogenic and aromatic amino acids, and altered the TCA cycle likely via suppression of fumarate activity. These metabolic changes may negatively affect carbohydrate metabolism and energy storage, and impair hormonal signaling in the worms. The total pool of free amino acids was depleted in nZnO exposures with potentially negative consequences for osmoregulation and protein synthesis. Exposure to nZnO led to accumulation of the lipid peroxidation products demonstrating high susceptibility of the cellular membranes to nZnO-induced oxidative stress. The nZnO-induced shifts in the metabolite profiles were more pronounced in the coelomic fluid than the body wall. This finding emphasizes the important metabolic role of the coelomic fluid as well as its suitability for assessing the toxic impacts of nZnO and other metabolic disruptors. The metabolic disruptions caused by environmentally relevant concentrations of nZnO can have negative effects on the organisms' fitness impairing growth and reproduction of the populations of marine bioturbators like the lugworms in nanoparticle-polluted sediments.

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1. Introduction

Engineered nanoparticles are widely used in industries, medicine and consumer products owing to their desirable novel properties that emerge as a result of size scaling (<100 nm in at least one dimension). The use of nanomaterials is increasing worldwide and results in increasing environmental loads due to the outdoor applications, unintentional release and improper wastewater treatment (Bundschuh et al., 2018). ZnO nanoparticles (nZnO) have the third largest production volume of all nanoparticles (Bondarenko et al., 2013) and a predicted global market growth of >13 % in 2022–2031 (Patwardhan et al., 2022). The high production volume and the broad use of nZnO results in the release of this nanomaterial into the environment raising concerns about its ecological toxicity (Blasco et al., 2020; Bundschuh et al., 2018; Yuan et al., 2022). Coastal marine ecosystems are major sinks for nanopollutants that accumulate in the water and sediments. Models predict the environmental concentrations of nZnO ranging from hundreds nanograms per liter up to 900 $\mu\text{g l}^{-1}$ nZnO in the surface waters, >100 $\mu\text{g kg}^{-1}$ in the sediments, and up to 65 mg kg^{-1} in the sewage sludge (Coll et al., 2016; Gottschalk et al., 2009; Yung et al., 2014). The currently predicted environmental concentrations of nZnO are well below LC_{50} for most aquatic organisms and, thus, are not expected to cause acute toxicity (except in the areas directly exposed to the sewage effluents) (Coll et al., 2016; Gottschalk et al., 2009). However, exposures to even low environmentally relevant nZnO concentrations can have negative effects on immunity, bioenergetics and redox status of marine organisms (Li et al., 2018; Mouneyrac et al., 2014; Noor et al., 2021; Vieira Sanches et al., 2021; Yung et al., 2015). These effects can weaken marine organisms making them more susceptible to disease or to abiotic stressors such as warming, acidification or salinity fluctuations and result in long-term negative effects on growth and performance of their populations (Noor et al., 2021; Wu et al., 2020; Wu et al., 2021a; Wu et al., 2022; Wu and Sokolova, 2021).

Understanding of the toxicity mechanisms and effects of nZnO in marine organisms remains challenging, partially owing to the lack of specific biomarkers for toxicity assessments (Falfushynska et al., 2022; Luo et al., 2020). Recently, non-targeted approaches such as transcriptomics, proteomics and metabolomics have been proposed for gaining mechanistic insights into the intracellular targets and modes of action of emerging pollutants including nanoparticles (Bannuscher et al., 2020; Canzler et al., 2020). Toxicometabolomics (i.e. simultaneous profiling of multiple metabolites in response to a toxic perturbation) is a rapidly growing field in environmental toxicology (Araújo et al., 2021; Schnackenberg et al., 2012). Toxicometabolomics can help identify new biomarkers for exposure and effects of nanopollutants (Araújo et al., 2021; Schnackenberg et al., 2012). This approach has been successfully used to determine the mechanisms of nanoparticle toxicity in model organisms and mammalian cell cultures (Cui et al., 2019; Jarak et al., 2017; Kumar Babele, 2019; Ratnasekhar et al., 2015) but its use in ecotoxicological research is still limited.

We used metabolomics to assess the metabolic impacts of environmentally relevant concentrations of nZnO on a sediment-dwelling marine annelid, the lugworm *Arenicola marina*. *A. marina* are common benthic species found worldwide in the temperate soft-bottom ecosystems. The lugworms are ecosystem engineers that play a key role in sediment reworking, nutrient, oxygen and water fluxes and biogeochemical cycles in the sediment (Volkenborn, 2006). They build deep U-shaped burrows in the sediments and feed by ingesting the sediment and extracting organic matter from it (Longbottom, 1970). This behavior makes *A. marina* susceptible to the nanoparticle exposure through a direct contact with contaminated sediment and the dietary consumption (Besseling et al., 2017; Browne et al., 2013; Campbell et al., 2014; Koelmans et al., 2014). Here we determined the effects of the prolonged (2 weeks) exposure to nZnO-contaminated sediments (100 and 1000 $\mu\text{g Zn kg}^{-1}$) on the oxidative stress markers (protein carbonyls and lipid peroxidation products) and the metabolite profiles of the lugworm *A. marina*. We focused on the free amino acids (FAAs) and intermediary metabolites in the bioenergetics (tricarboxylic acid cycle) and excretory (urea cycle) pathways to obtain a comprehensive insight into

the nZnO-induced shifts in the metabolism. Our findings show metabolic disturbances caused by the environmentally relevant nZnO concentrations with implications for amino acid homeostasis, osmoregulation and carbohydrate metabolism and indicate that metabolomics assessments can provide sensitive early-warning markers to assess the sublethal toxic effects of nanopollutants.

2. Materials and methods

2.1. Animal maintenance and experimental exposures

A. marina obtained from a local commercial supplier (Angelcenter Bastian, Rostock, Germany). The lugworms were collected in September 2019 from the intertidal zone of southern Netherlands and delivered to Rostock within three days of collection. The worms were immediately put into the sediment- and seawater-filled experimental mesocosms at salinity 24 and 15 °C. These conditions were similar to the habitat conditions at the time of collection as reported by the supplier. Seawater for animal maintenance, sediment preparation and experimental treatments was made of the natural filtered Baltic Sea water adjusted to salinity 24 with artificial sea salt (Pro-REEF Sea Salt, Tropic Marin). The water was continuously aerated. Three exposure treatments were conducted: control (no nZnO addition), low nZnO concentration (100 $\mu\text{g Zn kg}^{-1}$ sediment) and high nZnO concentration (1000 $\mu\text{g Zn kg}^{-1}$ sediment).

Sediment was collected in September 2019 at approximately 1.5 m depth near Heiligendamm (Germany) and sieved through 1 mm mesh to remove macrofauna and larger particles. The sieved sediment was thoroughly mixed and used to set up mesocosms for the three experimental treatments (control, low and high nZnO). The average water content of the sediment was 21.1 %, and the density was 1791 g l^{-1} wet mass. This sediment contained high fraction (>96 %) of grain sizes between 125 and 500 μm preferred by *A. marina* (Andresen and Kristensen, 2002). For nZnO exposures, the sediment was spiked with nZnO to achieve the nominal concentrations of 100 or 1000 $\mu\text{g Zn per kg dry sediment mass}$. Commercial ZnO nanoparticles (average size 30 nm, Sigma-Aldrich) were thoroughly mixed with the sediment for two days using an electrically powered mixing device under a thin layer of seawater at salinity 24 and 15 °C. Earlier studies showed that nZnO is stable and shows minimal dissolution in seawater within three weeks (Wu et al., 2020). Sediment without nZnO addition was used for control treatments. In this study, we report nominal concentrations of nZnO because direct detection of nanoparticles is technically difficult, and the measurements of sediment Zn concentrations cannot detect low exposure concentrations of nZnO against the high (mg kg^{-1}) natural Zn background of the Baltic Sea sediments (Shahabi-Ghahfarokhi et al., 2021). Twelve mesocosms (four replicates for each of the control and two nZnO exposures) were prepared. Each mesocosm (23 × 15 × 30 cm L x W x H) was filled with the sediment to a depth of 20 cm and incubated for two weeks under seawater (salinity 24) 15 °C without animals. Chloramphenicol (25 mg l^{-1}) was added to the seawater during sediment incubation and throughout all experimental exposures to minimize bacterial growth. Five worms were added to each experimental mesocosm. Only healthy, active individuals capable of digging into the sediment within 30 min were used for the study. *A. marina* were exposed in the control or nZnO-spiked sediments for two weeks. The water was exchanged in all mesocosms at least once a week, or every time if any signs of excessive bacterial growth were observed in at least one mesocosm. Chloramphenicol was added during every water change.

After two weeks of exposure, worms were carefully removed from the sediment and dissected on ice with an anteroposterior cut. The leaching coelomic fluid was collected individually in microcentrifuge tubes and shock-frozen in liquid nitrogen. The tail and the internal organs were removed, and the body wall of the anterior body (including head, thorax and abdomen) blotted dry and shock-frozen in liquid nitrogen. The coelomic fluid and the body wall tissues were stored at −80 °C until further analysis. Pilot analysis showed no significant variation in the studied traits between mesocosms within each treatment ($p > 0.05$; data not shown);

therefore, data on different mesocosms were pooled and individual worms treated as biological replicates. Sample size was 8 for the oxidative stress markers and 20 for metabolites.

2.2. Oxidative stress markers

For determination of protein carbonyl content, 200–300 mg of the body wall tissue were homogenized with a Potter-Elvehjem homogenizer in 1 ml of the ice-cold homogenization buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 125 mM KCl and 5.7 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was incubated for 30 min on ice and centrifuged for 15 min at $10000 \times g$ and 4°C . Protein content of the samples was determined using the Bradford assay (Bradford, 1976) and bovine serum albumin as a standard. Protein carbonyl content was measured in the supernatant at 380 nm (SpectraMax iD3, Molecular Devices, USA) using the 2,4-dinitrophenyl hydrazine (DNPH) method as described elsewhere (Levine et al., 2000). For each sample an individual non-DNPH treated blank was subtracted. Protein carbonyl content was calculated from the blank-corrected absorbance of the samples using the extinction coefficient $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{mol g}^{-1}$ protein.

Lipid peroxidation products were measured in the body wall tissue as described previously (Hermes-Lima et al., 1995). The homogenate was prepared using ~ 50 mg of the body wall tissue in $450 \mu\text{l}$ ice-cold methanol and centrifuged for 5 min at $10000 \times g$ and 4°C . An aliquot of the supernatant was incubated for 20 h in an assay solution containing 0.25 mM FeSO_4 , 25 mM H_2SO_4 and 0.1 mM xylene orange. The absorbance was measured at 580 nm, $5 \mu\text{M}$ cumene hydroperoxide was added to each sample, incubated for 40 min and the absorbance at 580 nm was measured again. Lipid peroxides content was calculated from the change in absorbance due to cumene hydroperoxide addition and expressed as $\mu\text{mol g}^{-1}$ wet mass. Sample size for oxidative stress markers was 8 per group.

2.3. Metabolite extraction and measurements

Metabolites were extracted from approximately 1 ml body fluids or 100 mg body wall homogenized in 1 ml ice-cold 80 % ethanol with $1 \mu\text{g ml}^{-1}$ 2-(N-morpholino)ethanesulfonic acid (MES) as an internal standard. All samples were obtained from individual worms. Samples were centrifuged for 10 min at $13000 \times g$ and 4°C to remove debris. The supernatant was freeze-dried using a cold trap (Unicryo MC 2 L, UniEquip, Germany) and stored at -80°C until further processing. The extracts were dissolved in LC-MS-grade water (ROTISOLV, Carl Roth) and sterile-filtered ($0.2 \mu\text{m}$) (Omnifix-F, Braun, Germany). The high-performance liquid chromatograph-mass spectrometer LCMS-8050 (Shimadzu) was used for measurements as described earlier (Haider et al., 2020). For metabolite identification and quantification the built-in LC-MS/MS package for primary metabolites (Vers. 2, Shimadzu, P/N 225–24,862-92) and the LabSolutions software package (Shimadzu) was used. The calibration was done using metabolite-specific standards from Merck and corrected for sample loading using the peak of the internal standard (2-(N-Morpholino)ethanesulfonic acid). In total, 32 metabolites including amino acids, tricarboxylic acid (TCA) cycle and urea cycle intermediates, adenosine monophosphate (AMP), carnitine and γ -aminobutyric acid (GABA) were measured in the body wall extracts and the coelomic fluid of *A. marina* (Supplementary Tables 1 and 2). Citrate and isocitrate cannot be distinguished with the method used here, and the concentrations of these metabolites are reported together. Sample sizes for metabolite analyses were 20 for the body wall and 18–19 for the coelomic fluid.

2.4. Statistics

Data distribution and variance were analyzed using a normal probability plot and Bartlett's test sensitive to the deviations from both normality and homoscedasticity. Outliers (no >1 –2 values per group laying outside 1.5 times of the interquartile range) were removed. Data that met the

assumptions of normality and homoscedasticity were analyzed using one-way ANOVA (with nZnO exposure as a fixed factor with three levels) followed by Tukey's Honest Significant Differences test for post-hoc comparisons of the group means. If ANOVA assumption(s) were violated, non-parametric Kruskal-Wallis test and Dunn's test for multiple comparisons were used. The analyses were conducted in GraphPad Prism v. 7.02 (GraphPad Software Inc., La Jolla, CA, USA). Factor effects and differences between the means were considered significant if the probability of Type II error was <0.05 .

The impacts of nZnO exposure on metabolism were analyzed using the pathway enrichment analysis as implemented in Metaboanalyst 5.0 (Pang et al., 2021) using the compound concentrations for the body wall tissue and the coelomic fluid. This analysis has high sensitivity and can identify subtle but consistent changes across compounds involved in the same biological pathway (Pang et al., 2021). The regression approach was used to integrate metabolic changes under different nZnO exposure concentrations (0, 100 and $1000 \mu\text{g Zn kg}^{-1}$ sediment). To avoid issues with handling zeroes in the regression analyses, 0.01 was added to all exposure concentrations. The citrate and isocitrate concentrations were treated together as citrate. Metabolic reference library of *Drosophila melanogaster* was used, with the Globaltest as the method of pathway enrichment analysis. The relative betweenness centrality was used as a node importance measure in the pathway topology analyses, and the concentrations of the compounds were auto scaled using means and standard deviations for each compound. The pathway was considered significantly enriched if the probability of the false discovery rate (FDR) was <0.05 . Significantly enriched pathways (FDR < 0.05) with the pathway impact >0 were considered in the further analysis and discussion. The correlation analysis and metabolite clustering for the body wall and the coelomic fluid were conducted in Metaboanalyst 5.0 (Pang et al., 2021).

3. Results

3.1. Oxidative stress markers

Concentrations of the lipid peroxidation products in the body wall of *A. marina* increased in the concentration-dependent manner with nZnO exposure (Fig. 1A). There was no evidence for the effects of nZnO exposure on the concentrations of protein carbonyls in the body wall (Fig. 1B) or methionine sulfoxide in the body wall and coelomic fluid (Supplementary Tables 1 and 2).

3.2. Free amino acid profiles

In the body wall of *A. marina*, Gly was a dominant FAA (76–77 % of the total pool), followed by Ala (11–12 %), Glu (3 %) and Tau (2–3 % of the total pool) (Fig. 2). All other FAA constituted <2 % each of the total FAA pool. All studied FAA found in the body wall of *A. marina* were also detected in the coelomic fluid (Fig. 2) except for taurine that was relatively abundant in the body wall (1.5 – $2.0 \mu\text{mol g}^{-1}$) but essentially absent from the coelomic fluid. Like the body wall, Gly was the most abundant FAA in the coelomic fluid representing 26–33 % of the total FAA pool (Fig. 2). The coelomic fluid also contained relatively high concentrations of Asp and Ala (8–10 % of the pool), Arg (7 %), Glu (5–7 %) and Val (5 % of the total FAA pool). The relative abundance of FAA in the coelomic fluid was more balanced resulting in a more diverse FAA pool than in the body wall ($H' = 2.5$ – 2.6 in the coelomic fluid compared with $H' = 0.95$ – 0.99 in the body wall).

