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Research Paper

An Assessment of Pb and Cu in Waters, Sediments, and Mud Crabs (*Scylla serrata*) from Mangrove Ecosystem Near Tanjung Api-Api Port Area, South Sumatra, Indonesia

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Abstract
Heavy metal pollution from anthropogenic activities can harm aquatic ecosystems. This study aims to determine the concentration of heavy metals (Pb and Cu) in waters, sediments, and mud crabs (*Scylla serrata*), and to analyze the relationship between environmental parameters and *S. serrata* which is consumed by humans. Samples were taken in the mangrove ecosystem around the Tanjung Api-Api port area in South Sumatra, Indonesia. Pb and Cu analysis used the Atomic Absorption Spectrophotometer (AAS). Pb and Cu linkages in waters, sediments, and *S. serrata* analyzed by SigmaPlot V12.5 and Principal Component Analysis (PCA) analyzed by XLSTAT 2022. The limit consumption of *S. serrata* was calculated using MWI (Maximum Weekly Intake) and MIT (Maximum Intake Tolerance). Based on the results, the heavy metal Pb in water was 0.1055 – 0.1322 mg.L^{-1}, and Cu was not detected. Furthermore, Pb in sediments ranged from 7.0104 - 11.8186 mg.kg^{-1}, Cu 3.7127 - 4.5347 mg.kg^{-1}, and Pb in *S. serrata* ranged from 0.0001 - 0.0021 mg.kg^{-1}, and Cu ranged from 0.03 – 0.0791 mg.kg^{-1}. The concentration of heavy metals in water, sediment, and *S. serrata* had not exceeded the specified quality standard, except for Pb in water. The principal component analysis obtained F1 (44.35%), F2 (27.53%) and F3 (17.83%) groups. Based on MWI and MIT values that *S. serrata* was still safe for human consumption.

Keywords
Anthropogenic Activities, Heavy Metals, Mud Crab, Sediment

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1. INTRODUCTION

The rapid economic development in coastal areas, such as industrial activities, household waste, agriculture, and port activities, produces substantial quantities of pollutants discharged into coastal waters (Apri et al., 2021; Rizk et al., 2022). The waste generated from these activities can cause a decrease in water quality, impacting aquatic ecosystems (Rozirwan et al., 2022). One of the causes of the decline in water quality is heavy metal pollution because it has toxic, persistent, and bioaccumulate characteristics in nature, which can have detrimental effects on global ecosystems and human health (Briffa et al., 2020; Rizk et al., 2022). Heavy metals belong to the group of pollutants because they are difficult to decompose (non-degradable) and are easy to accumulate with a weight of 5 g.cm^{-3} (Shrestha et al., 2021).

In general, heavy metals for the growth and development of organisms are divided into two categories: essential and non-essential heavy metals. Many essential heavy metals such as Cu, Fe, Mn, Co, Zn, and Ni are essential for maintaining the human body metabolism as long as they are not used excessively. Non-essential heavy metals such as Cd, Pb, Hg, Cu, and Al are not even needed in small amounts for every metabolic process and can cause poisoning (toxicity) (Bharti and Sharma, 2022). These metals pollute the waters and accumulate in sediments and organisms (Rizk et al., 2022). The high level of heavy metals in the waters negatively influences the biochemical and morphological traits of microbes, organisms, and the human body, causing many serious diseases such as cancer, paralysis, and carcinogens. Human well-being can be threatened due to heavy metals, which are considered the main components of pollutants in environmental waters (Briffa et al., 2020).

Organisms from the crustacean class can be used as bioindicators of heavy metal contamination in waters and sediments because of their ability to accumulate heavy metals. *S. serrata* lives in muddy substrates, so it has the potential as a bioindicator of heavy metal pollution (Soegianto et al., 2022). *S. serrata* is one of the highest export commodities in Indonesia and is among the most prominent fishery products in Banyuasin

Regency, South Sumatra. Monitoring heavy metals in water, sediment, and *S. serrata* is important to determine the potential for bioaccumulation and for environmental management. The interaction between biotic and abiotic in the waters creates an interconnected atmosphere (Maxwell et al., 2017). All anthropogenic that occurs in the environment seems to be a marker to know the status of the biotic group (Huggett, 2018; Upadhyay, 2020). Waters have a very dynamic and actual character so regular monitoring is needed to determine the latest environmental quality (Whitehead et al., 2019; Ustaoğlu et al., 2020).

Several researchers have studied heavy metal content, including Cu, Zn, Mg, Cd, and Cr, in crabs from mangrove ecosystems in Qi'ao, China (Zhang et al., 2019). The accumulation of heavy metal Cd in *S. serrata* in estuarine (Zhang et al., 2022b), and heavy metal health risk assessment in *S. serrata*, East Java Indonesia (Soegianto et al., 2022). Tanjung Api-Api mangrove areas have an essential role for the people of South Sumatra, such as shipping routes leading to the port and fishery activities. The difference in this study is not only analyzing *S. serrata* but also the relationship between heavy metals in the water and sediments where *S. serrata* is captured. This study aimed to analyze the heavy metal content of Pb and Cu in water, sediment, and *S. serrata* with water parameters and the maximum limit of *S. serrata* consumption.

2. EXPERIMENTAL SECTION

2.1 Study Area and Sampling Location

This research was carried out in the mangrove ecosystem near the Tanjung Api-Api port area in South Sumatra, Indonesia, at five observation stations with extensive mangrove vegetation (Figure 1). This location received seawater from the Bangka strait and freshwater from the Banyuasin river (Saputra et al., 2021; Almaniar and Rozirwan, 2021; Nugroho et al., 2022). Anthropogenic activities such as port, agriculture, industry, fisheries, and households could impact on these quality waters. Apri et al. (2021) stated that anthropogenic sources affect water environmental quality. Samples were taken during low tide conditions. The water conditions at the time were ideal for various crustaceans and gastropods, which dug and moved to terrestrial substrates in mangroves for protection (Rozirwan et al., 2022).

Water samples were taken on the surface of the water using a 500 mL polyethylene bottle and then preserved using HNO_3 until the pH reached ≤ 2 to prevent changes in organic matter by bacterial activity and transferred in an ice box (Rizk et al., 2022). Sediment samples were taken using sediment core, then put in plastic bags and chilled in an ice box (Apri et al., 2021). Crab samples were taken using traps carried out at low tide. All samples obtained were taken to the laboratory for further analysis.

2.2 Environmental Parameters

Water quality measurements were carried out in situ with three repetitions consisting of salinity using a refractometer, water temperature using thermometer, dissolved oxygen (DO) using DO meter (Hanna HI 98193), and pH using pH meter (Hanna HI 9811–5) (Apri et al., 2021).

Figure 1. Map of Sampling Location in Mangrove Areas Near the Tanjung Api-Api Port

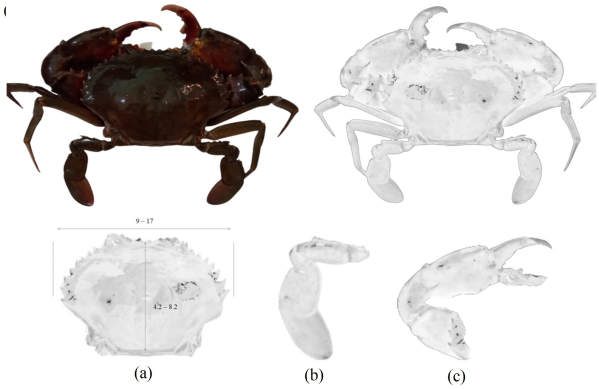

Figure 2. Details of *Scylla serrata* Morphological Characteristic; (a) Carapace, (b) Leg, (c) Cheliped

2.3 Sediment Grain Size Measurement

Sediment grain size analysis was carried out using wet sieving. Substrate type of sediment (gravel, sand, mud, and clay) analyzed using shepard triangle diagram with Microsoft Excel 2019. The type of sediment fraction was determined based on the most dominant values of the composition (Almaniar and Rozirwan, 2021).

2.4 Sample Preparation and Destruction

Water samples was filtered with 0.45 μm whatman filter paper, for sediment samples cleaned of foreign objects such as plastic pieces, and organic materials, to be further dried in an electric oven UF 55 Memmert at 60°C for 30 min and mashed into a powder with a mortar and pestle until fine particles and stored in polyethylene bottles (Yan et al., 2021). *S. serrata* was cleaned and mashed using a blender.

Destruction process were performed on heavy metals Pb and Cu for water, sediment, and *S. serrata* refers to (Rizk et al., 2022). Fifty milliliters of water sample was put into erlenmeyer and added 5 mL of HNO_3, heated with hotplate stirrer C-MAG HS 7 until the water sample reaches 15-20 mL. Furthermore, the sediment was acidly destroyed by putting ± 3 g of the sample in Erlenmeyer and adding 25 mL of distilled water to be heated on a hotplate with a temperature of 105°C-120°C. Mix HNO_3 as much as 5 mL and waited until the volume becomes 10 mL. After removal and cooling, added 5 mL of concentrated HNO_3 and 1 mL - 3 mL of $HClO_4$. The sediment sample was reheated with dampness until white smoke appeared, and the sample became clear, followed by heating for 30 min. After cooling, the sediment sample was filtered using quantitative filter paper.

Destruction of *S. serrata* samples was done using wet digestion to determined metal elements with low concentrations. The weighed sample was put into an Erlenmeyer, and HNO_3 (5–10 mL) and H_2O_2 (2 mL) were added. Digestion was carried out by setting up a microwave program. The result of the digestion was transferred into a 50 mL vial with ultra-distilled water and stored in polyethylene containers at room temperature until further measurements.

2.5 Atomic Absorption Spectroscopic Measurement

Measurement of heavy metal concentrations of Pb and Cu using an Atomic Absorption Spectrophotometer (Shimadzu AA-7000) with a wavelength of 283.3 nm for Pb and 324.7 nm for Cu.

2.6 Data Analysis

2.6.1 Bioconcentration Factor (BCF) Index

Bioconcentration factor (BCF) index were used to determine the pollutants bioaccumulation level in *S. serrata* mud crabs. The BCF calculated using the BCF formula.

$$\text{BCF index} = \frac{\text{Heavy metals concentration in biota}}{\text{Heavy metals concentration in water/sediment}} \quad (1)$$

2.6.2 Distribution Levels of Heavy Metals

The distribution level of heavy metals Pb and Cu in water, sediment, and *S. serrata* was analyzed using SigmaPlot V12.5.

2.6.3 Principal Component Analysis (PCA)

Principal component analysis determined the relationship between water parameters (DO, temperature, pH, and salinity) and the concentration of heavy metals in water, sediment, and *S. serrata*. This analysis was processed using XLSTAT 2022 (Apri et al., 2021).

2.6.4 Maximum Limit of Heavy Metals Consumption

Provisional Tolerable Weekly Intake was weekly intake accepted (without health effects) of trace and toxic metals through marine biota samples. The determination of the maximum limit of heavy metal consumption contained in *S. serrata* could be calculated using the following formula.

$$\text{Maximum Weekly Intake} = \text{Weight} \times \text{Provosional Tolerable Weekly Intake} \quad (2)$$

$$\text{Maximum Tolerable Intake} = \frac{\text{Maximum Weekly Intake}}{\text{Heavy metals concentration in biota}} \quad (3)$$

3. RESULTS AND DISCUSSION

3.1 Environmental Parameters

The environmental parameters were measured in situ in the sampling station (Table 1). The ecological parameters measured at five stations were pH, dissolved oxygen, temperature, and salinity.

Based on the results of measurements, the pH values at all stations tended to close fresh water in the range of 6.09 to 7.02. The highest value was station 2, and the lowest was station 5. DO had varying values ranging from 4.7 to 7.18 $mg.L^{-1}$. The highest value was station 2, and the lowest was station one. Temperature and salinity at all stations were not much different, the temperature ranging from 23.57 to 25.54°C and salinity from 11 to 12 ‰.

The Tanjung Api-Api has an extensive mangrove area, which is suitable for biota as a place to feed ground and a habitat for their life cycle. The high anthropogenic activity in this location cause changes in environmental water quality. According to Almaniar and Rozirwan (2021), water quality parameters around Tanjung Api-Api waters were classified as moderate to heavily polluted. Water quality parameter changes can be caused by mixing water masses. Based on the results of environmental parameters, good conditions for the growth of *S. serrata* were found in all station based on dissolved oxygen, temperature, pH, and salinity. Mud crabs have good adaptation in different salinity conditions ranging from 10 ‰ to 34 ‰, pH 8.0 to 8.5, temperature 23°C to 30°C, and dissolved oxygen more than 3 $mg.^{-1}$ (Pedapoli and Ramudu, 2014). A significant increase in temperature will cause higher evaporation and impact the drying of the sludge substrate. This situation makes it difficult for mud crabs develop biologically such as mating and molting (Leoville et al., 2021). Moreover, very alkaline or acidic pH conditions will be dangerous because they can cause metabolic and respiratory disorders (Pedapoli and Ramudu, 2014; Paran et al., 2022). Salinity is an essential factor for the spread of organisms in the sea, and oxygen is a limiting factor in determining the presence of organisms in the water (Nugroho et al., 2023). The variety of dissolved oxygen can be affected by waste materials or pollutants in the water. Environmental factors can affect heavy metals variations in chemical and physical parameters tend to affect the presence, displacement, and toxicity (Soegianto et al., 2022).

Table 1. The Measurement of Environmental Parameters in the Sampling Station

Stations	pH	DO (mg.L^{-1})	Temperature (°C)	Salinity (‰)
1	6.76 ± 0.03	4.70 ± 0.17	25.29 ± 0.33	12 ± 0.00
2	7.02 ± 0.05	7.18 ± 0.07	23.57 ± 0.49	11 ± 0.00
3	6.45 ± 0.02	5.91 ± 0.12	24.33 ± 0.03	12 ± 0.00
4	6.47 ± 0.03	6.25 ± 0.22	24.33 ± 0.08	11 ± 0.00
5	6.09 ± 0.05	5.48 ± 0.03	25.54 ± 0.08	11 ± 0.00

Data are shown mean ± SD with 95% confidence level

3.2 Description of *Scylla serrata*

There were two crabs collected in each station. The crab had size at 66.91 – 404.91 g in weight, carapace width ranging from 9 cm to 17 cm, and carapace length ranging from 4.2 cm to 8.2 cm (Figure 2). Morphologically, *S. serrata* had a green carapace with nine spines on the right and left, six spines between the eyes, a red pincer tip larger on the right than the left, and three pairs of walking legs and a pair of swimming legs.