Concentrations of most FAA in the body wall of *A. marina* remained stable during nZnO exposures (Supplementary Table 1). The only exceptions were the tissue levels of Ala, Asp, Gln, Gly and Lys that showed significant decrease in the body wall of the lugworms exposed to $100 \mu\text{g Zn kg}^{-1}$ sediment (Asp), $1000 \mu\text{g Zn kg}^{-1}$ sediment (Gln, Lys), or in both nZnO exposure groups (Ala, Gly) (Fig. 3A–E). As a result, the total tissue content of FAA decreased by 18–20 % in the nZnO-exposed groups compared with the control worms (Fig. 3F).

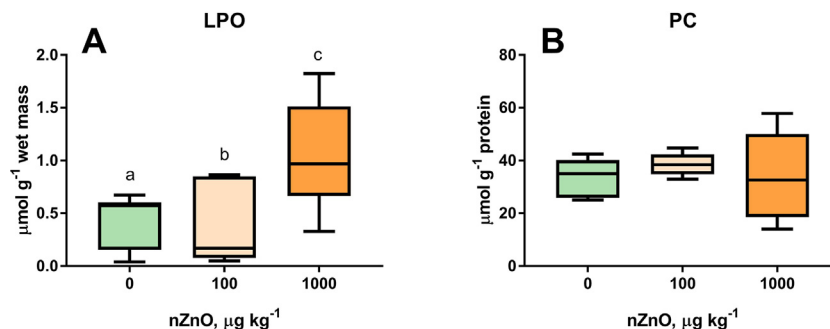


Fig. 1. Tissue levels of oxidative stress markers in the body wall of *A. marina* exposed to different nZnO concentrations (0, 100 or 1000 $\mu\text{g Zn kg}^{-1}$ sediment). (A) – lipid peroxidation products, (B) – protein carbonyls. Boxplots that do not share a letter are significantly different ($p < 0.05$). Absence of letters shows that no groups were significantly different ($p > 0.05$). $N = 6-8$.

In the coelomic fluid, concentrations of most FAAs decreased in nZnO-exposed *A. marina* relative to the control group (Figs. 4, 5). Thus, the lugworms exposed to the low concentration of nZnO (100 $\mu\text{g Zn kg}^{-1}$ sediment) showed lower levels of six FAAs (Lys, Asp, Ala, Gln, Gly and Glu) (Figs. 4C, 5B, C, D, E, G). In the coelomic fluid of the lugworms exposed to the high nZnO concentration (1000 $\mu\text{g Zn kg}^{-1}$ sediment), tissue levels of 15 FAAs (His, Lys, Phe, Val, Pro, Ile, Leu, Asp, Ala, Gln, Gly, Ser, Glu, Met and Tyr) decreased (Figs. 4B–D, G–J, 5B–I). As a result, the total concentration of FAA in the coelomic fluid decreased by 10 % and 45 % in the worms exposed to the low and high concentrations of nZnO, respectively (Fig. 5J).

3.3. TCA cycle intermediates

Of the five studied TCA intermediates (malate, (iso-)citrate, 2-oxoglutarate, succinate and fumarate), all five were detected in the body wall but only three (malate, (iso-)citrate, and succinate) were found in the coelomic fluid of *A. marina*. Exposure to 100 $\mu\text{g Zn kg}^{-1}$ sediment led to a decrease in the malate concentration in the body wall of *A. marina* (Fig. 6A) and elevated succinate levels in the coelomic fluid (Fig. 7C). Exposure to 1000 $\mu\text{g Zn kg}^{-1}$ sediment led to a decrease in the levels of 2-oxoglutarate and succinate and accumulation of fumarate in the body wall of *A. marina* (Fig. 6C–E). Concentrations of a neurotransmitter GABA

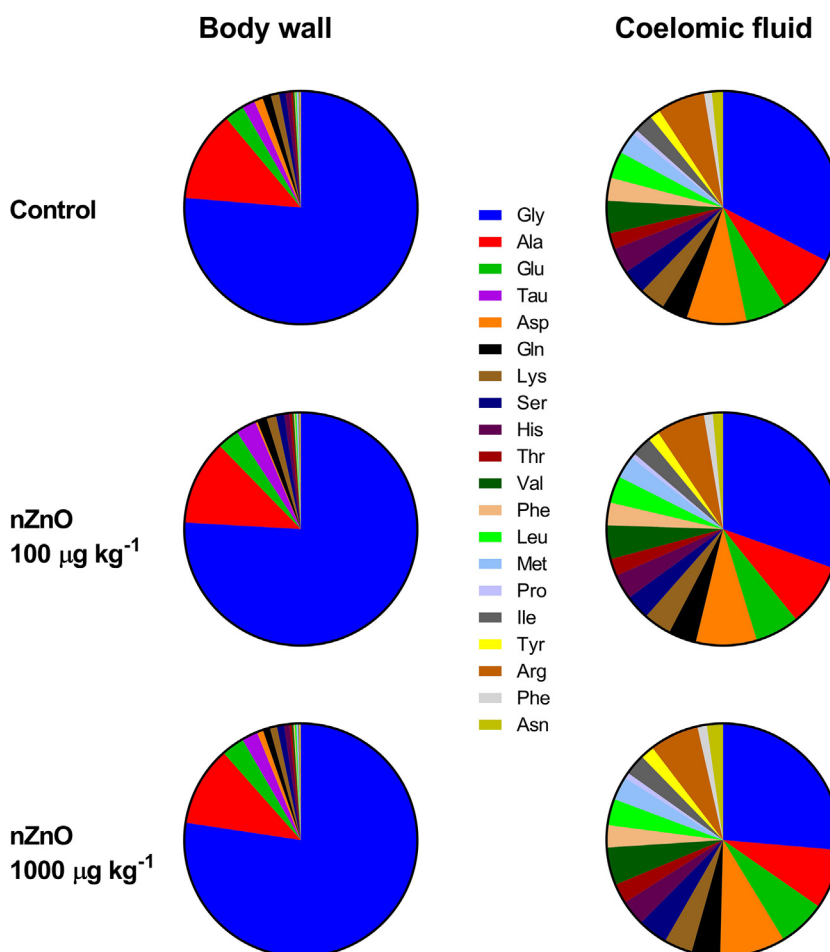


Fig. 2. Composition of the FAA pool (based on the molar concentrations of the respective FAAs) in the body wall tissue and the coelomic fluid of *A. marina* under the control conditions and after two weeks of exposure to nZnO (100 or 1000 $\mu\text{g Zn kg}^{-1}$ sediment) in the sediment.

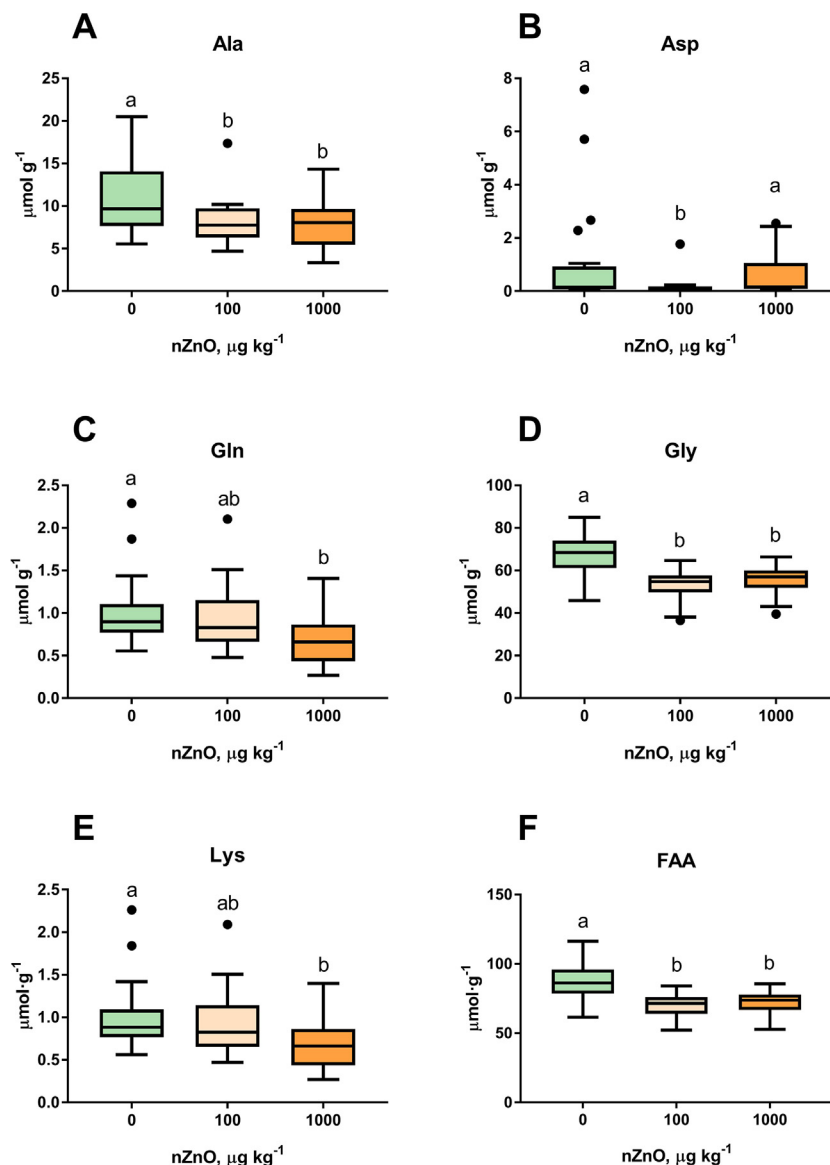


Fig. 3. Effects of nZnO exposure (0, 100 or 1000 $\mu\text{g Zn kg}^{-1}$ sediment) on the concentrations of free amino acids in the body wall tissues of *A. marina*. Only the amino acids that showed evidence of significant change ($p < 0.05$) in response to nZnO exposures are presented. Boxplots that do not share a letter are significantly different ($p < 0.05$). Concentrations of all other measured FAAs are given in Supplementary Table 1. $N = 20$.

in the body wall showed no evidence of the effects of nZnO exposure (Fig. 6F), whereas the GABA levels in the coelomic fluid decreased in both nZnO exposed groups (Fig. 7D).

3.4. Urea cycle intermediates

Concentrations of citrulline were reduced in the body wall and the coelomic fluid of the lugworms exposed to both studied nZnO concentrations in the sediment (Figs. 7E, 8A). Ornithine levels decreased in the coelomic fluid but not in the body wall of *A. marina* exposed to the high tested nZnO concentration (1000 $\mu\text{g Zn kg}^{-1}$ sediment) (Figs. 7F, 8C). Concentrations of Arg in the body wall or the coelomic fluid showed no evidence of change in response to nZnO exposures (Figs. 4A, 8B).

3.5. Carnitine

Exposure to nZnO led to a decrease in the carnitine levels in the body wall (at the low experimental nZnO concentration) and the coelomic fluid (at both tested nZnO concentrations) of *A. marina* (Figs. 7G, 8D).

3.6. Pathway enrichment analysis

Based on the changes in the metabolite profile in the body wall of *A. marina*, nZnO exposure significantly affected six metabolic pathways ($\text{FDR} < 0.05$) including pyruvate metabolism, glyoxylate and dicarboxylate metabolism, TCA cycle, Arg biosynthesis, Ala, Asp and Glu metabolism and Tyr metabolism (Table 1, Fig. 9A). Analysis of the coelomic fluid metabolome identified 14 metabolic pathways ($\text{FDR} < 0.05$) significantly affected by nZnO exposure including glutathione metabolism, Ala, Asp and Glu metabolism, Arg biosynthesis, Arg metabolism, glyoxylate and dicarboxylate metabolism, Gln and Glu metabolism, butanoate metabolism, Gly, Ser and Thr metabolism, His metabolism, Cys and Met metabolism, Phe, Tyr and Trp biosynthesis, Phe metabolism, Tyr metabolism and Trp metabolism (Fig. 9B).

3.7. Correlation analysis

Correlation analysis of metabolite concentrations across all experimental treatments revealed a cluster of 9 positively correlated metabolites in the

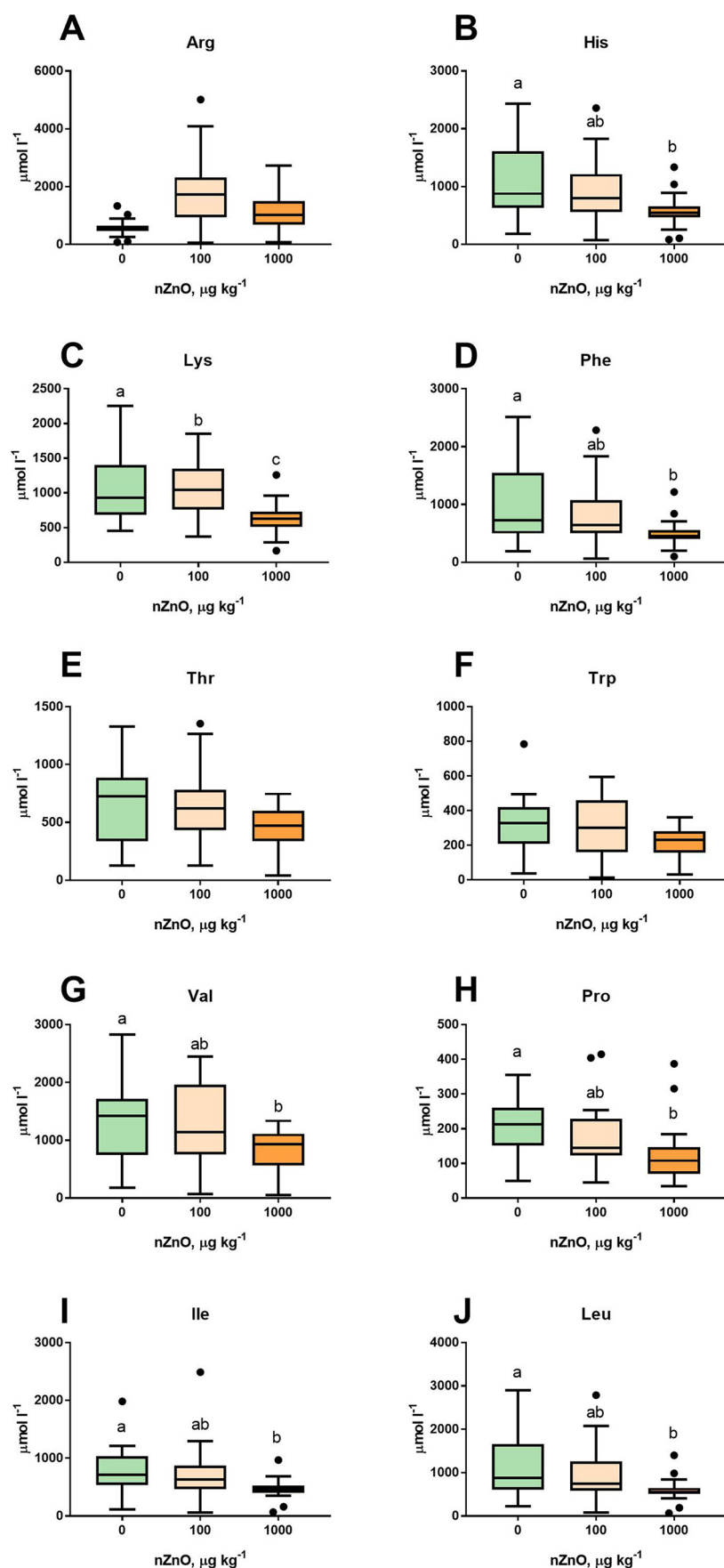


Fig. 4. Effects of nZnO exposure (0, 100 or 1000 $\mu\text{g Zn kg}^{-1}$ sediment) on the concentrations of essential free amino acids in the coelomic fluid of *A. marina*. Boxplots that do not share a letter are significantly different ($p < 0.05$). Concentrations of other measured FAAs are given in Supplementary Table 2. N = 17–19.

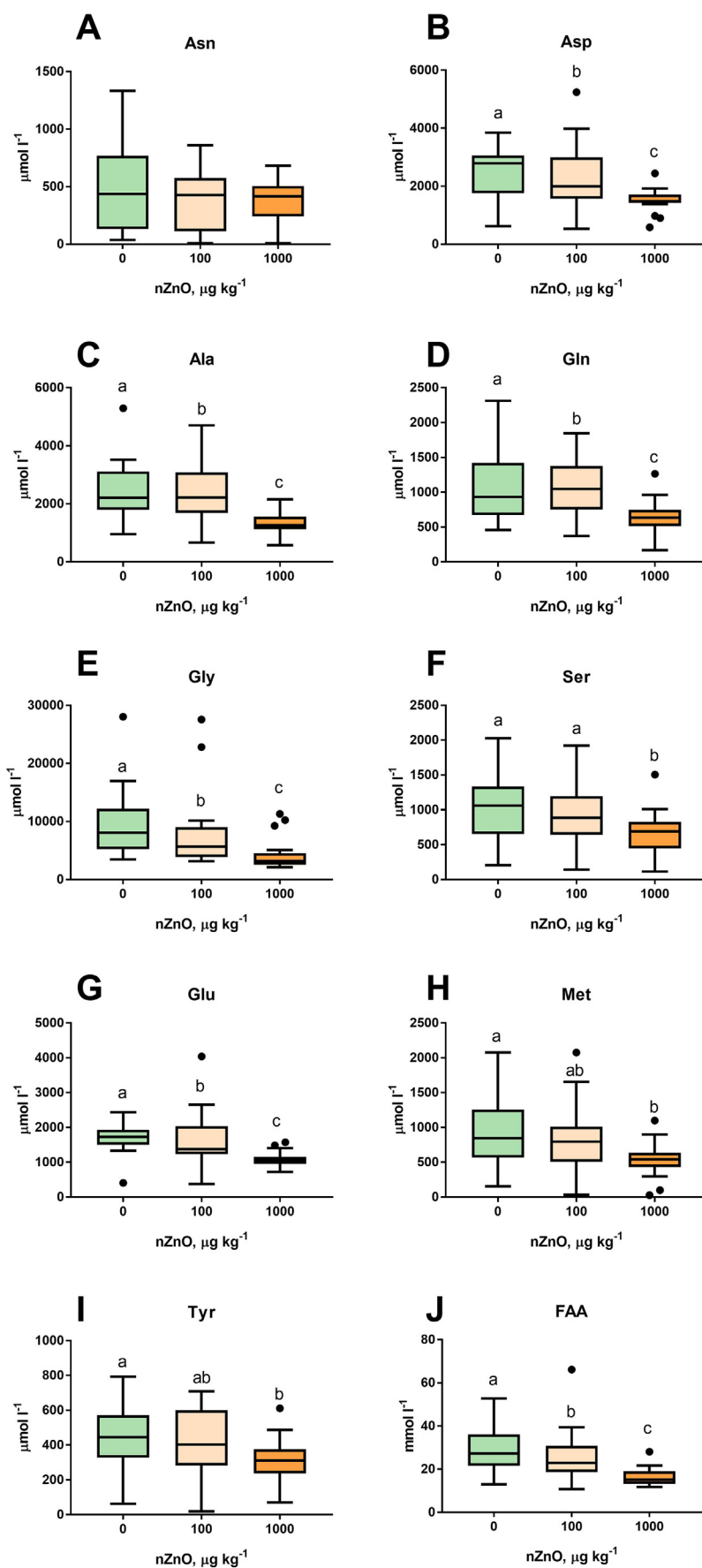


Fig. 5. Effects of nZnO exposure (0, 100 or 1000 $\mu\text{g Zn kg}^{-1}$ sediment) on the concentrations of non-essential free amino acids in the coelomic fluid of *A. marina*. Boxplots that do not share a letter are significantly different ($p < 0.05$). Concentrations of other measured FAAs are given in Supplementary Table 2. N = 17–19.

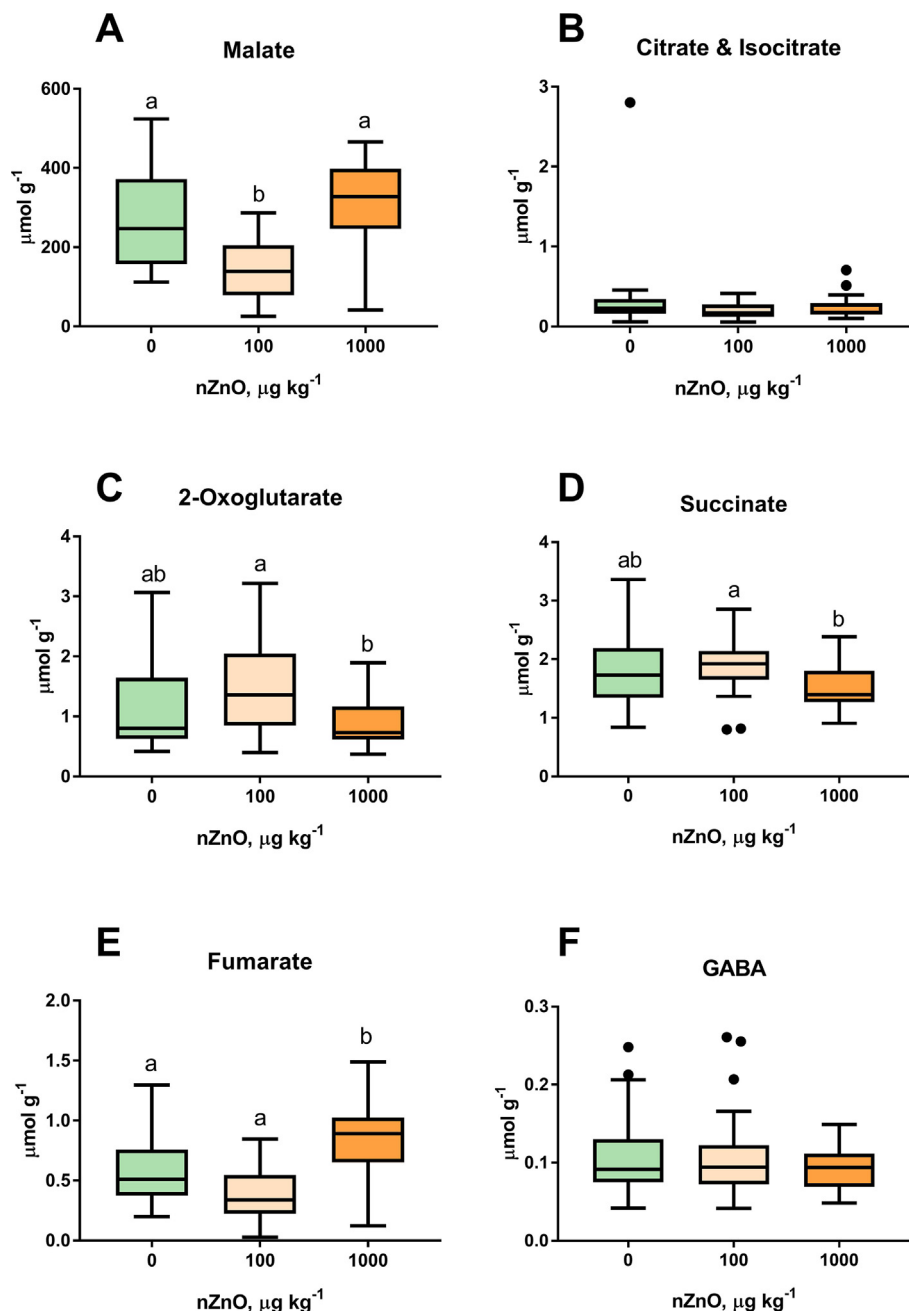


Fig. 6. Effects of nZnO exposure (0, 100 or 1000 $\mu\text{g Zn kg}^{-1}$ sediment) on the concentrations of TCA intermediates and GABA in the body wall of *A. marina*. Boxplots that do not share a letter are significantly different ($p < 0.05$). $N = 20$.

body wall of *A. marina* including aromatic amino acids (Try, Phe, Tyr), branch-chain amino acids (Leu, Ile, Val), Met, Arg and ornithine (Fig. 10A). The body wall concentration of taurine was negatively correlated with most studied metabolites (Fig. 10A). In the coelomic fluid, a large cluster of positively correlated metabolites was found, including 19 FAAs (Fig. 10B). This cluster involved aromatic amino acids (Try, Phe, Tyr, Thr), branch-chain amino acids (Leu, Ile, Val), Met, methionine sulfoxide, Asp, Asn, Ser, Pro, Glu, Gln, Ala, His, Arg and Lys (Fig. 10B). Similar to the body wall, taurine concentration was negatively correlated with most studied metabolites in the coelomic fluid (Fig. 10B). Concentrations of malate and (iso)-citrate were also negatively correlated with most other studied metabolites excluding Gly and GABA that positively correlated with malate (Fig. 10B).

4. Discussion

Exposure to nZnO-contaminated sediment led to oxidative damage in the cellular membranes of *A. marina* as indicated by accumulation of lipid peroxidation products. Oxidative stress is considered a key cellular event in the adverse outcome pathway of nZnO (Gerloff et al., 2017; Halappanavar et al., 2020; Pei et al., 2022) albeit molecular mechanisms of nZnO-induced oxidative damage are not yet fully deciphered. Mitochondrial damage and suppression of antioxidant defense have been proposed as possible mechanisms on nZnO-induced disruption of the redox balance (Pei et al., 2022; Saliani et al., 2016), although ROS production due to the direct catalytic activity of nZnO can also contribute (Biswas et al., 2022; Dutta et al., 2012). It is worth noting that in our present study no accumulation

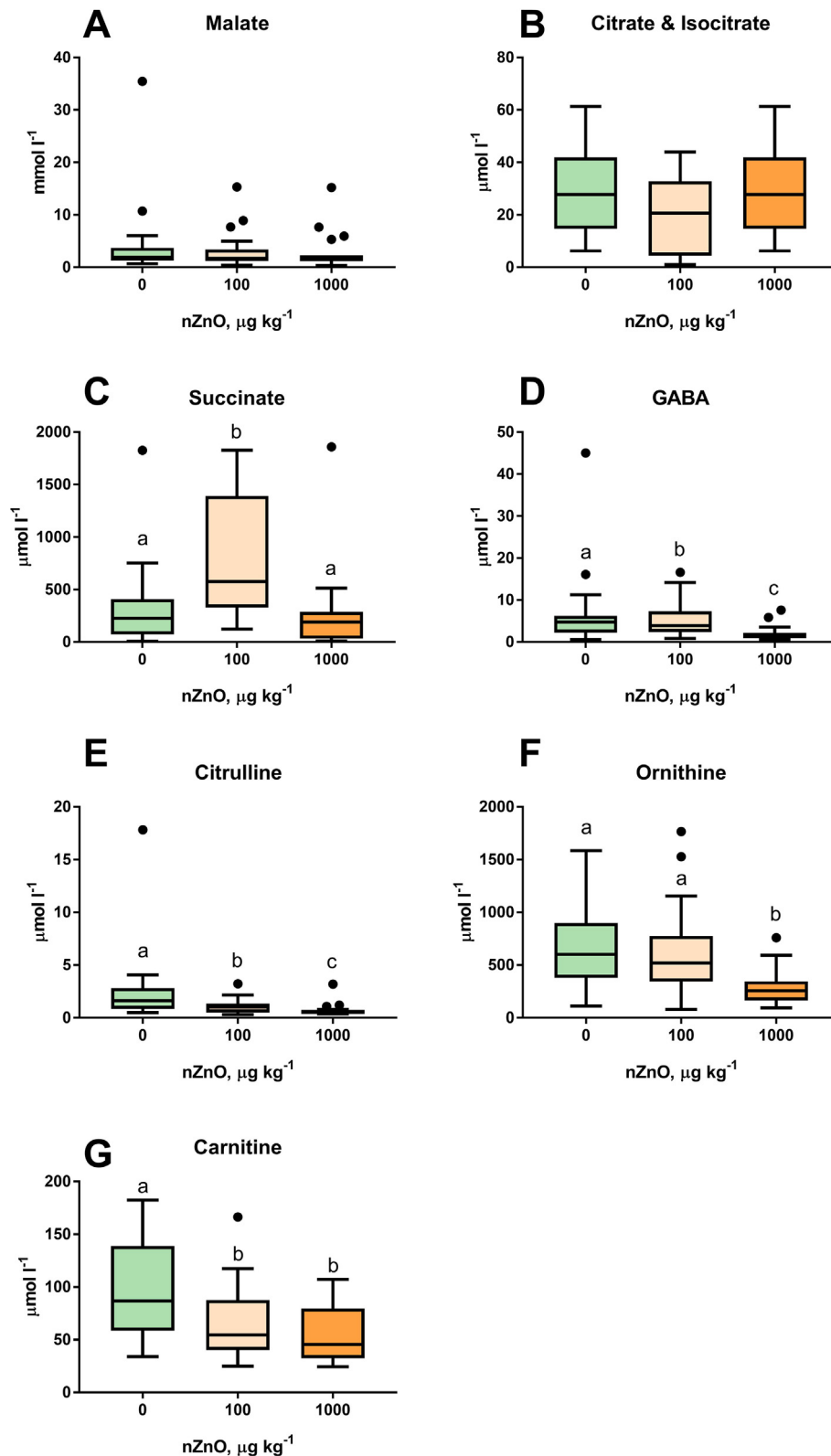


Fig. 7. Effects of nZnO exposure (0, 100 or 1000 µg Zn kg⁻¹ sediment) on the concentrations of TCA intermediates, GABA and urea cycle intermediates in the coelomic fluid of *A. marina*.