Many people eat mud crabs for seafood consumption. The selection of mud crabs as a measure of feasibility for consumption is also based on measuring the width of the carapace, divided into three phases. The juvenile phase has a carapace width of < 7 cm, the early stage has a carapace width of 7–12 cm, and the adult phase has a carapace width of >12 cm (Paran et al., 2022). Mud crabs have entered the adult phase, which is ready for consumption and has matured the gonads for reproduction. Mud crabs prepared to mate will enter mangrove forests. The environment of mangrove forests indicates a natural resource suitable for mud crabs. Nugroho et al. (2022) stated that the substrate on which mangrove vegetation develops is soft muddy, not hard soil.

3.3 Sediment Grain Size

The substrate type at the study site resulted from the Shephard Triangle Method (Figure 3). The determination of sedimentary substrates around the waters of Tanjung Api-Api mangrove areas were divided into four types (gravel, sand, mud, and clay). The results showed that the substrate type at all stations was clay. The sedimentary substrate around the Tanjung Api-Api mangrove areas was dominated by clay. The percentage of clay from all stations ranged from 83.04 to 91.79% (Table 2). The highest rate of clay was at station three, and the lowest was at station five. In other composition, sand percentage was from 3.04 to 8.37% and silt percentage was from 4.03 to 8.59%.

The sediment is the main accumulation point for metals in the aquatic environment. Heavy metal distribution in sediments is influenced by chemical sediment composition and grain size (Fitrah et al., 2020). *S. serrata* live in the area with clay substrate (Soegianto et al., 2022). Rozirwan et al. (2022) stated that mud crabs are often found in muddy substrates because suitable for their growth, provides a lot of food source and place to make holes to avoid predatory risk. Moreover, clay

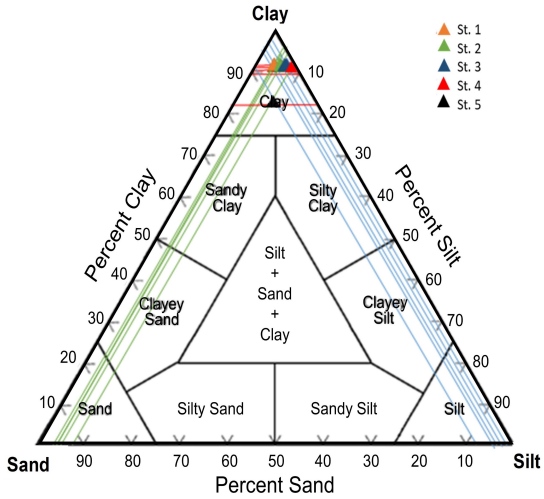

Figure 3. Classifications of Sediment Type with Shepard Triangle Method

Table 2. Sediment Grain Size in Each Station

Station	Sediment fraction (%)				Grain size
	Gravel	Sand	Mud	Clay	
1	0.00	6.74	4.89	88.36	Clay
2	0.00	5.56	4.03	90.41	Clay
3	0.00	3.04	5.17	91.79	Clay
4	0.00	4.30	4.31	91.39	Clay
5	0.00	8.37	8.59	83.04	Clay

substrate is easy to precipitate and accumulate because these particles have a high content of organic matter and high surface area for heavy metal absorption (Yan et al., 2021).

3.4 Heavy Metals Concentration

The results of heavy metal concentrations in water, sediments, and *S. serrata* were showed in (Table 3). Heavy metals in the water at all stations for Pb ranged from 0.1055 to 0.1334 mg.L^{-1}, and Cu was not detected (nd). Heavy metals in sediments for Pb ranged from 7.0104 to 11.8186 mg.kg^{-1}, and Cu ranged from 3.7127 to 4.5954 mg.kg^{-1}. Accumulation Pb in *S. serrata* ranged from nd at station 3 to 0.0021 mg.kg^{-1} at station 4 and

Cu ranged from 0.0300 to 0.0791 mg.kg^{-1}. The accumulation of heavy metal Pb in water had exceeded the quality standard value of 0.0044 mg.L^{-1} at all stations. Accumulation of Pb and Cu in sediments and *S. serrata* had not exceeded the quality standards. Based on the results of One-Way Anova analysis using a confidence level of 95% ($\alpha < 0.05$) showed significant difference in Pb concentration in sediment.

Aquatic ecosystems become ecosystems that are easily polluted by heavy metals because of mobility. Anthropogenic activities are one of the causes of water pollution (Zhang et al., 2022a). Heavy metal concentrations that have exceeded the quality standard allow for a decrease in water quality, sediments, and biota (Bharti and Sharma, 2022). Based on the analysis results, the concentration of Pb in the waters at each station has exceeded the threshold, and Cu was not detected. Avvari et al. (2022) stated that heavy metals in the water could disperse rapidly from their source because seawater is temporary and is affected by currents, tides, and other physical movements. Sampling in this study was carried out during low tide conditions. According to the conditions, the concentration of heavy metals tends to be higher because of the waters will experience a dilution process that will wash away the pollutants and affect their distribution (Rizk et al., 2022). Another activity factor that was estimated to have the potential to produce waste containing Pb was transportation and ports waste. Pb concentrations in waters are thought to have been influenced by ship maintenance activities and fuel oil spills from transportation activities (Briffa et al., 2020; Fitria et al., 2023).

3.5 Bioconcentration Factor (BCF) Indexs

Bioconcentration factors index (BCF) were used to determine the accumulation proportion of heavy metals Pb and Cu in *S. serrata* against heavy metals in sediment (Figure 4). Based on the results, the BCF value of heavy metals in *S. serrata* against in sediments ranged from 0 to 0.00024 (Pb) and 0.00690 to 0.01721 (Cu). The results showed that bioconcentration of Cu was higher than Pb in *S. serrata*. It could be concluded that the accumulation of Cu is higher than Pb in *S. serrata*.

The value of the BCF describes the ability of biota to accumulate heavy metals in the water and sediment in the environment (Bharti and Sharma, 2022; Rizk et al., 2022). The BCF of Cu is more incredible than Pb due to an essential metal organisms need in small amounts for metabolic processes. Still, if it exceeds the quality standards for heavy metals, it becomes toxic and harmful. High values of BCF indicate high levels of accumulated heavy metals and can have health implications that cannot be ignored for humans. According to Orabi and Khalifa (2020), a high BCF value in an organism indicates that the organism is capable of accumulating heavy metals. Bioaccumulation of heavy metals in organisms depends upon the concentration level in sediments, physiological factors, physicochemical properties, and biological activities in the ecosystem (Leoville et al., 2021; Bharti and Sharma, 2022).

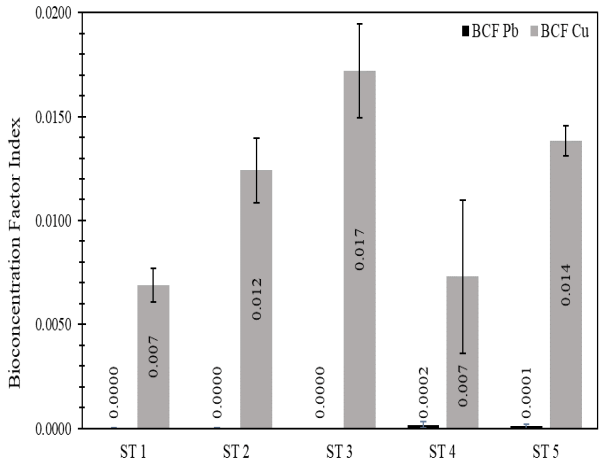

Figure 4. Bioconcentration factor (BCF) index of Pb and Cu in *S. serrata*

3.6 Distribution of Heavy Metals in Water, Sediment, and *Scylla serrata*

The distribution of Pb in waters, sediments, and *S. serrata* showed a parabolic line. In other media, Pb concentration showed fluctuation line in sediments and *S. serrata*. There was no distribution of Cu in the water because it was not detected at the time of measurement, whereas in sediment and *S. serrata* it formed a parabolic line distribution (Figure 5).

The distribution of Pb at the station 1 varied, sediment showed a high value, while water and *S. serrata* showed a low value compared to other stations. The heavy metal Pb at stations 2 to station 3 showed increasing pattern in sediments and waters, while decreasing in *S. serrata*. Furthermore, the distribution of Pb in water and sediment at station 4 had decreasing pattern, while at *S. serrata* it had an increasing pattern. At station 5, all accumulation media of Pb showed decreasing pattern. The distribution of Cu in sediments and *S. serrata* at station 1 showed a different pattern. Sediments showed the highest concentration compared to other stations, while *S. serrata* showed a low pattern. At station 2, the concentration of Cu in the sediment decreased, while at *S. serrata* it had an increasing pattern. The patterns between sediment and *S. serrata* was the same at stations 3, 4, and 5.

The concentrations of Pb and Cu in the sediments at all stations were still below the established quality standards, so it is still suitable for the habitat of *S. serrata*. Heavy metal concentrations in sediments are generally higher than in water (Bharti and Sharma, 2022). Heavy metals are also found to bind organic matter, settle to the bottom of the waters and blend with sediments (Algül and Beyhan, 2020; Rizk et al., 2022). Another factor, the research site is located in an estuary which forbidden place for waste containing heavy metals from anthropogenic activities (Niu et al., 2021; Zhang et al., 2022a). Moreover, the sedimentary substrate type influences the weight

Table 3. Pb and Cu Heavy Metals Accumulation in Water, Sediments, and *S. Serrata*

Sample	Heavy Metals	Quality Standard	Station				
			1	2	3	4	5
Water (mg.L^{-1})	Pb	0.0044	0.1055	0.1322	0.1334	0.1309	0.1192
	Cu	0.0013	nd	nd	nd	nd	nd
Sediment (mg.kg^{-1})	Pb	50	11.5922a	8.2391b	11.8186a	8.6954ab	7.0104c
	Cu	65	4.5347	4.2017	4.5954	3.7127	4.0055
S. serrata (mg.kg^{-1})	Pb	1.5	0.0002	0.0001	nd	0.0021	0.0008
	Cu	10	0.0313	0.0550	0.0791	0.0300	0.0624

Note: nd: not detected

of accumulated metals (Yan et al., 2021). The finer substrate has a high surface area and a stable ionic density to bind heavy metal particles. According to the grain size of the sediment, the smaller one has higher potential heavy metals concentration.

Several studies of heavy metal accumulation in *S. serrata* and other commercial crab species have been reported in *S. serrata* species recovered from the waters off Threspuram, Southeast Coast of India, showed Pb concentrations of 0.72 mg.kg^{-1} and Cu of 10.6 mg.kg^{-1} in gills (Yogeshwaran et al., 2020). From the coast of East Java, Indonesia, several locations have been reported to have *S. serrata* with Pb and Cu accumulation including from Solo River with Pb of 0.395 mg.kg^{-1} and Cu of 6.045 mg.kg^{-1}, Brantas River with Pb of 0.270 mg.kg^{-1} and Cu of 5.627 mg.kg^{-1}, and Banyuwangi Coastal with Pb of 0.260 mg.kg^{-1} and Cu of 5.142 mg.kg^{-1} (Soegianto et al., 2022). Commercial crab species *Portunus trituberculatus* from the coastal waters of Zhejiang Province showed the accumulation of Pb of 0.077 mg.kg^{-1} and Cu of 35.09 mg.kg^{-1} (Liu et al., 2020). In northern Bay of Bengal, *Portunus pelagicus* species contained Pb of 1.67 mg.kg^{-1} and Cu of 21.06 mg.kg^{-1} (Karar et al., 2019).

The presence of heavy metals in the study site will also affect heavy metal accumulation in biota ecosystems. The concentrations of Pb and Cu in *S. serrata* meat were below the quality standard. Although the heavy metal contents were much more significant in the sediment, this value does not mean that the *S. serrata* value was safe. Another factor that causes heavy metal accumulation in *S. serrata* was a detritivor (Pedapoli and Ramudu, 2014; Paran et al., 2022). Crustaceans eat Polychaeta and zooplankton in the sediment, which means they absorb a lot of heavy metals (Fitrah et al., 2020). The higher concentration of Cu compared to Pb is because the body of the organism contains the heavy metal Cu, which functions in metabolic processes and the formation of hemoglobin, and because of its physiology when added to the body of an organism (Harlyan et al., 2015; Rizk et al., 2022). Cu derived from water can increase its concentration.*S. serrata* can regulate the levels of essential heavy metals in their bodies but cannot limit non-essential heavy metals (Leoville et al., 2021; Soegianto et al., 2022).

3.7 Principal Component Analysis (PCA)

The principal component analysis showed three groups of analysis (Figure 6). The cumulative percentage of F1, F2 and F3 were 44.35%, 27.53%, and 17.83%. The biplot of PCA was presented in 6. Based on the biplot, F1 observation was station 1, 2, and 4 while the variable was dissolved oxygen, Pb in waters, Cu and Pb in sediment, salinity, and temperature. F2 observation was station 3 while the variable was Cu and Pb in *S. serrata*. F3 observation was station 5 while the variable was pH.

The variations in Pb and Cu distribution patterns in waters, sediments, and *S. serrata* in this study could be caused by environmental conditions. According to Avvari et al. (2022) and Rizk et al. (2022), distribution of heavy metals can be affected by temperature, dissolved oxygen, pH, brightness, and salinity. Oceanographic factors can also cause differences in heavy metal levels caused by current velocities. Warmer water temperatures provide a higher potential for heavy metal solubility than normal temperature conditions (Selvi et al., 2019; Algül and Beyhan, 2020). The results also showed that the high Pb in the waters was inversely proportional to the low Cu in the sediments. Due to the characteristics of heavy metals, which are generally higher in sediments, it is possible that Pb will be higher in sediments (Bharti and Sharma, 2022; Rizk et al., 2022).

3.8 Maximum Consumption of *Scylla serrata*

The consumption limit of *S. serrata* could be calculated using the MWI (maximum weekly intake) and MTI (maximum tolerable intake) (Table 4). The maximum consumption limit of *S. serrata* (MTI) from the analysis was 2.343 kg per week (Pb) and 4.072 kg per week (Cu).

The limit of consumption of *S. serrata* allowed per week is very high because the concentration of Pb and Cu in *S. serrata* is lower than quality standard. Therefore, *S. serrata* is still very safe for consumption. However, this does not rule out the possibility that the path of heavy metal pollution can disrupt aquatic ecosystems and harm humans if not addressed immediately (Soegianto et al., 2022; Zhang et al., 2022b). Therefore, efforts are needed by the government and society to reduce heavy metal pollution so that aquatic ecosystems can be

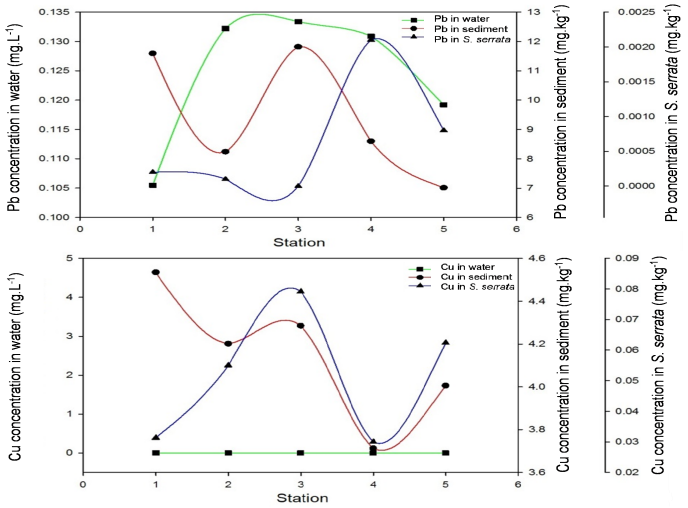

Figure 5. Distribution of Pb and Cu Heavy Metals in Each Station

Table 4. Maximum Consumption Limit of *S. serrata*

Heavy Metals	Average Concentration of Heavy Metals ($mg.kg^{-1}$)	PTWI ($mg.kg^{-1}$ per week)	Weight (kg)	MWI (mg per week)	MTI (kg per week)	Quality Standard (WHO, 1989) PTWI ($\mu g.kg^{-1}$ per week)	Quality Standard (WHO, 1989) PTWI ($mg.kg^{-1}$ per week)
Pb	0.00064	0.025	60	1.500	2.343	25	0.025
Cu	0.05156	3.500	60	210	4.072	3500	3.5

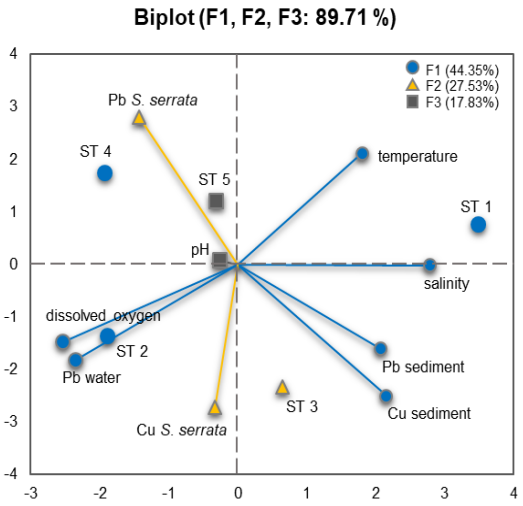

Figure 6. Principal Component Analysis of Environmental Parameters and Heavy Metals

sustainable (Briffa et al., 2020). Monitoring waste disposal into the waters regularly and inspecting ships to reduce oil spills into the seas are two forms of effort to control metal pollution. Outreach to the public about the survival of mud crabs and their relation to heavy metal waste in the waters is necessary to be safe for consumption.

4. CONCLUSION

The concentration of the heavy metal Pb in water has exceeded the quality standard while Pb and Cu concentrations in sediments and *S. serrata* has exceeded the quality standard. Based on the maximum limit value of meat consumption per week, *S. serrata* in Tanjung Api-Api is still safe for consumer. Future research should focus on analyzing heavy metals in another commercial marine biota.

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ARTICLES FOR FACULTY MEMBERS

INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

Bioaccumulation of heavy metals in edible tissue of crab (Scylla serrata) from an estuarine Ramsar site in Kerala, South India / Sherly Williams, E., Lekshmi Priya, V., & Razeena Karim, L.

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Bioaccumulation of heavy metals in edible tissue of crab (*Scylla serrata*) from an estuarine Ramsar site in Kerala, South India

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ABSTRACT

Wetlands are under severe threat due to anthropogenic activities and pollutants. Many pollutants such as heavy metals may accumulate to a hazardous level. In the present study, *Scylla serrata*, the mud crab and associated sediments were collected from three sites of Ashtamudi lake, the Ramsar site, to investigate the bioaccumulation of heavy metals Cadmium, Chromium, Copper, Lead and Zinc. Elemental analysis in the sediment sample showed that Cadmium and Chromium were found to be above USEPA and CCME limits and Copper was found to be above CCME limit on site 1. In site 2, Chromium was found to be above USEPA and CCME limits. In all the three sites, Lead and Zinc are below the permissible limit. Bioaccumulation status of heavy metals in the muscle of Scylla serrata with respect to the sites are in the order of site 1 > site 2 > site. The heavy metals Cadmium, Chromium and Lead were found to be above the permissible limit in site 1 and site 2, whereas Copper and Zinc concentrations in the muscle of crab from Ashtamudi wetland are below levels of concern for human consumption. A number of histological alterations such as splitting of muscle fibres, focal area of necrosis and muscular oedema were noticed in the samples. The result clearly indicates the fact that *S. serrata* undergoes intensified stress, when exposed to heavy metal contamination. Knowledge of metal concentrations in shell fish and fin fish are important both with respect to nature management and human consumption of fish.

1. Introduction

Metals are continually released into aquatic ecosystems, from natural to anthropogenic, sources and they cause serious threats because of their toxicity, bioaccumulation, long persistence, and biomagnification in the food chain (Erdoğrul and Ates, 2006). The pollution of brackish waters with an extensive range of heavy metals has become a matter of significant concern over the last new decades, because of the damage caused to the aquatic life. Metals like Copper, Zinc, and chromium are essential metals since they play an important role in biological system, while Cadmium and Lead are nonessential metals; as they have no identified role in biological systems.

When metals enter the aquatic ecosystems, they upset the ecological environment, and their ability to bioaccumulation may cause stress on fish and aquatic fauna (Olmedo et al., 2013; Mohammed Ali, 2016). Reported studies from the field and laboratory experiments showed that the accumulation of metals in tissues are mainly dependent upon water-metal concentrations, exposure period and type of tissue (Bochenek et al., 2008; Mansouri et al., 2011).

In aquatic systems, fish samples are observed as one of the most indicative organisms for the evaluation of metal pollution (Erdoğrul and Ates, 2006). Fish accumulates substantial amounts of metals in its tissues, especially in muscles, and thus, represents a major dietary source for humans and eventually affects their health (Sarkar et al., 2016; Dural et al., 2006). Fish contamination with metals was reported as a result of pollution of water with municipal solid waste, industrial effluents, and fertilizers containing metals (Dural et al., 2006). Contaminants in fish pose risks to human consumers as well as to piscivorous wildlife and are considered as a pathway for the transfer of heavy metals to higher trophic levels (Sanchari, 2017).

Benthic crustaceans such as crabs with detritus feeding habits may be sensitive to metal pollution because their life in the bottom sediments where chemical contaminants are mainly stored. To

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some extent, crabs can fulfill the main requirements of bioindicators, such as being relatively sedentary, easy to identify, abundant, long-lived, available for sampling all the year-round, and getting a wide distribution (Rainbow, 1995; Jake van, 2010). The pollution load of heavy metals in sediments of Ashtamudi estuarine system has been extensively studied and reported by Nagendra et al., 2017; Sherly et al., 2015; and Suma et al., 2012). Histological lesions can be used as indicators to identify the effects of various chemical contaminations on organisms such as fin fishes and shellfishes, and are reliable tools in controlled experiments and field studies (Lee et al., 2012). Histological analysis plays a pivotal role in determining the ultra-structure alterations that may occur in the tissues of organisms due to the toxicity of pollutants (Jaya and Shettu, 2015, Deore and Wagh, 2012, Geetanjali et al., 2018; Saravpreet et al., 2018; Mahmoud and Abd, 2017; Al-sawafi et al., 2017; Abalaka 2015). Knowledge of metal concentrations in fish is important both with respect to nature management and human consumption of fish (Ebrahimpour and Mushrifah 2010). Thus monitoring metals is of great significance to know the bioaccumulation potential of these pollutants and it is a key step in the management of aquatic ecosystems.

The Mud crab *Scylla serrata*, one of the prime shellfish food resources of humans in the domestic and international market was selected for the present study. This species is a very popular shellfish and it is extensively used as a candidate species in aquaculture practices. In this paper, the study focuses on the concentration of heavy metal accumulation in sediments, tissue, histological alternation of muscles of the representative crab *Scylla serrata*, the native species occupied in the muddy estuarine bottom.

2. Materials and methods

2.1. Study areas and sampling

Ashtamudi Lake, (Lat. 8°59′N; Long. 76°36′ E), the second largest wetland of Kerala, is palm shaped with eight prominent arms. Sampling was conducted between February 2017 to January 2018 from three sites along the estuary namely Kureepuzha (site 1), Perumon (site 2) and West Kallada (site 3) (Fig. 1). Effluents from Municipal Solid waste dumping site, mechanized fishing trawlers, fish processing and transportation industries, other small scale and large scale industries near to the site 1 and site 2 are the major sources of water pollution when compared to site 3, where the anthropogenic interferences are remarkably lesser. *Scylla serrata* of about 3 to 6 cms in carapace width and 220 to 270 gm weight (60 numbers) were collected from each study site.

2.2. Sample preparation

2.2.1. Heavy metals in sediment

Sediments were collected in separate polythene bags from the study sites. Samples were then digested with nitric acid and hydrofluoric acid in the ratio 5:2 for 30 min at 200 °C. After cooling the samples, the fluoride precipitates were dissolved by adding 0.8 g boric acid. The heavy metals such as Cadmium, Chromium, Copper, Lead and Zinc in sediment samples were determined by Atomic Absorption Spectrophotometer (APHA, 2012).

2.2.2. Heavy metals in crab

The collected live samples were brought to the laboratory, are thoroughly cleaned with water, abdominal muscles removed carefully and taken into petridishes, and kept in a hot air oven. The temperature was maintained at 60 °C for a period of 48 –72 h. Samples were fine powdered using mortar and pestle after complete drying. 0.5 gm of dried powder of each tissue samples were then digested in open beakers on a hot plate by adding nitric acid (Sigma-Aldrich, Germany) and perchloric acid (MERCK, Germany) in 4:1 ratio. After that, the samples were kept on hot plate and the temperature gradually allowed rising to 60 °C continue adding both acids in 4:1 ratio until the sample become colorless. The digested samples were allowed to cool. Then transferred to 25 ml volumetric flasks, and made up to mark with de-ionized water. The digests were store in plastic bottles for the analysis of Cadmium, Chromium, Copper, Lead, and Zinc using an Atomic Absorption Spectrophotometer (AAS, Pinnacle 900H, Perkin Elmer) (APHA, 2012). Metal concentrations were calculated in mg/kg.

2.2.3. Histological analysis

The tissues were cut into 1 mm pieces and fixed in 4 % glutaraldehyde solution for 24 h. One hour after initial fixation the tissues were rinsed in Cacodylated buffer (pH value of 7.2). Post fixation of tissues was carried out in 1 % osmium tetraoxide solution for 2 h. The tissues were then rinsed in distilled water and dehydrated through a graded series of ethanol. After that tissues were embedded in Epon 812. Ultrathin sections (0, 5–1 μm) were prepared for the thickness of 70 nms, collected on naked copper-meshed grids, and stained with uranyl acetate and lead citrate. The sections were examined and photographed using Transmission Electron Microscope (model TECNAI, Fei, Electron Optics) operating on 200 Kv at AIIMS, New Delhi.

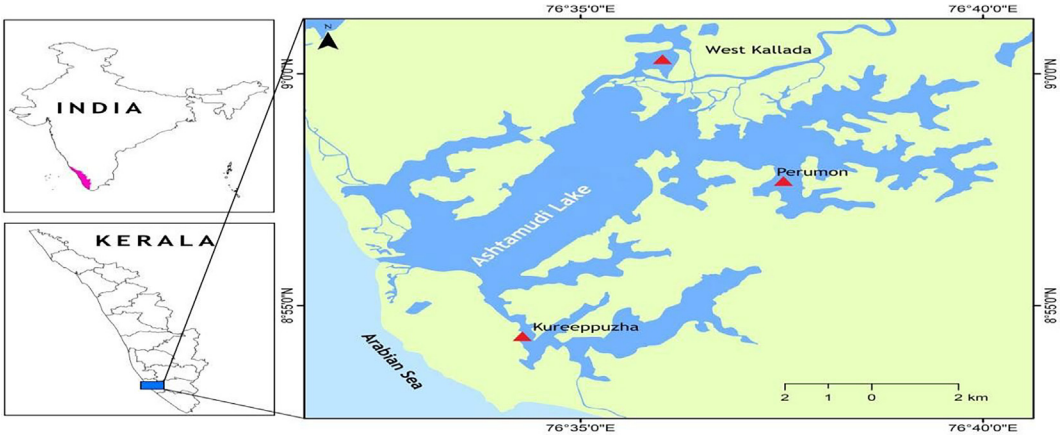

Fig. 1. Map of sampling sites in Asthamudi estuary.

2.2.4. Data analysis

Data generated from the study were analysed using the statistical package of SPSS 22. Analysis of variance (ANOVA) was used to test for significant differences in means in the elemental analysis. The Fisher's LSD post hoc test was used as basis of distinguishing mean differences, which were significantly different.