Boxplots that do not share a letter are significantly different ($p < 0.05$). N = 17–19.

of protein carbonyls was observed in nZnO-exposed worms. Low accumulation of the protein carbonyl levels in response to nZnO have been earlier reported for the marine and freshwater bivalves (Falfushynska et al.,

2019b; Wu et al., 2022). Overall, oxidative stress responses to nZnO measured by the levels of lipid peroxidation and protein carbonylation are variable and depend on the species as well as the concentration of the

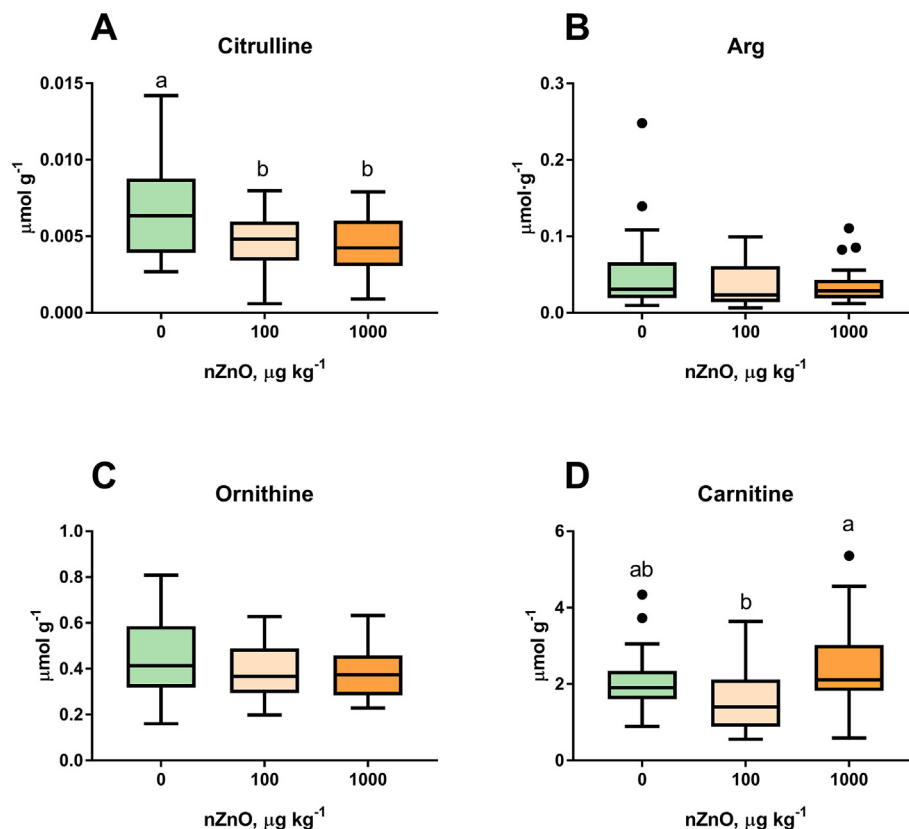


Fig. 8. Effects of nZnO exposure (0, 100 or 1000 $\mu\text{g Zn kg}^{-1}$ sediment) on the concentrations of urea cycle intermediates in the body wall of *A. marina*. Boxplots that do not share a letter are significantly different ($p < 0.05$). $N = 20$.

nanopollutant (Falfushynska et al., 2022). Lipids appear more sensitive to the nZnO-induced oxidative damage than protein, possibly due to the direct contact of ROS-producing ZnO nanoparticles with the cellular membranes (Biswas et al., 2022; Dutta et al., 2012).

4.1. Coelomic fluid is a metabolically important body compartment of *A. marina*

Our findings show that the coelomic fluid of the lugworms is an important site of metabolite transport and storage. The coelomic fluid contained

Table 1

Pathways modulated by the sediment nZnO exposures in the lugworms *A. marina*.

The pathway enrichment analysis was conducted using Metaboanalyst 5.0 software. Only significantly modulated pathways ($\text{FDR} < 0.05$) with impact > 0 are shown. Total: the total number of compounds in the respective pathway according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Hits: The number of metabolites analyzed in the present study that belong to the corresponding pathway. The metabolites identified as significantly affected by the experimental treatment ($p < 0.05$) are highlighted in bold.

Pathway	Total	Hits	Metabolites involved	FDR	Impact
Body wall					
Ala, Asp and Glu metabolism	23	10	Asp, Ala, Asn, Glu, Gln , pyruvate, fumarate , succinate , 2-ketoglutarate, GABA	0.011	0.80
Arg biosynthesis	12	8	Glu, Gln , Arg, Asp, 2-ketoglutarate, fumarate , citrulline, ornithine	0.011	0.63
Glyoxylate and dicarboxylate metabolism	24	5	Gly, Glu, Gln , (iso-)citrate, malate	0.048	0.17
TCA cycle	20	6	Malate , fumarate , 2-ketoglutarate, succinate, (iso-)citrate, pyruvate	0.011	0.30
Tyr metabolism	33	3	Pyruvate, fumarate , Tyr	0.018	0.19
Pyruvate metabolism	22	4	Pyruvate, malate , fumarate , lactate	0.011	0.21
Coelomic fluid					
Ala, Asp and Glu metabolism	23	7	Asp , Ala, Asn, Glu , Gln , succinate , GABA	0.0005	0.73
Arg biosynthesis	12	6	Arg, Asp , Glu , Gln , ornithine, citrulline	0.0005	0.63
Arg and Pro metabolism	31	5	Arg, Pro , Glu , GABA, ornithine	0.0005	0.42
Glyoxylate and dicarboxylate metabolism	24	5	Glu , Gln , Gly, (iso-)citrate, malate	0.0005	0.17
Glutathione metabolism	26	3	Gly, Glu , ornithine	0.0005	0.13
D-Gln and D-Glu metabolism	5	2	Glu , Gln	0.0005	1.00
Butanoate metabolism	14	3	Glu , succinate , GABA	0.0008	0.20
Gly, Ser and Thr metabolism	30	2	Gly , Thr	0.002	0.33
His metabolism	9	1	His	0.007	0.40
Cys and Met metabolism	32	1	Met	0.010	0.14
Phe, Tyr and Trp biosynthesis	4	2	Phe , Tyr	0.010	1.00
Phe metabolism	7	2	Phe , Tyr	0.010	0.38
Trp metabolism	30	1	Trp	0.017	0.21
Tyr metabolism	33	1	Tyr	0.017	0.15

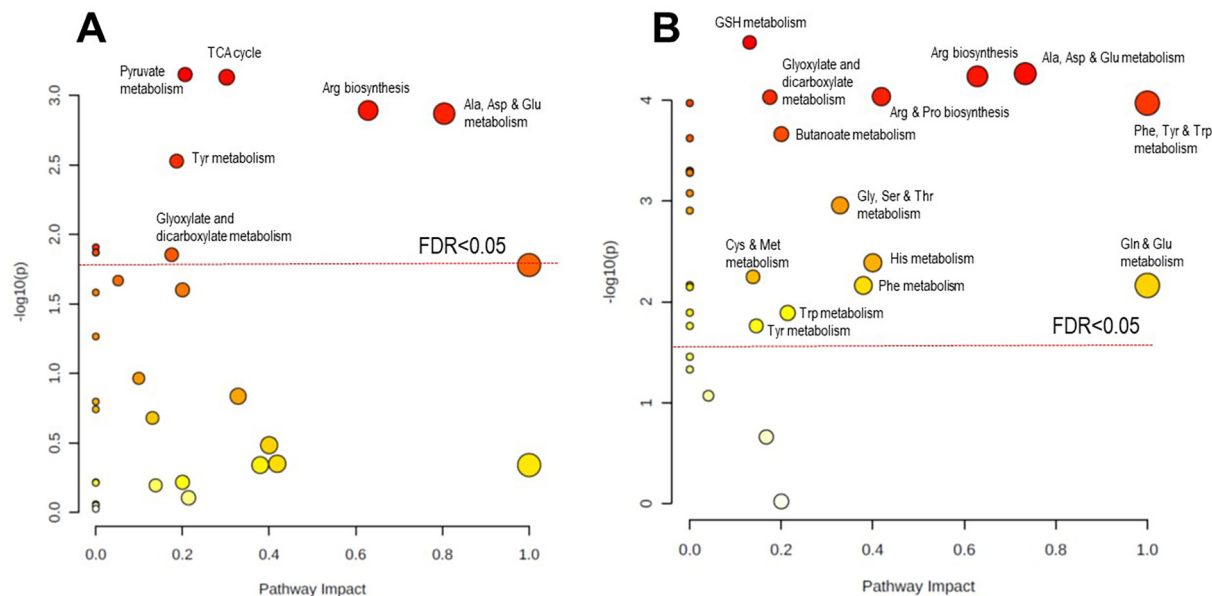


Fig. 9. Pathway enrichment analysis based on the metabolite profiles in the body wall (A) and the coelomic fluid (B) of *A. marina* exposed to control conditions and different nZnO concentrations. The Y axis - $-\log_{10}(p)$, X- axis- pathway impact, the horizontal red line corresponds to $FDR = 0.05$. The size of the symbol corresponds to the pathway impact, the color on the yellow to red scale reflects the relative significance of the enrichment (with red being the most significant).

high concentrations of FAAs and short-chain organic acids (often in high millimolar range), as well as a neurotransmitter GABA. The metabolome composition of the coelomic fluid differed from that of the body wall indicating that the metabolite secretion into the coelomic fluid is a selective process. Thus, we detected no AMP, fumarate and 2-oxoglutarate in the coelomic fluid despite relatively high concentrations of these metabolites in the body wall (588, 69 and $163 \mu\text{g g}^{-1}$ for AMP, fumarate and 2-oxoglutarate, respectively). Tau was found in very low concentrations in the coelomic fluid samples ($\sim 0.01 \mu\text{mol l}^{-1}$) and was below the detection limit in 35 out of 52 coelomic fluid samples. In contrast, Tau was the fourth

most abundant FAA in the body wall of *A. marina* with mean concentrations of $1550\text{--}2010 \mu\text{mol kg}^{-1}$ in different experimental groups. These findings indicate that Tau is selectively retained in the lugworm tissue and is not secreted into the coelomic fluid; the observed low Tau content in some of the coelomic fluid samples might be a result of contamination during tissue dissection. Furthermore, the relative concentrations of different amino acids were considerably higher and more balanced in the coelomic fluid compared with the body wall of *A. marina*. The concentration ratio of the most to the least abundant FAA was >2200 in the body wall (compared with only ~ 46 -fold difference in the concentrations of the most and the

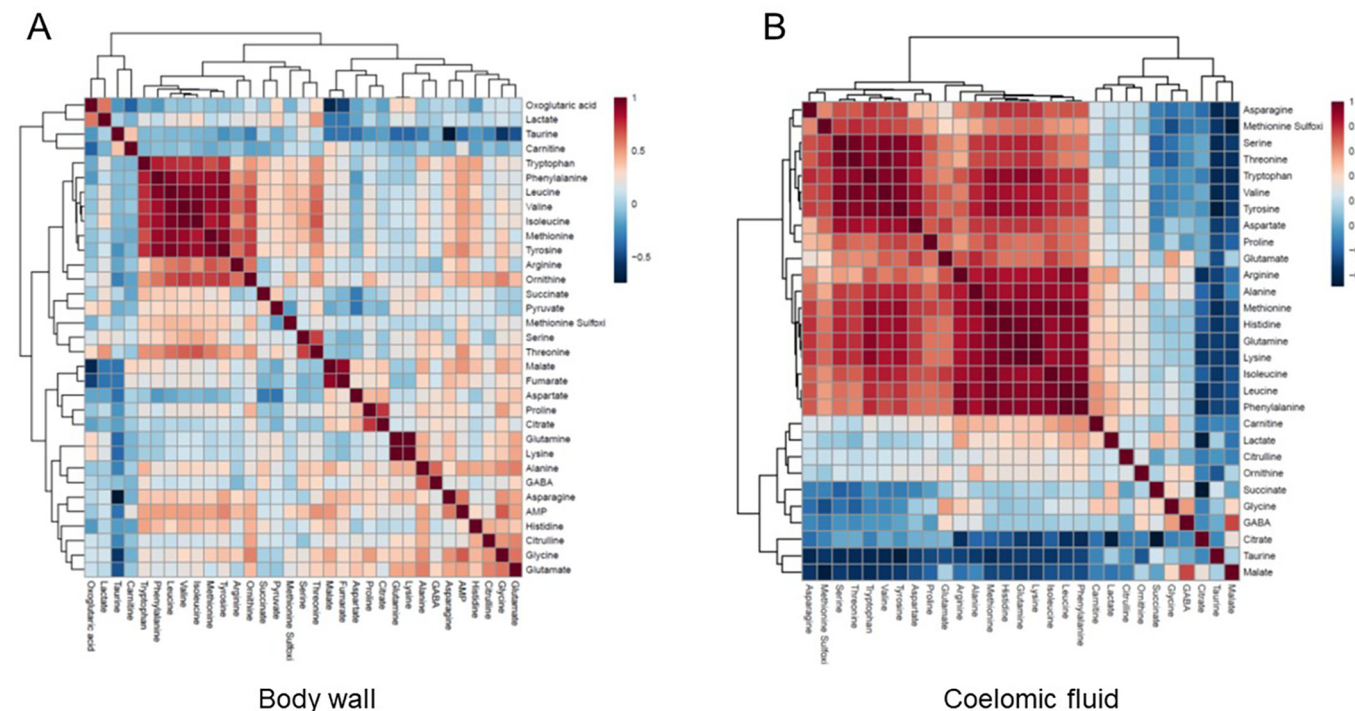


Fig. 10. Correlation analysis of the metabolite concentrations in the body wall (A) and the coelomic fluid (B) of *A. marina* under control and different nZnO exposure conditions.

least abundant FAA in the coelomic fluid). In the body wall, two amino acids (Ala and Gly) accounted for ~90 % of the total FAA pool, with Gly comprising >75 % of all free amino acids in the tissue. In the coelomic fluid, 13 FAAs made up ~90 % (Gly ~30 %) of the FAA pool. These differences in the metabolome composition between the body compartments indicates that metabolites are selectively transported into the coelomic fluid from the body tissues, and/or possibly metabolized in the coelomic fluid by coelomocytes. Regardless of the origin and transport mechanisms of coelomic fluid metabolites, these findings underscore the important role of the coelomic fluid plays in *A. marina* metabolism beyond the traditionally assumed passive role as the hydraulic skeleton (Zebe and Schiedek, 1996). Earlier studies showed involvement of the coelomic fluid in the lugworm immunity (Maltseva et al., 2014) supporting the notion of importance of this compartment in the vital functions of *A. marina*.

4.2. Impacts of nZnO on intermediary metabolism of *A. marina*

Metabolomics analysis showed that exposure to nZnO caused significant alterations in the amino acid metabolism, carbohydrate biosynthesis (as evidenced by the changes in glyoxylate and dicarboxylate metabolism and metabolism of gluconeogenic amino acids) and TCA cycle in *A. marina*. Modulation of some metabolic pathways (including glyoxylate and dicarboxylate metabolism, Ala, Asp and Glu metabolism, Arg biosynthesis, and Tyr metabolism) was detected both in the body wall and the coelomic fluid of the worms, whereas changes in the metabolic pathways involving Gly, Ser and Thr metabolism, His metabolism, Cys and Met metabolism and metabolism of aromatic amino acids were only detectable in the coelomic fluid. These findings demonstrate the importance of considering the coelomic fluid metabolome in ecotoxicological assessments of the metabolic impacts of pollutants in marine invertebrates. Generally, nZnO exposure led to a decline in the tissue and coelomic fluid concentrations of multiple amino acids resulting in an overall decrease of the FAA pool. FAAs play an important role as osmolytes in marine organisms including annelids (Yancey, 2005). The lugworms are osmoconformers, so that osmotic concentration of their intracellular and extracellular fluids follows changes in the osmotic concentration of the environment (Zebe and Schiedek, 1996). FAAs (particularly Ala and Gly) play a key role in intracellular isosmotic regulation of *A. marina* preventing excessive changes in the cell volume (Zebe and Schiedek, 1996). A decrease in the FAA pool (by ~20 % in the body wall and ~45 % in the coelomic fluid at the highest nZnO exposure concentration) may thus have an impact on osmoregulatory processes and the protein biosynthesis of these organisms.

The exposure to nZnO had a strong impact on the metabolism of the key gluconeogenic amino acids (including Ala, Asp, Glu, Arg, His, Gly, and Ser) as well as on glyoxylate and dicarboxylate pathway indicating interference of nZnO with carbohydrate metabolism. In particular, the concentrations of key amino acids (Gly, Gln, and Glu) that link alanine, aspartate and glutamate metabolism with glyoxylate and dicarboxylate metabolism were suppressed by nZnO exposure. Furthermore, concentrations of two other key gluconeogenic amino acids, Asp and Ala, decreased in response to nZnO in *A. marina*. In the baker yeast, decreases in Gly and Glu concentrations were observed during nZnO exposures whereas Asp and Ala content increased (Kumar Babele, 2019). Gluconeogenesis was also negatively affected by nZnO in a soil annelid *Enchytraeus crypticus* (He et al., 2020). Perturbations of carbohydrate metabolism by ZnO reported in such distantly related organisms as yeast (Kumar Babele, 2019), mammals (Lai et al., 2015), mollusks (De Marchi et al., 2017; Falfushynska et al., 2019a; Wu et al., 2021a) and annelids (He et al., 2020) (this study) demonstrate that this branch of cellular metabolism is an important and evolutionarily conserved target for nZnO toxicity. Earlier research in marine and freshwater bivalves showed depletion of carbohydrate (glycogen and glucose) reserves during exposures to ZnO nanoparticles (Falfushynska et al., 2019a; Wu et al., 2021a) and multi-walled carbon nanotubes (De Marchi et al., 2017). These changes were interpreted as an increase in glycogen utilization to cover energy costs of detoxification and stress response (De Marchi et al., 2017; Falfushynska et al., 2019a; Wu et al.,

2021a). Our present study suggests that nZnO-induced interference with gluconeogenesis might contribute to the depletion of the glycogen reserves in the exposed organisms. Given the importance of glycogen and glucose as rapidly mobilizable fuels used for reproduction and survival of abiotic stressors (such as hypoxia and anoxia) (Neuhoff, 1979; Pörtner et al., 1979; Zwaan, 1983), interference with carbohydrate metabolism caused by nZnO exposures might negatively impact these important fitness-related functions in *A. marina*.