3. Results and discussion

3.1. Elemental analysis in sediment

Concentration of heavy metals were observed as Cadmium 0.976 ± 0.754 mg/kg dw (site 1), 0.425 ± 0.180 mg/kg dw (site 2), 0.0242 ± 0.083 mg/kg dw (site 3); Chromium 47.430 ± 12.20 mg/kg dw (site 1), 40.349 ± 7.983 mg/kg dw (site 2), 0.824 ± 0.488 (site 3); Copper 16.533 ± 4.285 mg/kg dw (site 1), 10.773 ± 2.654 mg/kg dw (site 2) 2.795 ± 1.187 mg/kg dw (site 3); Lead 22.583 ± 10.784 mg/kg dw (site 1), 2.920 ± 1.302 mg/kg dw (site2) 0.699 ± 0.2474 mg/kg dw (site 3) and Zinc 92.853 ± 34.37 mg/kg dw (site 1), 32.683 ± 9.248 mg/kg dw (site 2), 14.375 ± 2.563 mg/kg dw (site 3) were depicted in Table 1. The accumulation order of heavy metals in sediment at all the three study sites were Cu > Cr > Pb > Zn > Cd. This result was similar to other reported studies (Razeena and Sherly, 2014; Geetha Bhadra, 1997; Tam and Wong, 2000; Defew et al., 2005). Heavy metal concentration in sediment at site 1 and 2 was above USEPA (2003) and CCME (2009) limit (Table 2). Site 3 was found to be below the permissible limit of international standards and this may be due to the absence of industrialization and urbanization around this site. Electroplating, alloy, metal industry, anthropogenic activities, fossil fuel burning, application of phosphate fertilizers, municipal waste water, sewage sludge, fly-ash, plastics, batteries and leather tanning were considered as the major sources of Cadmium which pollutes the water bodies. Results of ANOVA reveal that significant variations were observed with respect to Chromium in sediment ($F = 106.630$) samples among the three sites and showed significance at 5 % level ($p < 0.05$). Post hoc multiple comparison (LSD) further reveals all the three sites significantly vary among each other. Copper at site 1 in the sediment samples was found to be very higher than other two sites and was found to exceeding the limit of CCME (2009) and site 2 and 3 below the CCME (2009) and USEPA (2003) limit (Table 2). The area under present study is witnessing the presence of oil spills from mechanized boats, freezing and canning industry; fish processing units, welding and electroplating workshops; boat building yards; municipal solid wastes, plastics, discharge from hospitals and butcher shops may be attributed to this heavy metal load in sediment. The incidence of such industrialization will further boost the chance of crossing the permissible limit of copper especially at site 1 and 2. ANOVA reveal that significant variations was observed with respect to copper sediment ($F = 63.864$) among the three sites and showed significance at 5 % level ($p < 0.05$). Post hoc multiple comparisons (LSD) further reveals that site 1 significantly varies from each other with respect to sediment samples. Lead in the sediment ($F = 47.632$) among the three sites and showed significance at 5 % level ($p < 0.05$). All the three sites significantly vary from each other in heavy metal accumulation as per the Post hoc multiple comparisons (LSD). Electroplating industry, smelting and refining, mining, bio solids are mainly responsible to the release of zinc to the natural waters (Jamshed and VAmit, 2017). Results of ANOVA reveal that significant variations were observed with respect to zinc in the sediment ($F = 44.239$) among the three sites and showed significance at 5 % level ($p < 0.05$). Post hoc multiple comparisons (LSD) further reveals site 1 significantly vary with other two sites regarding the presence of heavy metals in the samples. Heavy metal distribution of sediments from 52 stations of Ashtamudi estuary reported that the Pb concentration ranged from 36.4 to 40.8 μg/g, Zn from 45 to 109.7 μg/g, Ni from 10.2 to 14.5 μg/g, Cu from 20 to 145 μg/g, Fe from 800 to 2500 μg/g and Hg from 0.001 to 0.020 μg/g (Nair and Abdul Azis, 1986). The higher concentration of heavy metals in sediment was due to the discharge of oil spills from mechanized boats and trawlers, automobile discharges and dumping of untreated wastes from industrial sources and intensive fishing operations prevailing in the areas.

3.2. Elemental analysis in Crab

Concentrations of Cd, Cr, Cu, Pd and Zinc in *Scylla serrata* from the site 1were found to be 1.104 ± 0 0.608 mg/kg dw, 1.604 ± 0 0.386 mg/kg dw, 8.141 ± 1.471 mg/kg dw, 6.850 ± 2.730, 15.50 ± 2.489, where as in the site 2 it was 0.725 ± 0.515 mg/kg dw, 0.675 ± 0.171 mg/kg dw, 3.383 ± 1.105 mg/kg dw, 0.816 ± 1.045 mg/kg dw, 10.191 ± 2.461 mg/kg dw and from site 3 showed the concentration of copper as 1.225 ± 0 0.616 mg/kg dw and zinc as 6.958 ± 0.701 mg/kg dw, whereas all other heavy metal were found to be Below Detectable Limit (Table 3). Concentrations of Cu, Zn, and Cd in the crab tissues from the mangrove wetlands in Qi'ao Island, South China ranged from 36 to 528, 44 to 280, 59 to 781 and 2.0 to 24 mg/kg dw (Zaiwan et al., 2019). It was stated that the levels of Cu and Zn in *S. dehaani* (69–128 mg/kg for Cu and 44–102 mg/kg for Zn) from the Changjiang River Estuary (Sun and Wang, 2003). The observed concentration of heavy metals accumulation in muscles of the present study was found to be lower when compared with the above studies, whereas when compared with international standard site 1 and site 2 showed the concentrations of the heavy metals Cadmium, Chromium and Lead above the permissible limit (Table 4). Economic development and anthropogenic activities have significantly contributed to the worldwide deterioration in water quality (Saiful islam et al., 2015; Simul Bhuyan et al., 2019; Balkis et al. 2010; Mokaddes et al., 2013). Result of Statistical analysis confirms bioaccumulation status of heavy metals in the muscles of *Scylla serrata*. One way analysis of variance (ANOVA) showed that selected heavy metals such as Cadmium ($F = 17.820$), Chromium ($F = 130.748$), Copper ($F = 119.606$), Lead ($F = 58.955$), and Zinc ($F = 52.526$), were found to be different in their values with respect to the sites and showed

Table 1
Analysis of variance (One-Way ANOVA) of heavy metals of the sediment samples of the Ashtamudi Lake.

Heavy metals mg/kg	Study sites			F value comparing study sites	P Value
	Site 1 (Mean ± SD)	Site 2 (Mean ± SD)	Site 3 (Mean ± SD		
Cadmium	0.976 ± 0.754[a]	0.425 ± 0.180b	0.0242 ± 0.083c	13.509	< 0.05*
Chromium	47.430 ± 12.20 [a]	40.349 ± 7.983[b]	0.824 ± 0.488[c]	106.630	< 0.05*
Copper	16.533 ± 4.285 a	10.773 ± 2.654[b]	2.795 ± 1.187[c]	63.864	< 0.05*
Lead	22.583 ± 10.784a	2.920 ± 1.302b	0.699 ± 0.247[b]	44.239	< 0.05*
Zinc	92.853 ± 34.37 [a]	32.683 ± 9.248[b]	14.375 ± 2.563[c]	47.632	< 0.05

* = p < 0 0.05, The mean difference is significant at 5 % level; SD – Standard deviation; a, b, c - Means within rows with differing subscripts are significantly different using Fisher's LSD post hoc test.

Table 2
Comparing the elemental analysis in the sediment samples with international standard.

Heavy metals	USEPA limits (mg/kg)	CCMElimits (mg/kg)	Present study – mean values (mg/kg)			Inference
			Site1	Site 2	Site3	
Cadmium	0.6	0.6	0.976	0.425	0.024	Site 1 above USEPA and CCME permissible limit and the other two sites below the limit
Chromium	25	37.3	47.430	40.349	0.824	Site 1 and 2 above USEPA and CCME limit and site 3 below the permissible limit
Copper	16	35.7	16.533	10.773	2.795	Site1 above the limit of CCME and site 2 and 3 below the CCME and USEPA limit
Lead	40	35	22.583	2.920	0.699	All sites below the permissible limit
Zinc	110	123	92.853	32.683	14.375	All sites below the permissible limi

Table 3
Analysis of Variance (One-Way ANOVA) of heavy metals of the muscles of *Scylla serrata* comparing study sites of the Ashtamudi Lake.

Heavy metals (mg/kg)	Study sites			F value comparing study sites	P Value
	Site 1 (Mean ± SD)	Site 2 (Mean ± SD)	Site 3 (Mean ± SD)		
Cadmium	1.104 ± 0.608[a]	0.725 ± 0.515[a]	0.00 ±.00[b]	17.820	< 0.001*
Chromium	1.604 ± 0.386[a]	0.675 ± 0.171[b]	0.00 ±.00[c]	130.748	< 0.001*
Copper	8.141 ± 1.471[a]	3.383 ± 1.105[b]	1.225 ± 0.616[b]	119.606	< 0.001*
Lead	6.850 ± 2.730[a]	0.816 ± 1.045[b]	0.00 ±.00[b]	58.955	< 0.001*
Zinc	15.50 ± 2.489[a]	10.191 ± 2.461[b]	6.958 ± 0.701[c]	52.526	< 0.001*

(* = p < 0 0.01, The mean difference is significant at 1 % level; SD – Standard deviation; [a, b, c] - Means within rows with differing subscripts are significantly different using Fisher's LSD post hoc test).

Table 4
Comparing the elemental analysis in the muscle of *Scylla serrata* with international standard – FAO (1984, 2012).

Heavy metals	FAO limits (mg/kg)	Present study- meanvalues (mg/kg)			Inference
		Site 1	Site 2	Site 3	
Cadmium	0.5	1.10	0.72	BDL	Site 1 and 2 above permissible limit
Chromium	0.2	1.60	0.67	BDL	Site 1 and 2 above permissible limit
Copper	30	8.14	3.38	2.7	All sites below permissible limit
Lead	0.5	6.85	0.82	BDL	Site 1 and 2 above permissible limit
Zinc	40	15.50	10.19	8.2	All sites below permissible limit

significance at 1 % level ($p < 0.01$). The results of the Fisher's LSD (Least significant difference) Post hoc multiple comparisons further reveal that site 1 and 2 significantly differ from site 3 with respect to the accumulation of heavy metal cadmium. With respect to copper and Lead at site 1 significantly differ with site 2 and 3; whereas for chromium and zinc, all the three sites were found to be significantly differing among each other (Table 3). Generally, bottom dwelling crabs are suitable to accumulate more heavy metals. Factors such as age, sex, size, tissue type, feeding habits, and growth rate could also affect the addition of heavy metal in crabs (Jennings and Rainbow, 1979; Turoczy et al., 2001; Mohapatra et al., 2009). In the present study the levels of metals in sediments were different at each sampling site. Sediment forms a major source of heavy metals and also food source for many benthic organisms (Zhang et al., 2014). Thus feeding habits might be an important factor affecting the bioaccumulation of heavy metals (Liu et al., 2017. It is believed that the crabs are capable of modifiable concentrations of essential metals in the body but are unable to regulate the levels of nonessential metals (Rainbow, 1985; Mohapatra et al., 2009). The results further confirm the pollution status of the Lake and accordingly the study sites can be classified in the hierarchical order as more polluted (site 1) > less polluted (site 2) > least polluted (site 3). It has been found that the concentrations of the heavy metals raised coastal ecosystems due to the release of industrial waste and agricultural activities. In order to ensure public health and safety standard, an evaluation of heavy metal pollution status of the tissues of edible aquatic organisms is very essential (Nasyitah et al., 2018; Rumisha et al., 2016). Many researchers have emphasized the bioaccumulation status of heavy metal pollution load in the tissues of shellfishes from polluted regions of the Lakes from different part of the world (Noor and Asmat 2017; Nascimento et al., 2017; Umunnakwe and Ogamba, 2013; Kaoud and Eldahshan, 2010). The pattern of heavy metal accumulation in the tiger prawn *Penaeus monodon* from different regions of Ashtamudi also revealed the presence of heavy metal like Cadmium, Chromium, Copper, Zinc and Lead (Sherly and Lekshmi, 2017). The Cadmium, Chromium and Lead concentrations in the muscle of crab from two sites of Ashtamudi wetland are above the levels of concern for human consumption as defined by the FAO (1984, 2012). The studied crab species in this Wetland

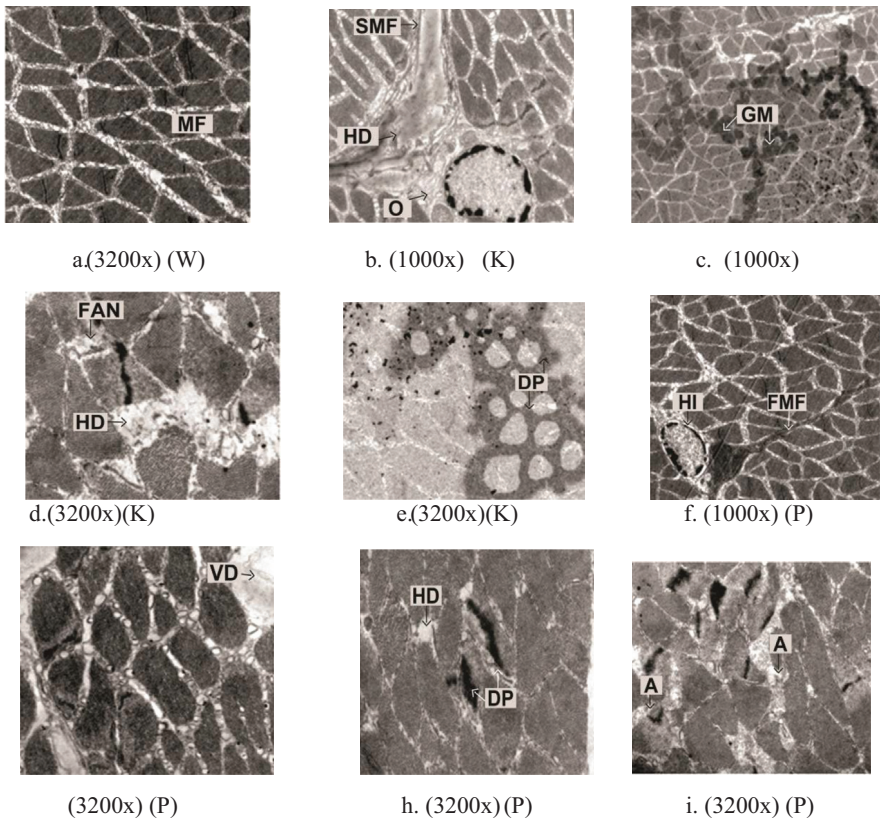

Fig. 2. (MF – Myofibrils; SMF – Splitting of muscle fibres; HD – Hyaline degeneration; O – Oedema; GM –Granular materials; FAN – Focal area of necrosis; DP – Dark patches; HI – Haemocytic intrusion; FMF – Fragmentation of muscle fibres; VD – Vacuolar degeneration; HD - Hyaline dedeneration; DP – Dark patches; A- Atrophy; W – West Kallada; K- Kureepuzha; P – Perumon).

system are being exploited for human consumption in indigenous market as well as export market. In the present study, muscle tissues of crab species were analyzed because these concentrations provide information on the potential risk to the crab themselves or to other organisms that consume them (especially humans). Heavy metals like Chromium and Cadmium which were found to be above the permissible limit in the sediment sample were also reflect in the muscle of the crab in higher concentrations.

Metal concentrations in the tissue of fish varies significantly among different studies, may be due to differences in metal concentrations and chemical characteristics of water from which fish were sampled, metabolism, ecological requirements, and feeding types of fishes and also the season in which studies were carried out (Canli and Atli, 2003, Erdoğrul and Ates, 2006). The principle pathways for metal uptake found to be highly variable over the range of metals, contaminant levels, specific ecological situation, and fish species studied. Thus, the metal concentrations in fish tissue may appear to be the result of a complex interaction of many factors (Alam et al., 2002).

3.3. Histological analysis

A number of histological alterations, which deviate from the normal structural patterns, were noticed in the muscle of *Scylla serrata* collected from the polluted regions of the Lake. Under Transmission Electron Microscopic investigation (TEM) muscles of *S. serrata* collected from site 3 revealed a normal histological architecture, made up of muscle cells containing contractile filaments. The thread like myosin filaments and proteins in the muscle tissue form a sheath of multi nucleate cells, which assemble into fibers, called myofibrils (Fig. 2a). Muscle of *S.serrata* collected from site 1 showed several structural changes under TEM analysis. Oedema, hyaline degeneration, and splitting of muscle fibers (Fig. 2b). Appearance of granular materials (Fig. 2c), focal areas of necrosis and hyaline degeneration were also noticed (Fig. 2d). Loss of normal state of myofibrils with appearance of dark circular patches was other predominant observations (Fig. 2e). Muscle of *S. serrata* from site 2 also reveals many abnormal features like fragmentation of muscle fibers, haemocytic intrusion (Fig. 2f), vacuolar degeneration (Fig. 2g), hyaline degeneration with appearance of dark patches (Fig. 2h) and atrophy (Fig. 2i). Many structural alterations such as atrophy, vacuolization, extensive necrosis and disrupted muscle bundles were observed in the muscle of *S. serrata* due to the effect of heavy metals like Cu, Pb, Zn Cd collected from Pulicat Lake (Lourduraj et al., 2014). Loosely packed and disintegrated muscle fibres were noticed in the selected fish species (Glenn et al., 2013). The heavy metals Cd and Pb caused similar type of alternations in liver, gills and kidney of *Leuciobarbus xanthopterus* (Sanaa, 2020). Morphological alterations caused by heavy metal pollution on gills and fins were noticed in tiger prawns *penaeus monodon* and *Etroplus suratensis* collected from the polluted regions of Ashtamudi Lake (Sherly et al., 2015; Sherly and Lekshmi, 2017). Zn accumulation resulted in significant alternations in the structure of the muscle such as disintegration of myofibrils, vacuolation in myofibrils, necrosis, atrophy in mud crab *Scylla serrata* (Ramya et al., 2019).

4. Conclusion

Growing pressure of anthropogenic influences continuously threatened the life of aquatic organisms inhabiting in the water system, which further leads to many toxic effects on them.

Bioaccumulation status of heavy metals in the muscle of *Scylla serrata* with respect to site is in the order of site1 > site2 > site 3. The shell fishes inhabiting in the polluted regions are more prone to heavy metal accumulation and subsequent alterations in its histological structure. If the discharge of aquatic pollutants into the water bodies goes on at an elevated level it will seriously affects the survival of the aquatic organisms. Knowledge of metal concentrations in shell fishes and fin fishes are important for both with respect to nature management and human consumption. Through the consumption of the affected shellfishes, pollutants can even reach higher organisms including humans. The future of mankind is inevitably linked to the protection of ecosystems. Hence, necessary actions should be taken up by the responsible authorities in order to safeguard the aquatic systems.

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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ARTICLES FOR FACULTY MEMBERS

INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

Do global environmental drivers' ocean acidification and warming exacerbate the effects of oil pollution on the physiological energetics of Scylla serrata? / Baag, S., & Mandal, S.

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RESEARCH ARTICLE

Do global environmental drivers' ocean acidification and warming exacerbate the effects of oil pollution on the physiological energetics of *Scylla serrata*?

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Abstract

Global climate change–induced ocean warming and acidification have complex reverberations on the physiological functioning of marine ectotherms. The Sundarbans estuarine system has been under threat for the past few decades due to natural and anthropogenic disturbances. In recent years, petroleum products' transportation and their usage have increased manifold, which causes accidental oil spills. The mud crab (*Scylla serrata*) is one of the most commercially exploited species in the Sundarbans. The key objective of this study was to delineate whether rearing under global environmental drivers (ocean acidification and warming) exacerbates the effect of a local driver (oil pollution) on the physiological energetics of mud crab (*Scylla serrata*) from the Sundarbans estuarine system. Animals were reared separately for 30 days under (a) the current climatic scenario (pH 8.1, 28°C) and (b) the predicted climate change scenario (pH 7.7, 34°C). After rearing for 30 days, 50% of the animals from each treatment were exposed to 5 mg L^{-1} of marine diesel oil for the next 24 h. Physiological energetics (ingestion rate, absorption rate, respiration rate, excretion rate, and scope for growth), thermal performance, thermal critical maxima (CT_{max}), acclimation response ratio (ARR), Arrhenius activation energy (AAE), temperature coefficient (Q_{10}), warming tolerance (WT), and thermal safety margin (TSM) were evaluated. Ingestion and absorption rates were significantly reduced, whereas respiration and ammonia excretion rates significantly increased in stressful treatments, resulting in a significantly lower scope for growth. A profound impact on thermal performance was also noticed, leading to a downward shift in CT_{max} value for stress-acclimated treatment. The present results clearly highlighted the detrimental combined effect of global climatic stressors and pollution on the physiological energetics of crabs that might potentially reduce their population and affect coastal aquaculture in forthcoming years.

Keywords Multiple stressors · Ocean acidification · Warming · Sundarbans · Oil spill · Crab · Scope for growth · CTmax

Introduction

In recent years, marine ecosystems are encountering numerous environmental challenges and myriad anthropogenic perturbations. It is now unequivocally acknowledged that the intense emission of atmospheric CO_2 is the prime cause of global climatic changes. Based on current trends, an escalation of 2–4°C average surface water temperature alongside a diminution by up to 0.4 pH units is predicted by the year 2100 under a business-as-usual scenario due to the unremitting rise of atmospheric greenhouse gas concentration (IPCC 2014, 2019). Hence, it is important to unravel the changes in the biological response under altered pH and temperature in contemporary natural variability as well as predicted future global changes (Hu et al. 2015). Temperature elevation is a crucial environmental factor regulating the physiological energetics of aquatic ectotherms (Huey and Kingsolver 1989; Hochachka and Somero 2002). Tropical invertebrates are more susceptible as they are the stenotherm with restricted thermal tolerance windows and reside close to their optimal thermal limit (Deutsch et al. 2008). Furthermore, it has been evinced that a rise in temperature also shifts animals' sensitivity toward other pollutants (Coppola et al. 2017; Andrade et al. 2019; Baag et al. 2021). Since the industrial revolution,

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global trade, predominantly petroleum products transportation, has rapidly soared. It has been estimated that about 380 million gallons of oil are discharged into the oceans yearly (Fiscella et al. 2010; Lu 2010; Pisano et al. 2016). Oil spill is one of the most deleterious anthropogenic threats to the coastal ecosystem (Marghany and Hashim 2015; Hawkins et al. 2017; Uno et al. 2017; Lumibao et al. 2018). The hefty traffic of small- and medium-size ships and fishing and tourist boats is accountable for the recurrent release of marine diesel oil (MDO) in coastal and estuarine environments.

Marine organisms experiencing severe environmental fluctuations have varied responses and adjustment potential (Madeira et al. 2018). In general, for aquatic ectotherms, compromising the energy budget is a fundamental survival strategy under stress (Baag et al. 2020). Recently, scope for growth (SfG) has emerged as an efficacious physiological index that appraises the energy allocation strategy for a particular animal (Bayne and Newell 1983; Baag et al. 2020). It estimates the surplus energy remaining for growth and reproduction after completing essential metabolic activities (Genoni and Pahi-Wohstl 1991). Elevated metabolic energy demand due to rising temperatures is accompanied by a decline in dissolved oxygen in the oceans and restricting animals' aerobic scope (Deutsch et al. 2015). Thermal performance curves are used to envisage an organism's response to stressors by quantifying the influence of body temperature on an ectotherm's performance and fitness (Sinclair et al. 2016). The assessment of critical thermal boundaries like the critical thermal maximum (CT_{max}), acclimation response ratio (ARR), Arrhenius activation energy (AAE), warming tolerance (WT), and thermal safety margin (TSM) also endows the eco-physiological status of aquatic organisms under stressors (Lutterschmidt and Hutchison 1997; Deutsch et al. 2008).

Although our knowledge regarding the biological consequences of various global and local environmental stressors is expanding distinctly, howbeit, their combined effects are still in its infancy. Global climatic stressors like ocean acidification and warming might severely jeopardize the fitness of coastal species, as these animals are already impoverished owing to numerous other local stressors like chemical pollution (Ghosh and Mandal 2021). Climate change factors might compromise the resilience potential of animals exposed to pollutants (Maulvault et al. 2019). Multiple environmental perturbations might cause substantial alterations in the energy budget of an organism owing to increased energy expenditure. It is conjectured that different categories of stressors, when combined, can have an even greater negative impact on the physiological responses of marine ectotherms (Arnberg et al. 2018). Global climatic factors affect organisms' sensitivity to oil pollution and alter their toxicity potential. Pollutants might interfere with the homeostasis of animals under climatic acclimation, which can potentially decrease the populations' perseverance and might cause additional ramifications on ecosystem functioning (Baag and Mandal 2022).

Decapod crustaceans play a pivotal role in coastal habitats by regulating trophic dynamics and nutrient cycling (Grilo et al. 2011; Pachelle et al. 2016; Madeira et al. 2018). They have been serving as excellent sentinel species and reliable environmental bio-monitors (Zheng et al. 2019). The mud crab (*Scylla serrata*) is one of the most commercially exploited species for aquaculture in coastal areas of India, especially the Sundarbans (Paital and Chainy 2014). Albeit its importance, very limited information is available on the ecophysiology of *S. serrata*. From the past few decades, the Sundarbans estuarine system (SES) has been under threat due to natural and anthropogenic disturbances like surface water warming, increased absorption of CO_2, and oil spill (Mitra et al. 2009; Panigrahy et al. 2014; Samanta et al. 2018; Sarma et al. 2021; Sridevi and Sarma 2021). Thus, it is obligatory to study the physiological energetics of *S. serrata* under global change scenarios and pollution, as it holds a crucial position in the aquaculture and rural economy of India.

The key objective of the present study is to compare and decode whether rearing under global environmental stressors (ocean warming and acidification) exacerbates the effect of local stressor (oil pollution) on physiological responses of *S. serrata*. Physiological energetics like ingestion, assimilation, absorption, respiration, and excretion rates were evaluated to compute the SfG. Furthermore, thermal performance and critical thermal maximum (CT_{max}) were also assessed. Several other parameters like the acclimation response ratio (ARR), Arrhenius activation energy (AAE), and temperature coefficient (Q_{10}) were calculated from the thermal performance data. All the variables were measured concurrently to get a comprehensive idea of the stress tolerance response about crabs against both medium-term and acute exposure to global environmental stressors and pollution. To the best of our knowledge, until date, no study has been conducted to evaluate the consequences of oil spill on physiological energetics in crabs acclimated under climate change conditions (combined ocean warming and acidification). The present study aimed to provide a clear perception on the physiological energetics of crabs under the co-occurrence of global climate change and oil spills explicitly in the tropical belts where animals are already residing at the margin of their thermal tolerance threshold with restricted acclimation plasticity.

Materials and methods

Species collection and experimental setup

Adult *Scylla serrata* (carapace length: 63.41 ± 4.08 mm, weight: 48.35 ± 4.79 g) were collected from the tidal creek of the river Matla in the Sundarbans, the largest mangrove ecosystem in the world, a UNESCO world heritage site since

1997 and recently has been declared as a Ramsar site in 2019 (Bhowmik and Mandal 2021). After sampling, the animals were transported to the laboratory and acclimated in aquaria filled with artificial seawater separately for two weeks (temperature: 27.95°C ± 0.36, DO: 7.88 mg L^{-1} ± 0.07, pH: 8.092 ± 0.025, and salinity: 22 psu). The crabs were fed with dried fish pellets until satiation, and unconsumed food particles were removed from the aquaria daily. Every day the animals were transferred to the tanks with freshly prepared artificial seawater after discarding previously used water.

After acclimation, 120 crabs were kept separately for 30 days under two scenarios (60 crabs in each scenario, 10 crabs in each aquaria): (a) present scenario (pH 8.1, 28°C) and (b) predicted climate change scenario for 2100 according to IPCC (2019) (pH 7.7, 34°C). The target temperatures were attained by immersing digitally controlled thermostats in water baths holding experimental aquaria for the entire experimental duration. Seawater acidification scenario was mimicked using a pH–stat system (Cole-Parmer, USA). The pH values were set and controlled by a computerized system connected to individual pH probes with an automatic cut-off mechanism controlling the CO_2 gas flux. After this experimental exposure period (30 days), half (30 crabs) of each treatment were exposed to 5 mg L^{-1} of marine diesel oil (MDO) for 24 h (maintaining the same water quality parameters for each treatment group), and the other half remained uncontaminated. The oil concentration was chosen to match a realistic oil spill scenario based on previous studies from different geographic locations (Bechmann et al. 2010; Sagerup et al. 2016; Mansour et al. 2017; Arnberg et al. 2019) and also from the present geographic location (Ghosh and Mandal 2021). This resulted in 4 different treatments (triplicate aquaria per treatment with 10 crabs per aquaria, i.e., 30 crabs per treatment): (1) pH 8.1 and 28°C in the absence of MDO (considered as a control condition in the experiment), (2) pH 8.1 and 28°C with MDO, (3) lower pH 7.7 and elevated temperature 34°C in the absence of MDO, and (4) pH 7.7 and 34°C with MDO.