Exposure to nZnO had negative impacts on Arg biosynthesis, and Arg and Pro metabolism in *A. marina*. It is worth noting that the effects of nZnO exposure on the metabolites in these two connected pathways were more evident in the coelomic fluid metabolome compared with that of the body wall. Exposure to nZnO led to a decrease in the concentrations of the Arg precursors (Gln, Glu, Asp) and urea cycle metabolites (ornithine and citrulline) in the lugworms. There was also a notable albeit statistically not significant tendency of decrease in Arg concentration in the body wall (by ~30 %) and the coelomic fluid (by ~10–40 %) of nZnO-exposed lugworms (Supplementary Tables 1 and 2). Arg is one of the most versatile amino acids that (besides its role as a building block for protein synthesis) serves as a precursor for the synthesis of urea, polyamines, proline, glutamate, creatine, as well as signaling molecules such as nitric oxide and agmatine (Morris, 2006). In many marine invertebrates including annelids Arg can also play a role as a precursor of a phosphagen phospho-L-arginine (PLA), an important cellular ATP buffer (Ellington, 2001). However, in *A. marina* phosphataurocyamine rather than PLA is the main phosphagen (Schiedek, 1997; Schiedek and Schoetter, 1990). Thus, whereas dysregulation of Arg metabolism by nZnO exposure might have negative consequences on cellular signaling, nitrogen excretion and protein biosynthesis of the lugworms, impacts on the energy storage in the form of phosphagens is unlikely.

An important cluster of the metabolic pathways dysregulated by nZnO exposure in *A. marina* involves metabolism of aromatic amino acids including Phe, Tyr and Trp biosynthesis and metabolism. Exposure to high concentrations (1000 $\mu\text{g kg}^{-1}$) to nZnO in the sediment led to decreased concentrations of metabolites involved in Phe, Tyr and Trp biosynthesis in *A. marina*, whereas low exposure concentrations (100 $\mu\text{g kg}^{-1}$ Zn) had less impact on these pathways. Phe, Tyr and Trp play an important role in protein synthesis and serve as precursors for hormones (including thyroid hormones and catecholamines) and neurotransmitters (such as dopamine and L-DOPA) in animals. These signaling molecules play a key role in immunity and development of annelids (Cuvillier-Hot et al., 2014; Flatt et al., 2006; Wilhelm et al., 2006), so that perturbation of the metabolism of aromatic amino acids might have negative consequences for hormonal and immune homeostasis and as a result, for survival and reproduction of these organisms.

Changes in the TCA cycle intermediates and the metabolic pathway enrichment analysis indicate a disturbance of the TCA cycle in the body wall tissue of *A. marina* by nZnO exposures. The observed changes are consistent with a suppression of fumarase activity by the nZnO exposures. Thus, at the low tested concentration of nZnO (100 $\mu\text{g Zn kg}^{-1}$ sediment), a decrease in malate, a product of fumarase activity was observed, while the concentrations of fumarate did not change. At the high tested concentrations (1000 $\mu\text{g kg}^{-1}$ sediment), accumulation of fumarate (accompanied by a decrease in the fumarate precursors 2-oxoglutarate and succinate) was found indicating a suppression of fumarate conversion by fumarase. Interestingly, malate concentrations remained stable in the body wall of the worms exposed to the high tested concentrations (1000 $\mu\text{g Zn kg}^{-1}$ sediment) of nZnO, possibly reflecting upregulation in malate dehydrogenase to compensate for the lower fumarase activity and supply malate for biosynthetic processes. The potential mechanisms of the nZnO-induced alteration of the TCA cycle in *A. marina* are not known but earlier studies showed that TCA cycle enzymes are highly sensitive to oxidative stress (Cherkasov et al., 2007; Daloso et al., 2015; Martínez-Reyes and Chandel, 2020). Oxidative stress is considered a key mechanism of toxicity of metal-containing nanoparticles, including nZnO (Falfushynska et al., 2022; Luo et al., 2020; Wu et al., 2021b). Thus, it is tempting to speculate

that the observed changes in the TCA intermediates in nZnO-exposed lugworms might reflect changes in activity of redox-sensitive TCA enzymes caused by oxidative stress. Earlier studies in model organisms showed that oxidative stress has opposite effects on fumarase (suppression) and malate dehydrogenase (activation) lending support to this hypothesis (Daloso et al., 2015; Shi and Gibson, 2011). However, in our present study the TCA enzyme activities have not been measured, and this hypothesis requires further investigation.

4.3. Conclusions and outlook

The present metabolomics study demonstrates that exposure to environmentally relevant concentrations of nZnO in marine sediments results in disruption of amino acid and short chain organic acids metabolism in the sediment-dwelling polychaetes *A. marina*. Given the high (mg kg^{-1}) natural Zn background of the Baltic Sea sediments (Shahabi-Ghahfarokhi et al., 2021), the observed metabolic shifts in *A. marina* are likely due to the toxicity of the nanoparticulate zinc rather than dissolved or sediment-associated zinc. The changes in the metabolic intermediates indicate dysregulation of gluconeogenesis, metabolism of aromatic amino acids and TCA cycle by nZnO exposures that have implications for energy metabolism, hormonal balance and cellular signaling. A depletion of amino acid pool in the nZnO-exposed lugworms can also have negative consequences for the protein synthesis. The metabolic shifts caused by nZnO are reflected in the metabolite profile shifts in the coelomic fluid that exceed the changes observed in the body wall tissue. The coelomic fluid is therefore a useful target tissue that can provide early warning signs of nZnO-induced metabolic disruption in the lugworms. Given high concentrations of the studied metabolites in the coelomic fluid, relatively small volumes ($\sim 100 \mu\text{l}$) would be sufficient for the metabolomics analysis, which makes possible a repeated non-lethal sampling from the same individual. Among the most sensitive signs of the metabolic toxicity of nZnO are the concentration-dependent decrease in the total concentration of FAAs in the coelomic fluid (which can be measured by metabolomics tools as well as by traditional approaches such as ninhydrin staining) and the decrease in the concentrations of aromatic and gluconeogenic amino acids that integrate the contaminant-induced shifts in amino acid and carbohydrate metabolism. The shifts in metabolite profiles in the coelomic fluid can therefore provide the means of detection of sublethal toxic effects of nZnO that can serve as additional or alternative biomarkers for the traditional (and more invasive) biomarkers used for toxicity assessment such as the oxidative stress markers. Given the central role of amino acid and tricarboxylic acid metabolism in the core metabolic pathways of the animals, the metabolic disruptions caused by the environmentally relevant concentrations of nZnO can have wide-ranging negative effects on the organisms' fitness impairing growth and reproduction of the populations of the sediment dwelling marine bioturbators like the lugworms in nanoparticle-polluted sediments.

Data availability

Data will be made available on request.

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.scitotenv.2022.160039>.

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II.3 Combined effects of organic and mineral UV-filters on the lugworm *Arenicola marina*

Contribution letter

The material of the manuscript titled “**Combined effects of organic and mineral UV-filters on the lugworm *Arenicola marina***” was published in 2024 in the journal *Chemosphere*.

My work was the major contribution to this manuscript. I was involved in the experimental design and planning, maintenance of the experimental animals, setup and execution of the experimental exposures and tissue sampling. Determination of the oxidative damage markers (lipid peroxidation and protein carbonyls) as well as the antioxidant system (superoxide dismutase and catalase activity as well as the total antioxidant capacity) was done in collaboration with Candela Sánchez-Girón Barba and Lilian König, respectively. I was involved in the initial adaptation of the methods and quality control. Furthermore, in collaboration with Prof. Dr. Inna Sokolova, I acquired support from the Deutscher Akademischer Austauschdienst which was granted in the form of the stipend of Ms. Sánchez-Girón Barba (grant number 91821143) to support her work on this manuscript. Determination of the metabolites was done in collaboration with Dr. Stefan Timm where I carried out the initial extraction of the metabolites. Further, I conducted the statistical and graphical analysis of the gained data and wrote the first draft of the manuscript. I contributed to the revision of the draft and to the responses to the reviewers during the submission process.

Based on the CRediT taxonomy (Brand et al., 2015), my contributions to this manuscript were as follows:

- Conceptualization
- Methodology
- Validation
- Formal analysis
- Investigation
- Data Curation
- Writing - Original Draft
- Writing - Review & Editing
- Visualization
- Funding acquisition

Signatures of consent

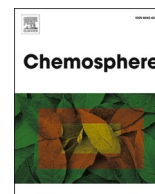
Prof. Dr. Inna Sokolova

(Supervisor)

Torben Bruhns

(PhD candidate)

Brand, A., Allen, L., Altman, M., Hlava, M., Scott, J., 2015. Beyond authorship: attribution, contribution, collaboration, and credit. *Learn. Publ.* 28, 151–155. <https://doi.org/10.1087/2015021>



Combined effects of organic and mineral UV-filters on the lugworm *Arenicola marina*

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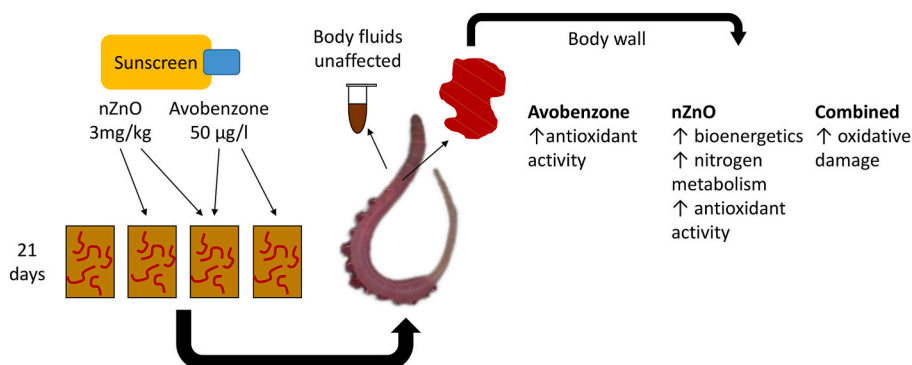
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HIGHLIGHTS

- *Arenicola marina* exposed to nZnO, avobenzone, and their combinations.
- nZnO exposure triggers amino acid and energy metabolism alterations.
- Metabolic shifts in both individual and combined exposures predominantly influenced by nZnO.
- Single UV filter exposures enhance antioxidant defenses.
- Concurrent nZnO and avobenzone exposure diminish antioxidants, leading to oxidative stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Pollution from personal care products, such as UV-filters like avobenzone and nano-zinc oxide (nZnO), poses a growing threat to marine ecosystems. To better understand this hazard, especially for lesser-studied sediment-dwelling marine organisms, we investigated the physiological impacts of simultaneous exposure to nZnO and avobenzone on the lugworm *Arenicola marina*. Lugworms were exposed to nZnO, avobenzone, or their combination for three weeks. We assessed pollutant-induced metabolic changes by measuring key metabolic intermediates in the body wall and coelomic fluid, and oxidative stress by analyzing antioxidant levels and oxidative lesions in proteins and lipids of the body wall. Exposure to UV filters resulted in shifts in the concentrations of Krebs' cycle and urea cycle intermediates, as well as alterations in certain amino acids in the body wall and coelomic fluid of the lugworms. Pathway enrichment analyses revealed that nZnO induced more pronounced metabolic shifts compared to avobenzone or their combination. Exposure to avobenzone or nZnO

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alone prompted an increase in tissue antioxidant capacity, indicating a compensatory response to restore redox balance, which effectively prevented oxidative damage to proteins or lipids. However, co-exposure to nZnO and avobenzone suppressed superoxide dismutase and lead to accumulation of lipid peroxides and methionine sulfoxide, indicating oxidative stress and damage to lipids and proteins. Our findings highlight oxidative stress as a significant mechanism of toxicity for both nZnO and avobenzone, especially when combined, and underscores the importance of further investigating the fitness implications of oxidative stress induced by these common UV filters in benthic marine organisms.

1. Introduction

Anthropogenic pollution represents a major threat to coastal marine ecosystems (Beiras, 2018). In recent years, the production, use and accumulation of personal care products (PCPs) has increased, raising concerns about their risks to the environment (Beiras, 2018; Fisch et al., 2017; Prichard and Granek, 2016). Sunscreens are common PCP pollutants that enter marine environments due to recreational activities or via wastewater (Beiras, 2018; Cuccaro et al., 2022; Fisch et al., 2017). Sunscreens comprise a complex mixture of active and inactive ingredients, including mineral (inorganic particulate) and organic UV filters. Mineral UV filters like ZnO nanoparticles (NP) are commonly used in sunscreens due to their high UV reflectance (Caloni et al., 2021; Osterwalder et al., 2014) and accumulate in marine sediments reaching concentrations from 6 µg/kg to 20 mg/kg (Gottschalk et al., 2013, 2015; Sun et al., 2016). These concentrations are concerning as nZnO can negatively affect marine organisms, causing oxidative stress, shifts in bioenergetics and disrupted metabolism (Bruhns et al., 2023b; Falfushynska et al., 2017, 2019; Gagné et al., 2016; Noor et al., 2021; Wong et al., 2010; Wu et al., 2020). The mechanisms of nZnO toxicity are not completely understood, but the physical interaction of the NP with the cells and the release of Zn ions likely contribute to the toxic effects (Ma et al., 2013; Mouneyrac et al., 2014; Reed et al., 2012; Wong et al., 2010).

Unlike mineral UV filters that reflect UV light, organic UV filters (e.g. avobenzone, octocrylene, oxybenzone and ensulizole) absorb UV radiation (Huang et al., 2021; Ramos et al., 2015; Serpone et al., 2007). Due to the established toxicity of some organic UV filters like oxybenzone, octinoxate and octocrylene to corals, unregulated marketing terms like “reef-safe” for sunscreens that do not contain these compounds became increasingly common (Miller et al., 2021; National Academies of Sciences Engineering and Medicine, 2022). An organic UV-filter which is frequently declared “reef-safe” is avobenzone (butyl methoxydibenzoylmethane; BMDM or BMDBM), despite the lack of evidence for its reef safety (Clergeaud et al., 2023; Danovaro et al., 2008; Fel et al., 2019; Miller et al., 2021; Mitchelmore et al., 2019). Recent studies report avobenzone concentrations in the range of 1–2431 ng/l in seawater (Bratkovics et al., 2015; Bratkovics and Sapozhnikova, 2011; Downs et al., 2022b; Poiger et al., 2004; Ramos et al., 2015; Tsui et al., 2019) and up to 28 µg/g for polluted beach sediments (Downs et al., 2022a). During the recreational season, peak concentrations of avobenzone might far exceed these values (Bratkovics et al., 2015; Labille et al., 2020; Picot-Groz et al., 2018). Studies show that avobenzone has negative impacts on metabolism, redox and hormonal homeostasis, as well as nervous function of crustaceans, bivalves and fish, questioning the safety of avobenzone for aquatic ecosystems (Bordalo et al., 2022; Boyd et al., 2021; Ka and Ji, 2022; Liu et al., 2022; Park et al., 2017). However, the ecotoxicological effects of avobenzone in marine organisms and its potential interactions of commonly co-occurring mineral UV filters like nZnO are not well understood and warrant further investigation.

Most recent studies examining ecotoxicity of UV filters in marine environments focus on filter feeders exposed to waterborne pollutants (Biswas and Sarkar, 2019; Caloni et al., 2021; Coll et al., 2016; Keller, 2023; Yuan et al., 2022). Although sediments are a sink for UV filters, ecotoxicology of UV filters in sediment-dwelling organisms that face

elevated risks due to the contact with and ingestion of polluted sediments is not well understood (Biswas and Sarkar, 2019; Bruhns et al., 2023b; Caloni et al., 2021; Ma et al., 2017; Pawlowski and Petersen-Thiery, 2020). Therefore, we aimed to assess single and combined toxicity of common UV filters, nZnO and avobenzone, to a sediment-dwelling polychaete *Arenicola marina* (the lugworm). *A. marina* are common in Northeast Atlantic and the Mediterranean and serve as important ecosystem engineers in soft bottom marine ecosystems (Hartmann-Schröder, 1996; WoRMS, 2023). The worms are infaunal sediment-feeders preferring sandy habitats that often overlaps with beaches used for recreational activities (Hartmann-Schröder, 1996). This makes *A. marina* a useful model to study ecotoxicity of UV filters.

We exposed *A. marina* to nZnO (3 mg/kg sediment) and avobenzone (~2 µg/l sea water) alone or in combination for three weeks. The selected UV filter concentrations were at the upper end of environmentally relevant range for polluted sediments and water (Buffet et al., 2012; Yung et al., 2013), mimicking acute exposures that might be expected at the peak of recreational activities (Afonso et al., 2014; Bratkovics et al., 2015; Downs et al., 2022a; Labille et al., 2020; Pawlowski and Petersen-Thiery, 2020; Picot-Groz et al., 2018). Using high concentrations of the studied UV filters was essential to detect the potential ecotoxic mechanisms of these pollutants and distinguish the stress-signatures of single and combined UV filter exposures (Boyd et al., 2021; Bruhns et al., 2023b; de Paula et al., 2022). We hypothesized that the exposure to avobenzone or nZnO alone will induce oxidative stress and metabolic disruption in *A. marina*, which will be enhanced in the combined exposure. To test this hypothesis, we measured oxidative stress markers (levels of antioxidants and oxidative damage to lipids and proteins) in the body wall of the lugworms exposed to nZnO, avobenzone and their combination. The activity of antioxidants was assessed by the total antioxidant capacity (TAOC) and glutathione content (GSH) of the body wall and by measuring activities of two key antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) that detoxify superoxide and hydrogen peroxide, respectively (Ahmad, 1995). The pollutant-induced metabolic shifts were analyzed using targeted metabolomics of key intermediates of tricarboxylic acid cycle, urea cycle and free amino acids in the body wall and coelomic fluid of lugworms.

2. Materials and methods

2.1. Maintenance and exposure

A. marina from southern Netherlands was obtained from a commercial supplier (Angelcenter Bastian, Rostock, Germany) in July 2021. The worms were transported and maintained at a salinity of 24 (practical salinity units) and 15 °C as described before (Bruhns et al., 2023b). The animals were active and visibly healthy. Sediment for the lugworm maintenance was collected near Heiligendamm (Germany) at ~1.5 m water depth. The sediment was sieved (1 mm) to remove macrofauna and stored with a layer of habitat seawater until further use. The water content was 13% and the wet density was 2.2 kg/l. The sediment contained ~93% of 125–500 µm sized particles, which are preferentially ingested by *A. marina* (Andresen and Kristensen, 2002). We conducted four different exposures: control (no UV filter additions), nZnO exposure

(3 mg Zn/kg dry sediment), avobenzone exposure (50 µg/l avobenzone in overlaying seawater) and combined exposure (3 mg Zn/kg of nZnO and 50 µg/l avobenzone in the sediment and water, respectively). All exposures were carried out in 23 × 15 × 30 cm (L × W × H) mesocosms (~7 l) filled with 20 cm of sediment and 2 cm of the overlaying water.

For nZnO exposure, the sediment was spiked with nZnO suspension as described elsewhere (Bruhns et al., 2023b). Briefly, commercial ZnO nanoparticles with an average size of 30 nm (Sigma-Aldrich) were thoroughly mixed with the sediment over two days using an electrically powered mixing device under a thin layer of seawater at a salinity of 24 and a temperature of 15 °C. The characteristics of nZnO used in these exposures were determined previously (Wu et al., 2022) and were as follows: the average particle size 30.1 ± 0.4 nm, dry surface area $35 \text{ m}^2/\text{g}$, and the hydrodynamic size $5.21 \pm 0.21 \text{ }\mu\text{m}$ (Wu et al., 2022). Previous studies have indicated that nZnO remains stable and exhibits minimal dissolution in seawater over three weeks (Wu et al., 2020). In this study, only nominal concentrations of nZnO are reported due to the technical challenges associated with detection of nanoparticles in the sediment. Additionally, measurements of sediment Zn concentrations cannot accurately discern low exposure levels of nZnO against the high natural Zn background found in Baltic Sea sediments (Shahabi-Ghahfarokhi et al., 2021). No avobenzone was added during the preparation of the nZnO-spiked sediments.

Avobenzone (Avo; Sigma Aldrich) was diluted in 98% ethanol (1 mg/ml), mixed with the artificial seawater (salinity 24; Pro-REEF Sea Salt, Tropic Marin) to a nominal concentration of 50 µg/l and added to the sediment-containing experimental mesocosms. Equivalent volumes of ethanol (4.9×10^{-3} % final concentration) were added to the controls and nZnO exposures as solvent controls. Overlaying water in all mesocosms was exchanged every second day with fresh artificial seawater containing appropriate amounts of avobenzone or ethanol. The measured avobenzone concentration in the exposure water (1.9–2.2 µg/l; see section 3.2) was similar to levels found in seawater near recreational beaches (up to 1.7–2.4 µg/l) (Bratkovics et al., 2015; Cadeña-Aizaga et al., 2020). Bioturbation and bioirrigation by *A. marina* constantly introduces fresh water into the burrow ensuring exposure (Gogina et al., 2022). The mesocosms were constantly aerated and kept under a day/night cycle of 12h/12h. For each experimental group, three replicate mesocosms were set up and incubated for 10 days without animals. Subsequently, five animals were placed into each mesocosm. Animals that failed to dig in within 30 min were replaced to ensure that only healthy and active animals were used in this study. After three weeks of exposure, the worms were removed from the sediment and dissected on ice (Bruhns et al., 2023b). The tissue and coelomic fluid samples were immediately frozen in liquid nitrogen and stored at –80 °C. For biochemical analyses, the tissues were ground under liquid nitrogen.

2.2. Avobenzone measurement

Water samples (250 ml) were taken weekly prior to the water exchange. Thus, the reported avobenzone concentrations corresponds to the levels detected in the water two days after the avobenzone addition. Water samples were stored at –20 °C until further analyses. For analysis, the samples were spiked with an internal standard (benzophenone-3-D5) and acidified to pH 3.0 with HCl (5 M). The samples were enriched and cleaned-up via solid phase extraction and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Supplementary Table 1) as described earlier (Fisch et al., 2021). Avobenzone was measured in positive mode. For data quantification linear calibration curves ($r^2 > 0.99$) from 0.01 to 50 µg/ml ($n = 10$) were applied. The method recovery rate was 100 ± 4.6 % ($n = 3$). Measured concentrations were corrected to the recovery rate and expressed as µg/l. The standards (avobenzone, benzophenone-3-D5 (BP-3-D5)) were produced by Dr. Ehrenstorfer GmbH. The solvents methanol and acetone were of analytical optigrade and acquired from Promochem (Germany).

The analytical grade acids were purchased from VWR (HCl, 5 M), acetic acid and formic acid from Sigma-Aldrich (Germany).

2.3. Oxidative stress markers

For determination of protein carbonyl (PCO) content and lipid peroxidation (LPO) levels, ~35 mg of tissue powder was mixed (1:10 w:v) with ice-cold 10 mM phosphate-buffered saline (PBS, pH 7.4) and split into two aliquots. The aliquots were mixed 1:2 (v:v) with 50% trichloroacetic acid (TCA) and incubated for 15 min on ice. The samples were then centrifuged for 10 min at $5000 \times g$ at 4 °C. Supernatants were separated from the pellet and combined. Pellets and supernatants were stored at –80 °C until further processing.

LPO was determined in the supernatant using the thiobarbituric acid (TBA) method (Ohkawa et al., 1979). TBA (21 mM in 0.1 M HCl) was added to the supernatants in 1:1 (v:v) ratio. The samples were incubated for 20 min at 90 °C and cooled for 5 min on ice. LPO was measured as thiobarbituric acid reactive substances (TBARS) at 532 nm and converted into malondialdehyde (MDA) equivalents using the extinction coefficient of $\epsilon = 1.56 \times 10^5 \text{ cm/M}$.

Protein carbonyls (PCO) were determined in the pellets using the 2,4-dinitrophenyl hydrazine (DNPH) method (Levine et al., 2000). Pellets from two aliquots of the same sample were treated either with DNPH (10 mM in 2 M HCl) and or with HCl (as a blank), and incubated at 37 °C for 1 h. The pellets were washed three times with 5 % TCA and dissolved in 8 M urea for 40 min at 90 °C. PCO was measured at 370 nm and calculated using the extinction coefficient of $\epsilon = 2.2 \times 10^4 \text{ cm/M}$. Protein carbonyls were standardized to the total protein content measured with the Bradford method and bovine serum albumin as a standard (Bradford, 1976).

For the measurement of CAT activity, SOD activity and TAOC, the ground tissues ($55.9 \text{ mg} \pm 3.8 \text{ mg}$) were incubated on ice for 10 min in phosphate buffered saline (PBS, pH 7.8, 50 mM) in the ratio of 1:15 (w:v). The supernatant (10 min, 4 °C, $5000 \times g$) was stored at –80 °C.

CAT activity was determined as described elsewhere (Hadwan, 2018). The samples were incubated with 6.7 mM H_2O_2 for 2 min at 37 °C. The incubation was stopped by adding a stopping solution (1.015 mg/ml $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.5 mg/ml $(\text{NaPO}_3)_6$, 81 mg/ml NaHCO_3) in the ratio 1:3.4 (v:v). After 10 min incubation in the dark at room temperature, the absorbance of carbonato-cobaltate (III) complex was measured at 440 nm and blank-corrected. CAT activity was expressed as U/mg wet tissue.

SOD activity was measured using the XTT-formazan method (Okado-Matsumoto and Fridovich, 2001a, 2001b; Ukeda et al., 1997). Xanthine (115 µM) and XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; 28.8 µM) were added to the sample, and the reaction started with xanthine oxidase (3.5 mU/ml). The final sample dilution was 1:8.7 (v:v). XTT-formazan formation was monitored for 20 min at 27–28 °C and 470 nm and calculated using $\epsilon = 21600 \text{ cm/M}$ at pH 7.8. SOD activity was expressed as mU/mg wet tissue.

Total antioxidant capacity was assessed using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method (Re et al., 1999). The samples were diluted with PBS (50 mM, pH 7.8) 1:20 (v:v), mixed with ABTS radical solution (final assay concentration 104 µM ABTS and 37 µM $\text{K}_2\text{S}_2\text{O}_8$; prepared fresh every second day at least 12 h before measurement) and incubated for 6 min at room temperature while shaking. Absorbance was measured and TAOC calculated as Trolox equivalents using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as a standard.

2.4. Metabolites

Metabolites were extracted from the coelomic fluid ($883 \text{ }\mu\text{l} \pm 169 \text{ }\mu\text{l}$) and ground body wall ($50 \text{ mg} \pm 6.5 \text{ mg}$) using LC-MS grade ethanol (1 ml; 80%) (Bruhns et al., 2023a, 2023b; Haider et al., 2020). The samples

were incubated for 5 min and the supernatants (10 min, 4 °C, 13.000×g) freeze-dried using a cooling trap (Unicryo MC 2 L, UniEquip, Germany). The samples were then dissolved in LC-MS-grade water (ROTISOLV®, Carl Roth) and filtered (0.2 µm, Omnifix®-F, Braun, Germany). Metabolite concentrations were measured using the liquid-chromatograph-mass spectrometer LCMS-8050 (Shimadzu) (Haider et al., 2020). The internal LC-MS/MS package for metabolites (Vers. 2, Shimadzu) and the LabSolutions software package (Shimadzu) were used for metabolite identification and quantification. 2-(N-morpholino)ethanesulfonic acid (MES) (1 µg/ml ethanol) served as an internal standard. Signals for citrate and isocitrate could not be distinguished, and these metabolites are reported together as (iso)citrate. For pathway enrichment analysis in MetaboAnalyst (iso)citrate was treated as citrate.

2.5. Statistics

Outliers >1.5x interquartile range were excluded from further analysis. Relevance of mesocosm replicate or the sampling time (for avobenzone content in water) was evaluated using a generalized linear model. Time of water sampling had no effect in the avobenzone content ($p > 0.05$). The avobenzone content between treatments was analyzed using Mann-Whitney-U test. All measured parameters (including metabolites) were not significantly affected by mesocosm distribution after Bonferroni correction ($p > 0.05$). Hence, values of different mesocosms were pooled.

Biomarkers were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) followed by one-way ANOVA and Bonferroni post-hoc. Biomarkers that violated these requirements were analyzed by non-parametric Kruskal-Wallis test with Dunn-Bonferroni post-hoc or Welch-ANOVA with Games-Howell post-hoc using IBM SPSS Statistics 27.

Metabolic profiles were analyzed using MetaboAnalyst 5.0 (Pang et al., 2021). The data was normalized using auto scaling and missing values were filled by the k-nearest neighbors' method. Pathway enrichment analysis was conducted using *Drosophila melanogaster* as the reference library, relative-betweenness centrality for topology analysis and global test as the enrichment method. The different exposures were compared to the control. Pathways with an impact = 0 were excluded from further analysis and significant pathways selected by recalculating the false discovery rate (FDR) cutoff using the Benjamini-Hochberg procedure with $\alpha = 0.05$ (Benjamini and Hochberg, 1995). To reduce the dimensionality of the dataset, partial least squares-discriminant analysis (PLS-DA) was conducted. Metabolites contributing to the separation were selected for further investigation using the loadings cutoff ± 0.3 . Further, a feature wise Pearson r correlation analysis was conducted across all treatments.

3. Results

3.1. Mortality

One worm died in each of the three exposure groups, corresponding to a mortality rate of 6.7% ($n = 15$) in all exposure groups. In the control group all worms survived.

3.2. Avobenzone content

No avobenzone was detected in the water in the control and nZnO exposures. Average avobenzone content of the water was similar in avobenzone and combined nZnO + Avo exposures (mean $\pm$ SEM: 2.2 ± 0.40 µg/l, $n = 6$ and 1.9 ± 0.31 µg/l, $n = 9$, respectively) ($p > 0.05$).

3.3. Antioxidant system and oxidative damage

Total antioxidant capacity (TAOC) increased significantly in the

body wall of the lugworms exposed to either avobenzone or nZnO alone (Fig. 1 A). The TAOC levels in the body wall of the worms co-exposed to avobenzone and nZnO were not significantly different from the control and lower than in two other exposure groups (Fig. 1 A). No significant change in SOD activity was found in individual pollutant exposures relative to the control (Fig. 1 B). Co-exposure to avobenzone and nZnO led to a significant decrease of superoxide dismutase (SOD) activity compared to the control and exposure to either UV filter alone (Fig. 1 B). CAT activity in the experimental exposure groups did not change relative to the control baseline (Fig. 1 C). However, higher CAT activity was detected in the body wall of the worms exposed to nZnO than in those exposed to avobenzone or nZnO + Avo (Fig. 1 C). GSH content of the body wall was not significantly affected by any of the experimental treatments (Supplementary Table 2).