The temperature, pH, and salinity of artificial seawater were monitored continuously throughout the experimental duration. Total alkalinity was measured twice weekly using an alkalinity checker (HANNA HI755). CO_2SYS software (Lewis and Wallace 1998) was used to calculate the carbonate chemistry parameters with constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) (Table S1 for a summary of water quality parameters).

Thermal performance

On the 31st day of the experiment, the aerobic metabolic performance of crabs was assessed using an increasing thermal ramp of 2°C h^{-1} from 24 to 42°C and measuring the respiration rate at every temperature for both the treatment groups separately following Leung et al. (2017) and Leung et al. (2018). The respiration rate was calculated following Baag et al. (2020). Changes in the animals' locomotory behaviour and motor coordination (separate crabs) were observed carefully throughout the ramping period. The point at which motor coordination got disorganized (crabs were forcefully turned upside down at each temperature, and if they were incapable of being back upright, they were considered to have reached the end-point) was designated as the upper thermal tolerance level or CT_{max} (Lutterschmidt and Hutchison 1997). Acclimation response ratio (ARR) was calculated as the change in CT_{max} (ΔCT_{max}) per degree change in acclimation temperature (ΔT) (Claussen 1977). Warming tolerance levels were measured as the difference between CT_{max} and average habitat temperature ($CT_{max} - T_{hab}$) following Deutsch et al. (2008). Thermal safety margins were also estimated by the difference between thermal optimum and average habitat temperature of the animals ($T_{opt} - T_{hab}$) following Deutsch et al. (2008). Arrhenius activation energy (AAE) was calculated for all the ramping temperature ranges with respect to the acclimation temperatures from http://www.calctool.org/CALC/chem/kinetics/act_en and was expressed as kJ mol^{-1}. Furthermore, the temperature coefficient (Q_{10}) was also measured across all the ramping temperature ranges using the Van't Hoff equation (Schmidt-Nielsen 1999):

$$Q_{10} = (K_2/K_1)^{10/(t_2-t_1)}$$

where K_1 and K_2 are the respiration rates at temperatures t_1 and t_2, respectively.

Physiological variables and energy budget

Physiological variables were measured on the 32nd day of the entire experiment with a different set of crabs which were not used in the thermal performance test. *S. serrata* from each replicate treatment were provided with dried fish pellets in individual feeding chambers for 6 h. After feeding, the unconsumed tissue was removed and oven-dried at 60°C for 24 h. Ingestion rate (I) was calculated as the dry weight of food consumed (difference between initial feed dry weight and unconsumed feed dry weight) per individual per hour. Furthermore, the ingestion rate was converted into its energy equivalent by using its calorific value. After 24 h of surplus food removal, faeces were collected from feeding chambers and dried.

The assimilation efficiency (AE) was assessed by a formula:

$$AE(\%) = [F' - E'/(1 - E')(F')] * 100$$

F′ and E′ represent the organic fraction of food and faecal matter, respectively, calculated by dry weight to ash-free dry weight ratio (combusting for 6 h at 450°C) for both food and faeces (Conover 1966).

Finally, absorption rate (AR) was calculated as the product of ingestion rate and assimilation efficiency.

$$AB = AE * I$$

To measure the respiration rate (RR), individual crabs were transferred into airtight containers of 300 ml each with oxygen-saturated water and were kept enclosed for 1 h in respective treatment temperature water baths. Initial and final dissolved oxygen concentrations were measured using a DO meter (Orion Star A223 dissolved oxygen/RDO portable meter kit) which was calibrated according to the manufacturers' instructions at room temperature. Blank samples without animals were used in every treatment for accurate measurements. For calculating the excretion rate (ER), 25 ml of water was collected from each feeding chamber, and the phenol-hypochlorite method (Solorzano 1969) was followed to evaluate the ammonia concentration in the water. The values were converted into energy equivalent using a conversion factor of 14.14 J mg O_2^- and 0.025 J μg^{-1} NH_4 for RR and ER, respectively (Elliott and Davison 1975).

The scope for growth (SfG) was calculated and expressed as J ind^{-1} h^{-1} using a formula:

$$SfG = AB - (RR + ER)$$

following Widdows (1985).

Statistical analysis

The effect of climate change (CC) stressors (ocean warming and acidification) and oil, as well as their interaction, was investigated on ingestion rate, assimilation efficiency, absorption rate, respiration rate, excretion rate, and scope for growth. A two-way permutational analysis of variance (PERMANOVA) was applied. It tests differences between the groups with many variables and permutations to avoid possible biases. The main test was performed using climate change stressors and oil as fixed factors, and whenever significant interactions were found between these factors, a paired test was conducted for each factor separately in each level from another factor. The effect of climate change factors was also assessed by PERMANOVA on the thermal performance of the test species. All the analyses were performed in PRIMER 6 (Clarke and Gorley 2006; Clarke et al. 2008) with PERMANOVA + add on (Anderson et al. 2008).

Results

Thermal performance

A significant effect ($p < 0.01$) of ramping temperature, acclimation conditions (pH 8.1, 28°C and pH 7.7, 34°C) as well as their interaction was evident on the thermal performance of *S. serrata* (Fig. 1, Table 1). Acclimation conditions indicated a significant impact in the pairwise test. Respiration rate ranged from 0.60 to 0.91 mg O_2 gm^{-1} h^{-1} and 0.39 to 0.91 mg O_2 gm^{-1} h^{-1} for pH 8.1, 28°C and pH 7.7, 34°C acclimated crabs, respectively (Fig. 1). At the individual level of each ramping temperature, there was a significant difference between the acclimated conditions (except at 36°C). A downward shift in CT_{max} was noted in pH 7.7, 34°C acclimated animals (stress group). Critical thermal limit arrived at 36°C for stress-acclimated crabs, whereas in the control group CT_{max} arrived at 38°C. Thus, CT_{max} reached 2°C earlier in stress group animals than in the control animals reflecting a narrowed down thermal tolerance threshold. The acclimation response ratio (ARR) value was calculated as 0.33. The respiration rate of *S. serrata* was significantly higher in pH 7.7, 34°C acclimated crabs than in control conditions throughout the initial ramping temperatures (i.e., from 24 to 34°C). A sharp increase in RR was observed at 34 °C for pH 7.7, 34°C acclimated animals which then abruptly declined at 36°C. The stress-acclimated animals exhibited a rapid decline in RR from 36°C, and a stark drop was noted at 42°C albeit, the decline in RR was not that steep for control animals. Even at 42°C, the animals of control treatments tried to maintain a certain level of respiration through the locomotory movements seized. Motor coordination was lost at 36°C and 38°C for CC treatment (pH 7.7, 34°C) and control (pH 8.1, 28°C) acclimated crabs, respectively. The calculated warming tolerance levels were 10°C and 8°C; however, thermal safety margins were 8°C and 6°C for control and stress-acclimated animals, respectively. The Q_{10} and values ranged from 1.01 to 1.65 and 0.28 to 2.05 for control and stress-acclimated animals, respectively. The Q_{10} and AAE values are represented in Table 2.

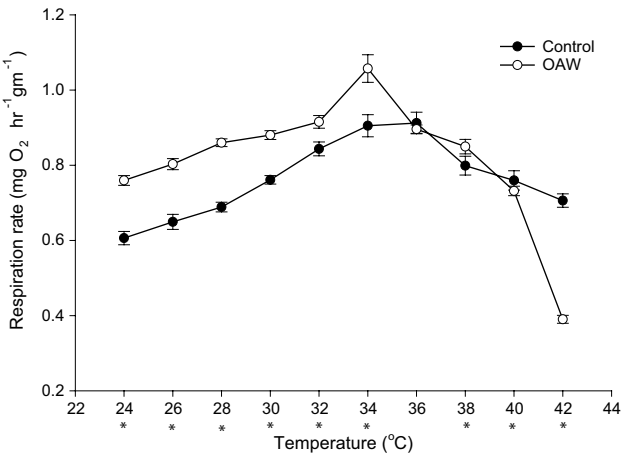

Fig. 1 The thermal performance curve of *Scylla serrata* along an increasing thermal ramp (+2°C h^{-1}) from 24 to 42°C after 30 days of exposure to different treatments indicating CT_{max}. The values represent the mean ± SD ($n = 6$). The temperature levels marked with "*" indicate a significant difference between the two acclimated groups

Table 1 Summary of the main test of PERMANOVA exposed to different treatments ($p \leq 0.05$)

	Ingestion rate			Assimilation efficiency			Absorption rate		
Source of variation	MS	F	P	MS	F	P	MS	F	P
CC	551.76	50.811	0.001	0.63226	52.286	0.001	716.17	132.1	0.001
Oil	2717.4	250.24	0.001	4.4362	366.86	0.001	3389.9	625.29	0.001
CCXOil	86.964	8.0085	0.008	0.19628	16.232	0.001	149.75	27.623	0.001
	Respiration rate			Excretion rate			Scope for growth		
Source of variation	MS	F	P	MS	F	P	MS	F	P
CC	2199.8	1953.1	0.001	2160.3	1080.8	0.001	1492.8	201.01	0.001
Oil	588.89	522.85	0.001	1138.8	569.77	0.001	4131.8	556.37	0.001
CCXOil	184.56	163.87	0.001	780	390.24	0.001	177.98	23.966	0.001
	Thermal performance								
Source of variation	MS	F	P						
Ramp Temp	225.78	610.51	0.001						
Acc Temp	47.895	129.51	0.001						
Ramp TempXAcc Temp	109.12	295.05	0.001						

Energy budget of crabs

After the 4-week-long experimental duration, there was no significant effect on mortality and overall growth (changes in length, width, body weight) of adult crabs. Ingestion and absorption rates were significantly reduced by the effect of climate change stressors ($p<0.01$) and oil ($p<0.01$), as well as their interaction ($p<0.01$) (Fig. 2a, b, Table 1). Significant differences were found among all the treatment groups. Amid stress treatments, the highest ingestion and absorption rates were found in the treatment group with only the OAW scenario (lower pH 7.7 and elevated temperature 34°C in the absence of MDO), whereas the lowest in the combined OAW scenario and oil treatment (pH 7.7 and 34°C with MDO). The assimilation efficiency was also found to vary significantly with CC stressors, oil, and their interaction ($p<0.01$). Significant variations were found among all the treatment groups in the pair-wise test except between the MDO-contaminated animals under the two climate scenarios.

Respiration rate was also found to be significantly different ($p<0.01$) with climate change stressors and oil as well as their interaction (Fig. 2c, Table 1). There was a significant difference among all the treatment groups. RR decreased significantly when exposed to MDO in the present climatic scenario (pH 8.1 and 28°C with MDO). However, RR significantly increased in OAW conditions both in the presence as well as the absence of MDO compared to the control conditions. The highest RR was found in the treatment group with a lower pH of 7.7 and an elevated temperature of 34°C in the absence of MDO.

Similarly, a significant variation ($p<0.01$) was also recorded for excretion rate under different climate change stressors, oil as well their interaction (Fig. 2d, Table 1). A significant difference between all the treatment groups was noted. The excretion rate was significantly higher ($p<0.01$) in all the stress treatment groups compared to control animals.

There was a significant effect ($p<0.01$) of climate change stressors, oil, and their interaction on the scope for growth (SfG) of *S. serrata* (Fig. 3, Table 1). SfG significantly reduced with MDO exposure irrespective of the climatic treatments in which the animals were exposed to. Although attenuated, SfG was highest in the CC scenario (lower pH 7.7 and elevated temperature 34°C in the absence of MDO) among all stress treatments compared to the control. The cross-stress treatment group (pH 7.7 and 34°C with MDO) clearly exhibited significant signs of exacerbated effect of stressors with the lowest SfG among all the treatments.