Lipid peroxidation increased in the body walls of worms co-exposed to avobenzone and nZnO compared to the controls and avobenzone exposure (Fig. 2 A). Furthermore, LPO levels in the tissues of the worms exposed to nZnO were higher than in worms exposed to avobenzone. Exposure to avobenzone, nZnO or their combinations showed no

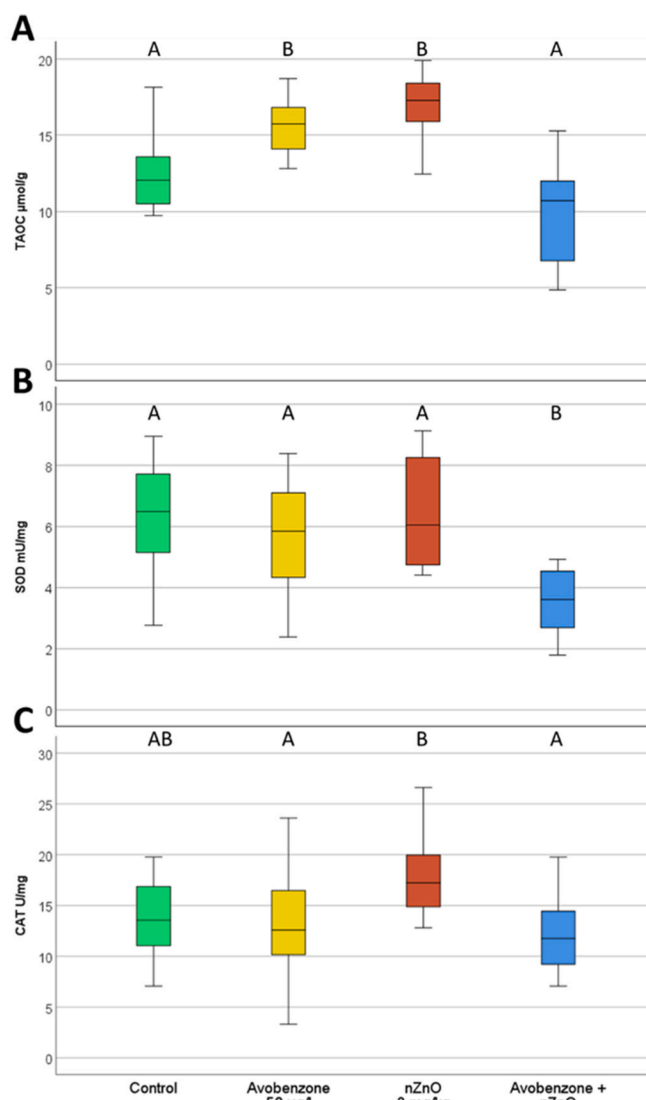


Fig. 1. Antioxidant system: Total antioxidant capacity (TAOC, A) in Trolox equivalents, superoxide dismutase activity (SOD, B) and catalase activity (CAT, C) are shown and scaled to fresh tissue mass. Boxplots show median, inter-quartile range (box) as well as minimum and maximum (whiskers). Columns that do not share the same letter are significantly ($p < 0.05$) different.

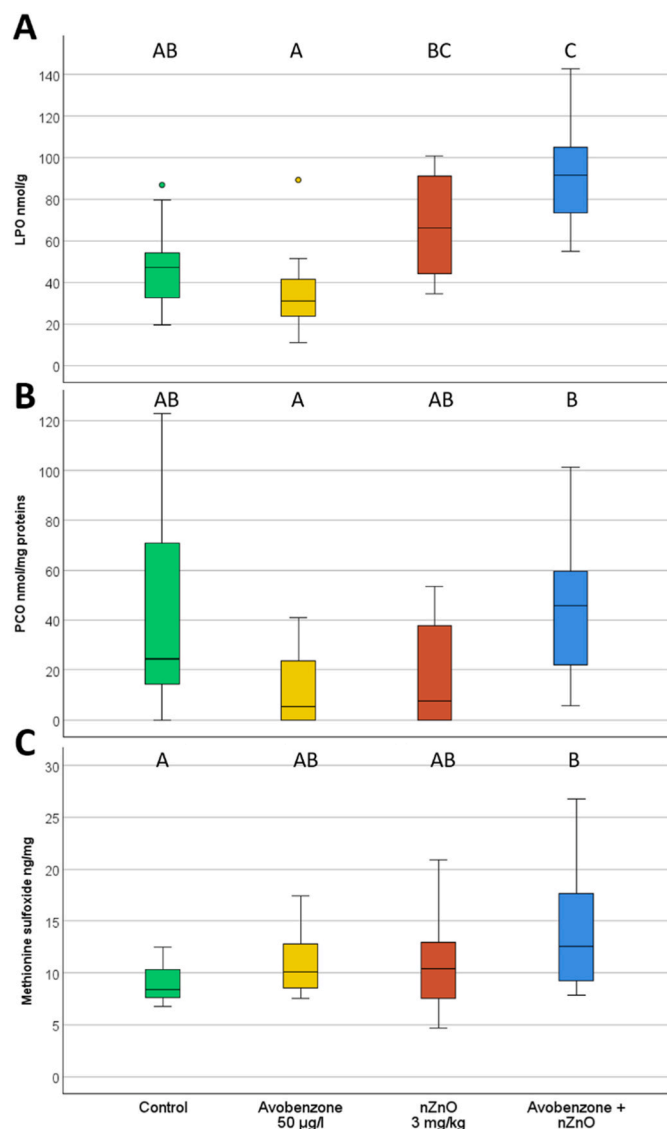


Fig. 2. Oxidative stress markers: lipid peroxidation (LPO, A) in malondialdehyde equivalents, protein carbonyls (PCO, B) and methionine sulfoxide (MetO, C) are shown and scaled to fresh tissue mass (LPO and MetO) or protein content (PCO). Boxplots show median, interquartile range (box) as well as minimum and maximum (whiskers; considering <1.5x interquartile range). Columns that do not share the same letter are significantly different ($p < 0.05$). Circles indicate outliers >1.5x interquartile range.

evidence of effect on PCO content compared to the control (Fig. 2 B). However, PCO content was higher in the body wall of worms co-exposed to avobenzone and nZnO compared to avobenzone alone (Fig. 2 B). Co-exposure to avobenzone and nZnO also led to significant accumulation of methionine sulfoxide (MetO) in the body wall (Fig. 2C). MetO content of the worms exposed to either avobenzone or nZnO alone was intermediate between the control and the combined exposure. MetO content was not altered in the coelomic fluid of exposed worms.

3.4. Metabolite profiles body wall and coelomic fluid

A total of 39 metabolites was measured in the body wall and the coelomic fluid of *A. marina* (Fig. 3, Supplementary Table 2, Supplementary Table 3). Fumarate, GSH, NAD and Pro were only found in the body wall, whereas Cys and cystine were only detected in the coelomic fluid.

Correlation analysis showed a cluster of positively correlated

metabolites in the body wall including branched chain amino acids (BCAAs) Leu, Ile and Val, aromatic amino acids (AAAs) Phe, Trp and Tyr as well as Thr, Gln, Lys, Met, MetO and Arg (Fig. 3 A). 2-oxoglutarate and Asp were negatively correlated with most of the studied metabolites (Fig. 3 A). In the coelomic fluid, correlation analysis showed a cluster of positively correlated metabolites including the BCAAs Leu, Ile and Val, the AAAs Phe, Tyr and Trp as well as His, Ala, Glu, cystine, MetO, Asn, Asp, Gln, Lys, Met, Thr, Ser and Cys (Fig. 3 B). AMP and malate were negatively correlated with most studied metabolites (Fig. 3 B).

PLS-DA analysis revealed that the first two components explain 26.7% and 15.6% of the variation in the metabolite concentrations in the body wall of *A. marina* (Fig. 4). There was a shift in the metabolite profile of the nZnO-exposed groups (nZnO and nZnO + Avo) relative to the controls and avobenzone-exposed groups along the 1st principal component (Fig. 4). The metabolite profile of the body wall tissue of the worms exposed to avobenzone alone was shifted along the 2nd component relative to the metabolite profiles from the worms exposed to nZnO or co-exposed to nZnO + Avo. However, all groups showed partial overlap of the metabolite profiles plotted in the plane of the two first principal components.

Based on the loadings (cutoff ± 0.3), Gln, Lys and Leu were associated with the 1st component whereas Pro, AMP, 2-oxoglutarate, malate, succinate, pyruvate and L-arginosuccinate were associated with the 2nd component (Table 1).

PLS-DA analysis of the metabolite profiles in the coelomic fluid revealed that the first three components explain 52.5%, 6.9% and 7.9% of the metabolic profiles, respectively (Supplementary Fig. 1; Table 1). The metabolite profiles of the coelomic fluid of different experimental groups showed less separation on the PLS-DA biplots than those in the body wall (Supplementary Fig. 1).

Pathway enrichment analysis revealed six pathways that were significantly altered in the body wall of the worms exposed to nZnO including Ala, Asp, and Glu metabolism; Arg biosynthesis; TCA cycle, D-Gln and D-Glu metabolism; glyoxylate and dicarboxylate metabolism; and purine metabolism (Table 2). Avobenzone exposure or the combined exposure to nZnO + Avo did not significantly affect metabolic pathways in the body wall of the worms (data not shown). There were no detectable pathway enrichments in the coelomic fluid in any treatment group relative to the control (data not shown).

Besides MetO, six metabolites in the body wall and one metabolite in the coelomic fluid showed significant differences in the mean concentrations between the experimental treatment groups (Table 3). Thr and 2-oxoglutarate increased in the body wall of the lugworms co-exposed to avobenzone and nZnO (Table 3). Asp, citrate and citrulline increased in the body wall after nZnO exposure, whereas L-argininosuccinate increased as a result of avobenzone exposure (Table 3). In the coelomic fluid, Trp was significantly decreased by nZnO exposure (Table 3).

4. Discussion

4.1. Combined exposures to nZnO and avobenzone enhances oxidative stress in *A. marina*

Our study showed disturbances of the redox balance in the worms exposed to two common UV filters. Exposures to avobenzone or nZnO alone led to a significant increase in TAOC indicating upregulation of cellular antioxidants to counteract the pollutant-induced ROS formation (Hermes-Lima, 2005). No accumulation of oxidative lesions (LPO, PCO, MetO) was observed in single pollutant exposures indicating that elevated TAOC was sufficient to prevent oxidative damage. This indicates that the tested concentrations of avobenzone ($\sim 2 \mu\text{g/l}$) and nZnO (3 mg/kg) do not cause significant oxidative stress in *A. marina*. In Mediterranean mussels and freshwater fish (zebrafish and crucian carp), exposure to avobenzone (10–182 $\mu\text{g/l}$) altered activity of SOD and CAT, although the direction of change (increase or decrease) was inconsistent

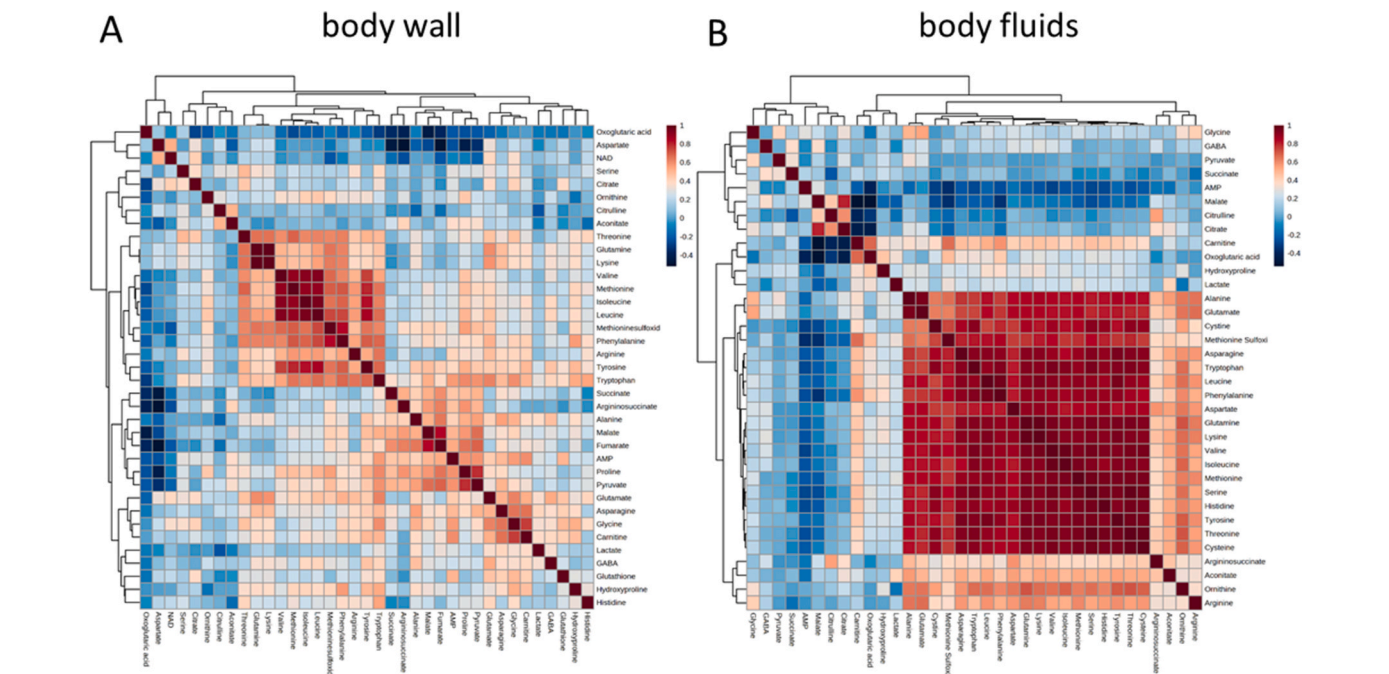


Fig. 3. Correlation heat maps for body wall (A) and coelomic fluid (B). Correlation coefficient is indicated by color with red being highly positive correlated and blue higher negatively correlated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

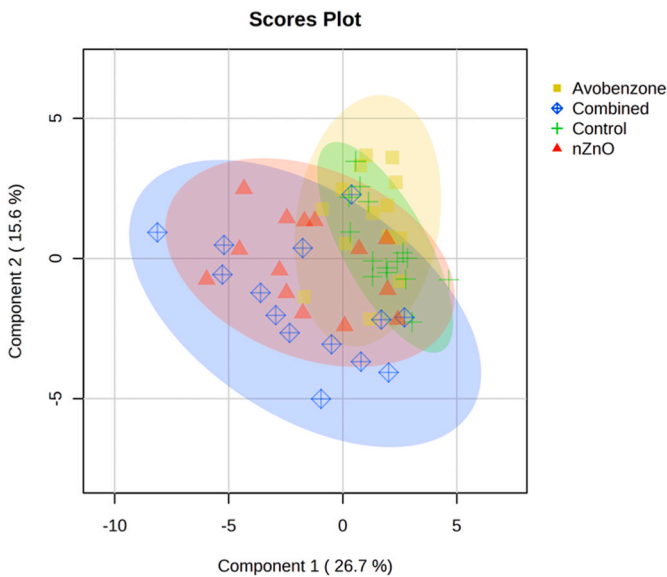


Fig. 4. PLS-DA 2D scores plot for the body wall including the first two components.

among these studies (Bordalo et al., 2022; Liu et al., 2022; Ma et al., 2017). Interestingly, an earlier study on *A. marina* found significant LPO accumulation after exposure to 1 mg nZnO/kg for two weeks (Bruhns et al., 2023b). In contrast, our present study using 3 mg/kg nZnO showed only a weak (statistically non-significant) trend of increased LPO formation in the body wall of *A. marina*. Possibly, longer (three weeks) exposure used in our present study allowed sufficient time for antioxidant system upregulation, thereby minimizing oxidative damage (Amiard et al., 2006; Marisa et al., 2016; Ng and Wang, 2004). Taken together, our findings indicate that exposure to avobenzone (~2 µg/l) and nZnO (3 mg/kg) alone lead to mild redox misbalance but do not cause oxidative damage in the tissues of *A. marina*. In contrast, the combination of avobenzone and nZnO exposure caused a significant

Table 1
Loadings of PLS-DA analysis for the first two components and 1st and 3rd component of the body wall and coelomic fluid, respectively. Only metabolites with absolute values of the loadings ≥ 0.3 (marked in bold) in PC1, PC2 or PC3 are shown.

body wall	Comp 1	Comp 2
Glutamine	-0.308	-0.026
Proline	-0.118	0.326
Lysine	-0.306	-0.032
AMP	-0.118	0.315
Leucine	-0.301	0.093
2-oxoglutarate	-0.022	-0.326
Malate	-0.066	0.363
Succinate	0.017	0.336
Fumarate	-0.022	0.335
Pyruvate	-0.090	0.323
Argininosuccinate	0.009	0.364
coelomic fluid	Comp 1	Comp 3
Citrulline	-0.002	0.389
Malate	-0.043	0.547
Citrate	0.017	0.560

decrease in SOD activity and a modest decrease in TAOC accompanied by significant increases in the oxidative damage (LPO, MetO). Hence, oxidative stress caused by the combined nZnO + Avo exposure appears to exceed the capacity of the antioxidant system leading to accumulation of oxidative damage in lipids and proteins.

4.2. nZnO drives metabolic shifts in the body wall tissues

Correlation analysis detected two clusters of positively correlated metabolites in the body wall of *A. marina* that mostly consisted of BCAAs, AAAs and essential FAAs (such as Arg, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp and Val) (Fitzgerald and Szmant, 1997). Free amino acids serve as important intracellular osmolytes and building blocks for proteins in marine polychaetes, so that changes in FAA levels induced by pollutant exposures may have implications for intracellular osmoregulation and protein biosynthesis (Fitzgerald and Szmant, 1997; Li et al., 2021). PLS-DA analysis showed high similarity of metabolite profiles of

Table 2

Pathway enrichment analysis: Significantly altered metabolic pathways in the body wall after exposure to nZnO alone ($p < \text{FDR threshold}$, $\text{impact} > 0$). Total Cmpd = total number of metabolites in the respective pathway; Hits = metabolites of the respective pathway detected in this study. Significantly changed metabolites might differ from single metabolite analysis in Table 3.

Pathway	Total Cmpd	Hits	p	FDR threshold	Impact	measured total	Significant (Metaboanalyst $p < 0.05$)
Glyoxylate and dicarboxylate metabolism	24	6	0.001	0.00263	0.2125	Aconitate, Citrate, Glu, Malate, Gln, Gly	Citrate, Glu, Gln
Arg biosynthesis	12	9	0.003	0.00526	0.94286	Glu, Arg, Citrulline, Asp, Gln, 2-Oxo, Ornithine, Fumarate, l-arginosuccinate	Glu, Asp, Gln
D-Gln and D-Glu metabolism	5	3	0.006	0.00789	1	Gln, Glu, 2-Oxo	Gln, Glu
Citrate cycle (TCA cycle)	20	7	0.007	0.01052	0.35197	2-Oxo, Succinate, Malate, Aconitate, Citrate, Pyruvate, Fumarate	Succinate, Citrate
Purine metabolism	63	2	0.008	0.01315	0.05148	Gln, AMP	Gln
Ala, Asp and Glu metabolism	23	11	0.010	0.01578	0.82433	Asp, Asn, l-arginosuccinate, Ala, 2-Oxo, Gln, glu, Butanotate, Fumarate, Pyruvate, Succinate	Asp, Gln, Glu, Succinate

the body wall tissues in control and avobenzone-exposed worms indicating that avobenzone exposure ($\sim 2 \mu\text{g/l}$) does not lead to significant alterations of intermediary metabolism in lugworms. This conclusion is supported by the pathway enrichment analysis that did not identify any significantly altered metabolic pathways in avobenzone-exposed worms.

Unlike single avobenzone exposure, nZnO exposure alone or in combination with avobenzone led to a shift of the metabolic profiles in the body wall of *A. marina*. The metabolic shifts were stronger in single nZnO exposure relative to the combined nZnO + avobenzone exposure. This is shown by a significant shift in metabolic pathways related to energy metabolism (Ala, Asp, and Glu metabolism, TCA cycle, glyoxylate and dicarboxylate metabolism, and purine metabolism) and ammonia detoxification (Arg biosynthesis and D-Gln and D-Glu metabolism) in the nZnO-exposed worms. These findings are consistent with the results of our previous study using short-term (2 weeks) exposure of *A. marina* to 1 mg/kg nZnO that showed shifts in the same metabolic pathways (except for the purine metabolism) (Bruhns et al., 2023b). Analysis of individual metabolites identified (iso)citrate and Asp as the most significant contributors to the metabolic shifts in the body wall observed in the present study, consistent with earlier studies showing shifts in the levels of these metabolites under environmental stress (Bruhns et al., 2023b; Fritzsche and von Oertzen, 1995; Liu et al., 2009; O'Malley and Terwilliger, 1975).

Unexpectedly and unlike the single pollutant exposures, the combined exposure to nZnO and avobenzone did not led to significant shifts in the metabolic pathways in *A. marina*. This suggests that co-exposure to avobenzone might mitigate the metabolic disturbances caused by nZnO in *A. marina*. Existing studies on combined UV filter effects indicate that antagonistic interactions between different filters are not uncommon. Thus, a study on *Daphnia magna* showed lower toxicity of mixtures of three organic UV-filters (ethylhexyl methoxycinnamate, octocrylene, and avobenzone) compared to the single toxicity tests (Park et al., 2017). Organic UV filters also showed antagonistic effects in mixtures in standard bacterial toxicity tests (Kudlak et al., 2022; Marcin and Aleksander, 2023). Furthermore, studies on human skin and fish gill cells demonstrated mitigating effects on toxicity and inflammation for some combinations of organic and mineral UV filters (Martin et al., 2022; Migdal et al., 2010). Martin et al. (2022) suggest that the mitigating effect might be derived from adsorption of the organic UV filter by the NP. It remains to be determined whether this mechanism also plays a role in our exposures, since the sediment provides abundant alternative sites for avobenzone adsorption (Apel et al., 2018a, b; Cadena-Aizaga et al., 2020; Ma et al., 2017; Pawlowski and Petersen-Thiery, 2020). Alternatively, the mitigating effects of the combined exposures might be due to the cellular antagonism of the two studied UV filter at the level of uptake or interaction with intracellular targets. Regardless of the specific mechanisms of antagonism, these findings highlight the urgent need for further assessments of the ecologically realistic mixtures of the UV filters, as the exclusive focus on

single compound toxicity might overestimate the ecotoxicological hazards for some common UV filter combinations.

4.3. Conclusion and outlook

Our study revealed distinct metabolic effects of two common UV filters, avobenzone and nZnO, on the intermediary metabolism of *A. marina*. Exposure to nZnO altered multiple pathways associated with bioenergetics and nitrogen excretion, consistent with previous reports identifying nZnO as a metabolic disruptor in this species (Bruhns et al., 2023b). Conversely, avobenzone demonstrated no significant effect on *A. marina* metabolism when applied alone, showed a mitigating effect on metabolic disturbance when co-exposed with nZnO. Our study showed negative effects of the two studied UV filters on the redox balance of *A. marina* tissues but the degree of disturbance differed between the single and combined pollutant exposures. Thus, single exposures to avobenzone and nZnO showed a mild oxidative stress response compensated by upregulation of antioxidants, whereas the combined exposure to avobenzone and nZnO resulted in enhanced oxidative stress and damage to the lipids and proteins. Comparison of our present study with a similar research using shorter (two week) exposure to nZnO showed milder oxidative stress in longer exposures. This indicates that exposure duration plays an important role in toxicity of nZnO, possibly due to the time-dependent activation of detoxification and damage repair mechanisms (Brausch and Rand, 2011; Marcin and Aleksander, 2023; Ramos et al., 2015). Given the diverse ingredients in sunscreens (Osterwalder et al., 2014), additional studies involving different combinations are necessary to assess the environmental impact of this particular PCP type. Further, other environmental factors such as temperature or radiation could be taken into focus to address possible effects on the toxicity of the UV filters (e.g. due to degradation) or the detoxification response of the lugworm (Afonso et al., 2014; Theede, 1980; Vieira Sanches et al., 2021).

CRedit authorship contribution statement

Torben Bruhns: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Candela Sánchez-Girón Barba:** Writing – review & editing, Investigation, Data curation. **Lilian König:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Stefan Timm:** Writing – review & editing, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Kathrin Fisch:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Inna M. Sokolova:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Table 3

Summary of the individual analysis of the significantly affected metabolites of the body wall (BW) and body fluids (BF). AN = ANOVA, WA= Welch-ANOVA, KW= Kruskal-Wallis test, ND = not detected, SEM = standard error of mean, Avo = avobenzene, Combi = combined exposure. Significant statistics and corresponding mean values are highlighted in bold. Different letters indicate significant differences within each sample type. See [Supplementary Table 2](#) and [Supplementary Table 3](#) for non-significant metabolites.

	Test	Statistic	Post hoc		Mean BW (ng/mg ± SEM)	Mean BF (ng/mg ± SEM)
			BW	BF		
2-oxoglutarate			A	Control	96.1 ± 7.8	0.15 ± 0.014
BW	WA	F(3,27.588)=3.401 p=0.032	A	Avo	89.4 ± 5.9	0.12 ± 0.012
BF	KW	Chi2 = 2.427 P = 0.489	B	Combi	177 ± 26.5	0.15 ± 0.013
Argininosuccinate			AB	nZnO	98.6 ± 8.2	0.15 ± 0.015
BW	WA	F(3,27.19)=2.992 p=0.048	AB	Control	2.62 ± 0.25	0.01 ± 0.001
BF	AN	F(3,52) = 0.257 p = 0.856	B	Avo	3.73 ± 0.51	0.01 ± 0.002
Aspartate			AB	Combi	2.29 ± 0.37	0.01 ± 0.001
BW	AN	F(3,52)=4628 p=0.006	A	nZnO	2.10 ± 0.21	0.01 ± 0.001
BF	AN	F(3,51) = 1.095 p = 0.360	A	Control	174 ± 36.5	19.5 ± 2.1
Citrate			A	Avo	142 ± 35.2	19.1 ± 2.0
BW	KW	Chi2=18.147 p<0.001	AB	Combi	178 ± 27.2	23.3 ± 2.9
BF	KW	Chi2 = 1.774 P = 0.621	B	nZnO	301 ± 29.0	17.9 ± 1.8
Citrulline			A	Control	5.42 ± 0.76	1.21 ± 0.27
BW	KW	Chi2=18.147 p<0.001	AB	Avo	8.43 ± 0.92	1.07 ± 0.25
BF	KW	Chi2 = 1.774 P = 0.621	A	Combi	5.64 ± 0.69	0.86 ± 0.175
Citrulline			B	nZnO	10.6 ± 1.0	0.85 ± 0.239
BW	AN	F(3,51)=5.099 p=0.004	A	Control	0.23 ± 0.023	0.05 ± 0.007
BF	KW	Chi2 = 7.750 P = 0.432	A	Avo	0.23 ± 0.019	0.05 ± 0.009
Cystine			AB	Combi	0.29 ± 0.023	0.03 ± 0.004
BW	ND	ND	B	nZnO	0.33 ± 0.024	0.04 ± 0.005
BF	WA	F(3,25.114)=4.610 p=0.011	A	Control		1.21 ± 0.3
Glutamine			A	Avo		1.12 ± 0.35
BW	WA	F(3,26.155)=3.94 p=0.019	A	Combi		2.22 ± 0.65
BF	KW	Chi2 = 2.554 P = 0.466	A	Zinc		0.49 ± 0.117
Isoleucine			A	Control	34.8 ± 2.6	11.9 ± 1.6
BW	WA	F(3,26.155)=3.94 p=0.019	A	Avo	37.4 ± 3.5	11.1 ± 1.8
BF	KW	Chi2 = 2.554 P = 0.466	A	Combi	54.2 ± 6.8	14.2 ± 2.4
Lysine			A	nZnO	51.0 ± 5.6	8.55 ± 0.54
BW	KW	Chi2 = 3.137 p = 0.371	A	Control	4.71 ± 0.47	15 ± 2.7
BF	WA	F(3,25.758)=3.751 p=0.023	A	Avo	4.70 ± 0.38	15.7 ± 3.5
Phenylalanine			A	Combi	6.59 ± 1.04	17.3 ± 3.6
BW	KW	Chi2 = 3.137 p = 0.371	A	nZnO	6.30 ± 0.84	8.25 ± 1.25
BF	WA	F(3,25.758)=3.751 p=0.023	A	Control	36.6 ± 2.7	12.6 ± 1.7
Serine			A	Avo	39.9 ± 4.0	11.8 ± 1.9
BW	WA	F(3,25.93)=4.08 p=0.017	A	Combi	57.4 ± 7.3	15 ± 2.6
BF	KW	Chi2 = 2.688 P = 0.442	A	nZnO	54.5 ± 6.0	9.03 ± 0.58
Threonine			A	Control	9.83 ± 0.82	31.3 ± 4.9
BW	KW	Chi2=8.403 p=0.038	A	Avo	11.5 ± 0.5	30.1 ± 4.7
BF	KW	F(3,51) = 1.007 p = 0.397	A	Combi	17.0 ± 2.8	35.5 ± 5.4
Tryptophan			A	nZnO	14.0 ± 1.6	24 ± 3.4
BW	AN	F(3,51) = 0.719 p = 0.545	A	Control	20 ± 2.5	5.47 ± 0.8
BF	WA	F(3,25.144)=4.548 p=0.011	A	Avo	24.3 ± 1.7	5.05 ± 0.9
Valine			A	Combi	22.3 ± 1.6	6.59 ± 1.25
BW	AN	F(3,51) = 0.719 p = 0.545	A	nZnO	23.7 ± 2.9	3.22 ± 0.33
BF	WA	F(3,25.144)=4.548 p=0.011	A	Control	9.50 ± 0.55	5.38 ± 0.88
Threonine			A	Avo	11.6 ± 0.7	4.99 ± 1.02
BW	KW	Chi2=9.277 p=0.026	AB	Combi	12.7 ± 0.7	6.76 ± 1.4
BF	KW	Chi2 = 2.000 p = 0.572	B	nZnO	12.3 ± 1.2	3.25 ± 0.23
Tryptophan			AB	Control	1.51 ± 0.10	11.9 ± 2.2
BW	WA	F(3,24.806) = 2.136 p = 0.121	A	Avo	1.67 ± 0.07	11.7 ± 2.5
BF	WA	F(3,24.272)=6.829 p=0.002	A	Combi	1.95 ± 0.28	14.9 ± 3.3
Valine			B	nZnO	2.1 ± 0.24	4.93 ± 0.72
BW	AN	F(3,49) = 2.089 p = 0.114	A	Control	5.31 ± 0.44	13.1 ± 2.3
BF	WA	F(3,23.409)=4.012 p=0.019	A	Avo	6.09 ± 0.55	12.2 ± 2.6
			A	Combi	7.75 ± 1.03	16 ± 3.5
			A	nZnO	7.63 ± 1.13	7.65 ± 0.59

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2024.142184>.

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I hereby declare that the present thesis is entirely my original work unless stated otherwise. Information or work that is derived from other publications or authors is referenced accordingly. I am aware of the university's regulations regarding plagiarism.

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