Discussion

Bioenergetics-based studies are considered powerful tools to disentangle the fitness and energy budget of marine organisms under multiple environmental stressors (Sokolova

Table 2 Temperature coefficients (Q_{10}) and Arrhenius activation energy (AAE) recorded in *Scylla serrata* at different temperatures ranging from 24 to 42°C after 30 days of exposure to different treatments

		24°C	28°C	28°C	30°C	32°C	34°C	36°C	38°C	40°C	42°C
28°C, pH 8.1	Q_{10}	1.375932	1.342744	-	1.648548	1.659787	1.576798	1.421012	1.159961	1.085045	1.017846
	AAE	23.7445	22.0754		37.9449	38.7144	35.0233	27.1988	11.5606	6.39991	1.39584
34°C, pH 7.7	Q_{10}	1.391975	1.410365	1.41114	1.581749	2.054995	-	0.43685	0.57885	0.541465	0.287554
	AAE	25.0973	26.2688	26.4867	35.4986	56.1301		−65.3839	−43.4424	−49.061	−100.309

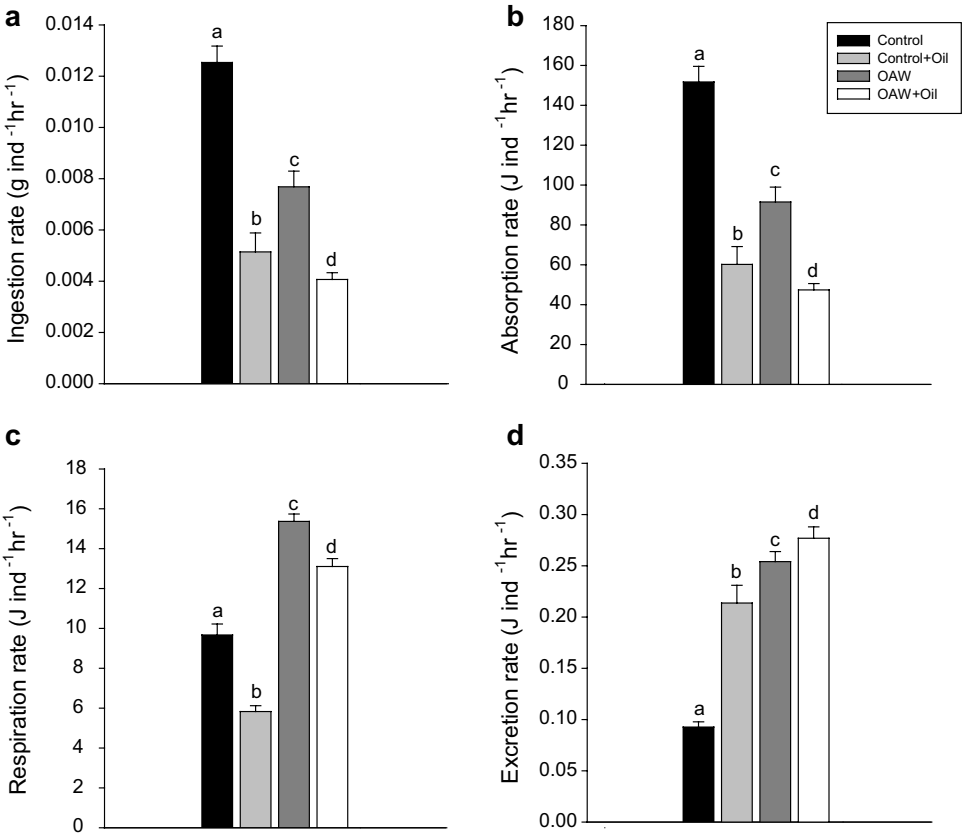

Fig. 2 Ingestion rate (**a**), absorption rate (**b**), respiration rate (**c**), excretion rate (**d**) of *Scylla serrata* under different treatments of the experiment. Values represent mean ± SD ($n = 9$, $p < 0.05$). Different letters (**a**, **b**, **c**, **d**) indicate significant differences between treatment groups

2013). Physiological alterations in response to abiotic factors are crucial for proper ecological forecasting to understand the impact of multiple stressors on organisms with economic importance. The Sundarbans estuarine system (SES) is vulnerable to coastal ocean acidification and warming (OAW) due to enhanced atmospheric CO_2 levels as well as oil spill events. In the present study, we aimed to delineate the effects of 3 major abiotic stressors (coastal ocean acidification, warming, and oil pollution) for the first time on *S. serrata*.

Thermal performance

As the global environment changes, concerns regarding the ability of ectothermic animals to thrive in these fluctuating conditions are also rising. In ectotherms, physiological tolerance levels define the thermal niche and probable response of populations to climate change (Deutsch et al. 2008; Sunday et al. 2011; Huey et al. 2012). Thermal tolerance levels tend to shift when they reside in altered environmental conditions by acclimation (Somero 2005). Thermal performance curves (TPCs) estimate the physiological proficiencies of ectotherms as a function of temperature (Huey and Stevenson 1979). Acclimation can shift the TPC (broadening or narrowing) after exposure to different environmental conditions (Huey et al. 2012). A directly proportional relationship between CT_{max} and acclimation temperature is more common

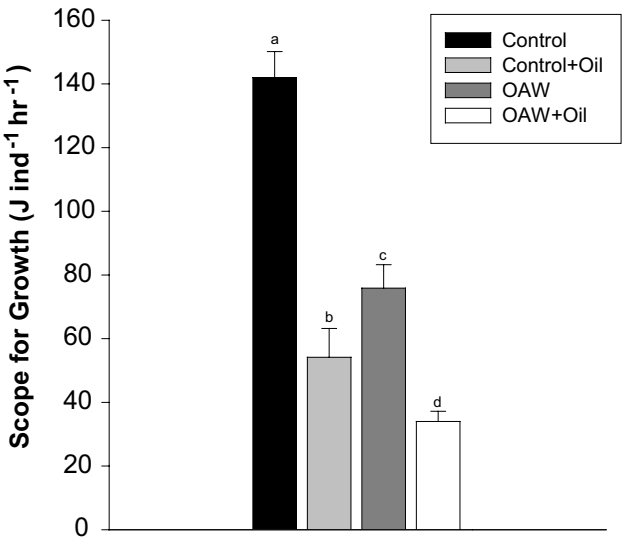

Fig. 3 Scope for growth of *Scylla serrata* under different treatments of the experiment. Values represent mean ± SD ($n = 9$, $p < 0.05$). Different letters (**a**, **b**, **c**, **d**) indicate significant differences between treatment groups

in crustaceans, including crabs (González et al. 2010; Kumlu et al. 2010; Hyde et al. 2012; Ravaux et al. 2012; Re et al. 2012; Darnell et al. 2015; Cumillaf et al. 2016). A higher

CT_{max} is necessary for evolutionary adaptation and population perseverance, as acclimation does not necessarily stimulate adaptive responses in all organisms (Huey and Berrigan 1996). Our findings show that combined OAW conditions result in a narrowing (downward shift of CT_{max}) (Fig. 1) of the thermal tolerance window in *S. serrata*. A similar downward shift in thermal limits was observed on the crab *Cancer pagurus* (Metzger et al. 2007), larval stages of the spider crab *Hyas araneus* (Schiffer et al. 2014), and oyster *Saccostrea glomerata* (Parker et al. 2017) exposed to OA conditions. While working with a tropical shrimp (*Lysmata amboinensis*), Rosa et al. (2014) reported a significant decrease in CT_{max} values when animals were reared under predicted warming conditions (30°C) in contrast to the current temperature (24–27°C). These observations corroborate the theory that combined OAW would hamper the energy metabolism of marine ectotherms and will narrow down their thermal tolerance window (Pörtner and Farrell 2008). In the present study, the observed constraint in the TPC clearly indicates warming conditions were outside the tolerance range of the crab, and an incomplete compensation of extracellular acidosis occurred due to OA resulting in early metabolic depression. The ARR value of 0.33 was within the range (0.30–0.50), which is generally found in estuarine species (Re et al. 2005), but was on the lower edge due to poor acclimation capacity. Critical thermal limits were found to be between 37 and 38°C in isolated gill mitochondria of *S. serrata* (Paital and Chainy 2014), which validates our results. The abridged warming tolerance levels and thermal safety margins in *S. serrata* under stress treatment imply a potent risk of extinction in the imminent future. The Q_{10} and AAE values were lower in the control treatment until RR increased with increasing temperature until CT_{max} reached. This phenomenon clearly signifies crabs were better acclimated to control conditions and maintained homeostasis, countering the stress conditions. A higher value of Q_{10} and AAE under stress-acclimated conditions mirrors stenothermic behaviour in the crabs. In stress treatments, beyond CT_{max}, the Q_{10} values became < 1, and the AAE value became negative, accentuating complete temperature-independent behaviour. Thus, our study again substantiates the theory that tropical species may face greater vulnerability to climate change as their tolerance limits are closer to optimal conditions (Stillman 2003; Deutsch et al. 2008).

Energy budget of crabs

Although feeding poses the capacity to alleviate the adverse effects of stress by supplying extra energy, it is an energetically exorbitant process (Pörtner et al. 2004). As metabolic expenditure in animals increases due to stressors for their sustenance, organisms may cope by reducing their food consumption which lowers energy costs related to digestion as well (McGaw 2006). This phenomenon was noted in our study and mirrored in previous studies which are discussed below. Effects of oil and OAW were investigated in shrimp *Pandalus borealis*, where a reduction in feeding activity was noticed with exposure to only oil (Arnberg et al. 2018). Exposure to oil also decreased food consumption rates in other shellfish (Stickle et al. 1984; Gilfillan et al. 1986; Widdows et al. 1987; Jeong and Cho 2007). Additionally, in some other molluscs like gastropods and bivalves, acidification and warming caused a significant decrease in feeding rates (Wang et al. 2015; Zhang et al. 2015; Lemasson et al. 2018). A significant combined effect of temperature and oil was also recorded in the assimilation efficiency of *Daphnia magna* (Ullrich and Millemann 1983). These results accentuate the prominence of natural stressors, like temperature, which exacerbate the toxicity potential of chemical pollutants in aquatic organisms.

Under stressful environments, aerobic metabolism must be facilitated for the disbursal of adequate metabolic energy required in molecular defence mechanisms (Leung et al. 2018). Some species follow this strategy and elevate metabolism to counterbalance surplus energy demand (Lannig et al. 2010), whereas few try to conserve energy by minimizing their metabolic rate for survival (Guppy and Withers 1999). Both these strategies were taken up by the crabs in our study under different treatments, which is clearly reflected in our results. RR decreased only in the oil exposure treatment. This phenomenon was also observed in other ectotherms species like crabs and fish (Camus et al. 2002; Dal Pont et al. 2019). This can be attributed to the oil-induced stress and impaired oxidative metabolism in those species. Generally, exposure to oil leads to a state of hypoxic condition in the aquatic environment. The reduced respiration may be caused due to reduction in gill permeability. The response of *S. serrata* could be regarded as an avoidance response in this case. This behaviour would minimise the interaction with the external medium. The low oxygen uptake would reduce oil uptake through the gills. Acute exposure to oil might have inhibited aerobic metabolism and activated anaerobic metabolism as a strategy to cope with sudden toxic environmental change. In contrast, RR significantly increased in OAW conditions both in the presence and absence of oil. A similar increase in metabolic rates under combined elevated temperature and high pCO_2 conditions was noticed in several other shellfishes (Comeau et al. 2010; Leung et al. 2020; Saba et al. 2021). The oxygen saturation level of water steadily drops with a simultaneous rise in temperature levels. This impedes oxygen transport to tissues and also the oxygen uptake efficacy by gills (Frederich and Pörtner 2000). To deal with such adverse conditions, RR is raised with increasing temperature. Under OA conditions, an increase in RR signifies higher costs for the maintenance of cellular homeostasis required in acid–base regulations. Our observation implies that the species tries to partially compensate for the higher energy costs under OAW conditions,

and thus, crabs are negatively impacted. The combined effect of OAW and oil elevated the RR in *S. serrata*. This might have been caused due to disruption in the histological integrity of gill membranes owing to the combined stress, which increases their penetrability in the organ and inhibits major ion transport enzymes in the gill membranes. This resulted in a higher metabolic cost for osmotic and ionic homeostasis, as indicated by elevated RR.

Ammonia is the chief excretory substance of aquatic organisms formed due to protein degradation and is regarded as a useful stress indicator in bioenergetics studies. Increased nitrogenous waste excretion was noticed in stress treatment groups, probably due to the additional expenditure of cellular energy by catabolizing stored reserves (Jeong and Cho 2007). Similar to our findings, previous studies have also indicated that temperature significantly affects the ammonia excretion rate in Decapoda (Chen and Chia 1996; Thomas et al. 2000; Crear and Forteath 2002; Nandy et al. 2021). Studies on the combined effects of OAW on the excretion rate of other shellfishes also show a significant increase in ammonia excretion levels in elevated temperature conditions but not affected by OA (Navarro et al. 2016; Zhang et al. 2016; Leung et al. 2020). Studies like Zhang et al. (2015) have shown increased excretion rate under combined exposure to OAW. Similar to our results, ammonia excretion was elevated in the embryonic and larval stages of an ectotherm *Coryphaena hippurus*, where temperature and oil cross stress elevated nitrogenous excretion (Pasparakis et al. 2016). Thus, our results infer the possibility of toxicant-induced alterations in metabolism, which finally intensifies catabolism in *S. serrata*.

Physiological energetics-based integrative indices like SfG elucidate the adaptable responses of organisms to the shifting oceanic climate (Leung et al. 2020). Scope for growth constitutes a robust tool for evaluating an animal's instantaneous energy status under a stressful environment (Navarro et al. 2016). Our study indicates a strong correlation between increased stress and a decline in the energy budget for the studied species. In accordance with the present study, lower SfG has been reported under combined OAW conditions (Wang et al. 2015; Leung et al. 2017, 2020) and oil exposure (Jeong and Cho 2007) in many crustaceans besides other shellfishes. Most of these studies have shown diet to be the determining factor affecting SfG for the studied animal. Reduced feeding and energy assimilation reflect a substantial decrease in overall energy gain, which was also noticed in our study. Low energy gain and higher metabolic cost ultimately reduce the energy budget. Although low, positive SfG values were recorded under stressful treatments which might be due to the high energy content in the food provided (dry fish) during our study. Thus, high calorific value food intake might provide some alleviation under stress by boosting acquired energy. Crabs used metabolic depression as a survival strategy when exposed to oil to balance energy requirements, but the energy budget remained relatively poor due to the low feeding rate in that condition. Previous literature on the combined effect of OAW has shown disagreement with our results where SfG were not reduced under stress (Zhang et al. 2015, 2016; Navarro et al. 2016). According to those authors, these observations were justified as the animals already encounter the experimental stressor levels in their normal habitat regime. Such pre-exposure to oscillations in the daily environment would confer strength for coping with changes. Thus, the energy requirement could keep pace with the supply provided. In our study, the stress levels chosen are not experienced by the animals in their habitat but are conjectured to face in the near future. The lowest SfG value was observed under the combined stress of OAW and oil. Therefore, we can conclude that additional stress from oil pollution can exaggerate the already low energy budget under global climatic stressors (OAW).

Conclusion

This study confirms that the combined effects of global environmental drivers OAW exacerbate the impact of acute oil spill and diminish the energy budget of mud crab *S. serrata*. Energy availability is crucial in determining the adaptive responses of marine organisms under altering oceanic climates. Suboptimal environmental conditions tax the energy budget, and as a result, curtailed energy is left for compensatory responses under additional stress, eventually sensitizing animals to pollutants. The present study confirms that combined OAW conditions narrow down the tolerance levels and upsurge the expended energy necessary for homeostatic function in this species. Our work emphasizes the significance of including environmentally relevant multiple stressors in toxicological studies. These results highlight the importance of natural stresses in the enhancement of pollutant toxicity to aquatic animals. The combined effects of environmental stress and pollution showed a reduced energy budget at the organismal level, which might lead to long-term repercussions at the population level. This adverse result implies a constricted geographical distribution of this species in predicted future climate change scenarios. *S. serrata* is an integral component of aquaculture in the Sundarbans, and with the predicted risk of OAW and oil spills in this region, the population might face local extinction without long-term adaptation and thermal tolerance. This will significantly jeopardize the rural economy as well as the ecological balance of the largest mangrove ecosystem in the world. Future research on the impact of global environmental stressors combined with contamination on crab ecophysiology would help better understand future stock production and envisaging consequences in future oceans. We advocate

this study as an effective framework for the management of commercially exploited crab populations and could be potentially beneficial in forecasting the status of coastal biodiversity in the context of future climate change scenarios.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

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Consent to participate Not applicable.

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ARTICLES FOR FACULTY MEMBERS

INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

Feeding habits and the occurrence of anthropogenic debris in the stomach content of marine fish from Pattani Bay, Gulf of Thailand / Soe, K. K., Hajisamae, S., Sompongchaiyakul, P., Towatana, P., & Pradit, S.

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Feeding Habits and the Occurrence of Anthropogenic Debris in the Stomach Content of Marine Fish from Pattani Bay, Gulf of Thailand

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Simple Summary: In this work, the feeding behaviour of fish from a natural bay environment and the ingested anthropogenic fragments in a fish community in relation to their feeding habits and habitats were investigated. The identification of 34 fish species and analysis of their stomach content by visual inspection were carried out. The ingestion of anthropogenic debris by fish differed between season and their feeding features. The planktivorous fish having higher ingestion of anthropogenic debris than other species were found. The study results enhance the understanding of the spatiotemporal variation of feeding habits of fish communities and support future alerts relating to the risk of anthropogenic pollution in marine food webs.

Abstract: This study assessed the feeding habits and ingestion of anthropogenic debris in 34 marine fish species from the southern Gulf of Thailand. A total of 5478 fish samples of 12 families were categorised into seven groups: planktivore, *Lucifer* feeder, fish feeder, *Acetes* feeder, shrimp feeder, piscivore, and zoobenthivore fish. A total of 2477 anthropogenic debris items were extracted from 12 fish species by visual inspection. Their ingestion of anthropogenic debris was influenced by season ($p < 0.0001$), with the highest ingestion during the northeast monsoon season. Furthermore, planktivorous fish displayed more ingested anthropogenic debris than the other investigated species ($p = 0.022$). Blue-coloured anthropogenic debris was commonly detected in the stomachs of fish and significantly differed between species ($p > 0.001$). Water depth and season significantly influenced the availability of food types (AF) for fish ($p < 0.001$). These findings provide evidence of the ingestion of anthropogenic debris by fish inhabiting a natural bay and signal the future anthropogenic pollution of marine fish.

Keywords: feeding features; microplastic; food type; season; water depth

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1. Introduction

In the marine ecosystem, fish are major top predators and important for aquaculture and conservation management [1]. Fish stomach content is important primary data to directly study the feeding ecology of fish [1,2] and can be quantitatively or qualitatively presented [3,4]. To determine the feeding habits of fish, the index of relative importance (%IRI) is most frequently used by about 30% of citations examining the stomach content of fish [1]. It can be calculated from the weight or volume of a prey item and the percentages of number and frequency of occurrence [5]. It is important fundamental information to

understand the functional role of the fish community in aquatic ecosystems [1,2,6] and useful to understand the interspecific interaction of resource partitioning (e.g., habitat, food) between species [7–9]. Fish show a narrow range of feeding adaptation, though there are some overlaps in food selection between niches in tropical estuaries [2] and nontropical estuaries [9–13]. There are some studies of diet overlap, food selection, and resource partitioning of fish in tropical and nontropical regions. Examples include the fish community inhabiting the bay mouth region in Thailand [14], short mackerel (*Rastrelliger brachysoma*) in a tropical estuarine environment [15], estuarine–reef habitat fish in Brazil [9], demersal fish on the continental shelf of the East/Japan Sea [10] and Southern Tyrrhenian Sea [16], deep-sea fish community in the benthic layer of the Mediterranean Sea [11], deep-sea shark (*Galeus melastomus*) in the Mediterranean Sea [12] and southern Tyrrhenian Sea [17], and diet overlap between jellyfish and juvenile fish in Alaska [13].

Further, fish diet composition fluctuates by season and spatiality [2], including food availability and reproductive activity [18]. Studying diets to obtain information about the food composition and feeding behaviour of species is critical to examine the ecosystem role and position of species in the ecosystem food web [19]. This information is crucial to support the management of aquatic life, especially fisheries, aquaculture, and the conservation among many species of aquatic ecosystems, in addition to supporting food security. Fish are the most important predators and have a determinant status in the trophic position of aquatic ecosystems. Many fish species play an important role in the economy of many countries around the world. Hence, information on the diet composition and feeding features of fish near Thailand is necessary.

Fish are a useful bio-indicator of contamination of anthropogenic debris (microplastics making up the main content) to assist food security valuations [20]. However, Santana et al. [21] reported there was no evidence of plastic particle persistence in aquatic organism tissue, but consumption of fish with microplastics can lead to human health risk if toxic substances adhere to microplastics [22]. Meanwhile, consumption of microplastics can have negative effects on growth, reproduction, and survival evidence, although most of the effects are sublethal [23,24]. In addition, soft or thin plastic fragments on muddy beaches are found in higher amounts than other debris types that may have a harmful impact on marine organisms [25]. Specifically, plastic debris from anthropogenic activity occurs regardless of the season, area, or ontogenetic phase and may be passed through direct consumption and prey items [26].

Microplastics are defined as any plastic particle smaller than 5 mm [27] and have been widely distributed in the ocean and sediments worldwide in recent years [28], including all water of pelagic and benthic marine organisms [16,17,29–32]. Microplastics can be found in all living organisms, from tiny animals such as zooplankton [31], mysid larvae [24], and bivalves [33,34] to top predators [16,18,21,30,32,35–42]. The first report of microplastics in plankton tows was reported by Carpenter et al. [43] in North America, which later caused concern for massive water bodies [44]. For instance, microplastics have accumulated in oceans and sediment with concentrations of 3 to 102,000 m^{-3} and 1 to >1000 m^{-2}, respectively [28]. On the contrary, Md Amin et al. [45] stated that the average abundance of microplastics in surface seawater of the southern South China Sea was 0.003 m^{-3}. Microplastics pose increasing threats to the food web [38] and are transferred from prey to predator [21,31,46]. In particular, low trophic fauna is mostly affected by microplastics through ingestion [28]. For example, plastic shaped similar to algae was mistaken for food by suspension feeders [47]. When comparing the concentration of microplastics in the young and adult stages of mugilids, the early development stage of fish had a greater concentration than the older stage [37], and small-sized shellfish contained more particles than large-sized fish [34]. Therefore, there was a significant effect of prolonged exposure to microplastic harm related to age and size specific to organisms [24].

In the lower part of the Gulf of Thailand, some sciaenid fish show higher ingestion of mesoplastics than micro- and macroplastics [35]. Fishing net fibres were the major types of plastic found in the stomachs of some commercial marine fish, and of those, 80% were

microplastics (<5 mm), whereas the rest were mesoplastics (5–25 mm) [36]. In addition, higher ingestion of microplastics was found in some commercial shrimp than in fish, most of the microplastics being fibres from textiles and fishing nets in Thailand [39]. However, the occurrence of ceramic and glass debris is greater than plastic and other debris in beach sediment due to shoreline and recreational activities [25].

Microplastics enter the marine environment by different pathways [42], and the ingestion of microplastics by aquatic organisms is related to their feeding habits and habitats [30]. Information on the diet composition, feeding features, and potential threats of plastic debris in marine creatures in Thailand is necessary. The present study aimed to evaluate the feeding habits of those fish and their potential contamination in environments by determining the following: (1) the occurrence of anthropogenic debris, including microplastic-like debris, in wild fish from the natural bay environment; (2) the anthropogenic debris ingestion of fish dependent upon their feeding features, water depth, and season; and (3) the variety of food types of fish at different depths or in different seasons.

2. Materials and Methods

2.1. Study Area

The survey site is situated off Pattani Bay with a surface area of 74 km^2, located in the lower part of the Gulf of Thailand at latitude 06°52′5.3″ N and longitude 101°15′0.3″ E (Figure 1). The bay is semienclosed by a 12 km long sand spit on the northeast side. In general, the seasonal pattern of southern Thailand is influenced by the sea on both sides and heavy rains throughout the year. Based on the rainfall level, Pattani province has three seasons: dry season from January to May, moderate rainfall season (southwest monsoon) from May to September, and heavy rainy season (northeast monsoon) from September to December [14,15,48,49]. On average, Pattani province has seven months of rainfall and five months of drought due to the northeast monsoon (November–February) and southwest monsoon (May–September) [48]. The main site of this study comprised the area around the vicinity of the mouth of the bay, called Rusamilae fishing village. This area was selected because it is locally known as a major fishing ground by local fisherfolk [15,49].

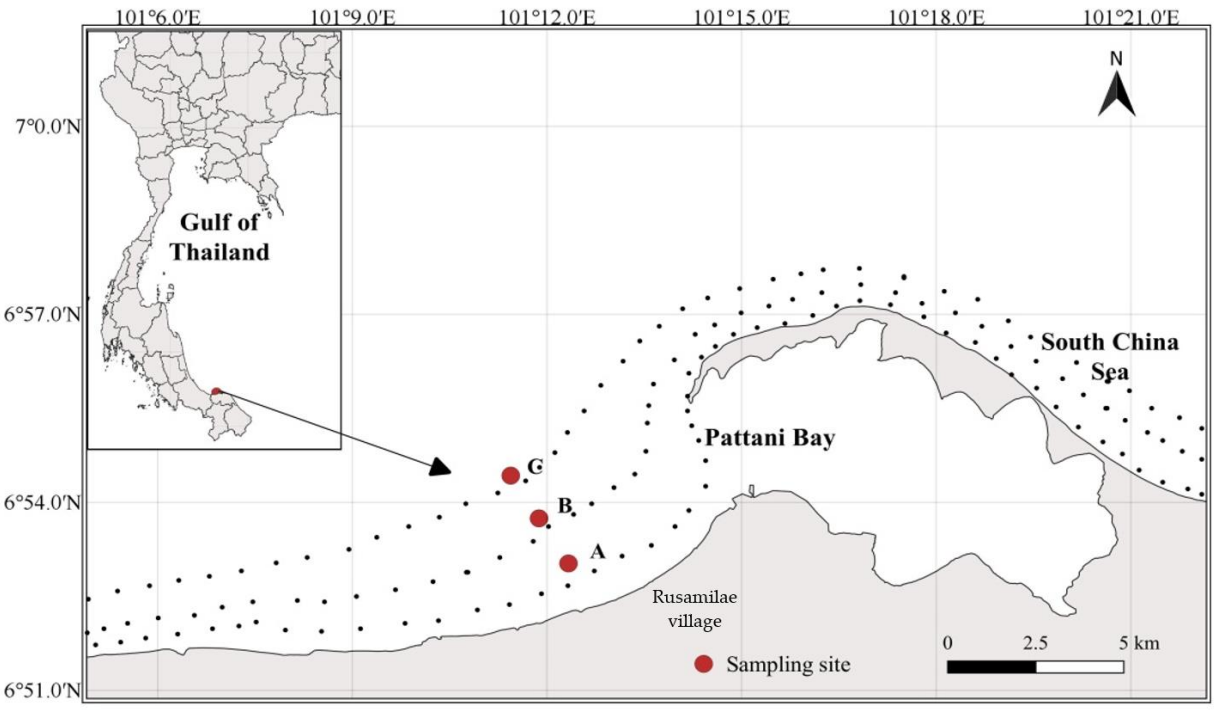

Figure 1. Fish sampling sites off the mouth of Pattani Bay in the lower Gulf of Thailand. The red circles represent the fish sampling sites between the water depth contours of 2, 4, and 6 m.

2.2. Sample Collection and Storage

Altogether, 13-month fish sampling was conducted from February 2019 to February 2020. Fish were caught at three water depth contours of 2, 4, and 6 m using a set of multiple-mesh-sized mackerel gill nets with 3.0, 4.0, and 4.5 cm stretched mesh-sizes, 3.5 m deep and 540 m long; therefore, each mesh was 180 m long [14,15]. At each sampling station, a set of multiple-mesh-sized nets were hauled and left to drift for around one hour between 18:00 and 19:00. Most fish died immediately after being caught and were preserved in iceboxes as soon as possible before transportation to the laboratory at the Faculty of Science and Technology, Prince of Songkla University. Specimens were sorted and identified immediately. Fifty individual fish per species were randomly collected from each of the three sampling stations and preserved with 10% formalin for four days before being transferred to 70% ethanol for further analyses.

2.3. Diet and Anthropogenic Debris Identification

In the laboratory, a diet analysis of 5478 fish samples was performed. Total length was measured from the tip of the snout of fish to the tip of the caudal fin. Then, the fish stomach was removed from the body cavity and opened with surgical scissors. During processing, stomach content was carefully taken apart, and all identifiable prey from the 3236 nonempty stomachs were counted and specified to the lowest possible taxa with pertinent literature [50–53]. The feeding functional group was classified according to dietary preference [54]. There were seven main feeding guilds based on their %IRI: (1) planktivorous, which feeds mainly on phytoplankton and copepod zooplankton; (2) *Lucifer* feeder; (3) fish feeder, which feeds mainly on fish but also feeds on phytoplankton and copepod zooplankton; (4) *Acetes* feeder; (5) shrimp feeder; (6) piscivorous, which feeds mainly on fish; and (7) zoobenthivorous, which feeds mainly on polychaetes.

The prey types for piscivorous fish, shrimp feeder, and zoobenthivorous fish were examined under a stereomicroscope. For planktivorous fish and *Lucifer* feeder, the stomach content was put in a 15 mL measuring cylinder filled with water. The 1 mL subsample was later taken and placed on a Sedgwick Rafter chamber. Thereafter, diets were identified and counted under a light microscope. To reduce misidentification between plastic-like debris and the broken cell structure of natural prey items, nonorganic fibre was considered as plastic-like fibre, while nonorganic hard material was considered as fragments [37,42,55]. To distinguish between organic and nonorganic materials, we followed the rules of Hidalgo-Ruz [56]: Rule 1, no cellular or organic structures visible; Rule 2, fibres should be equally thick throughout their entire length; Rule 3, particles should exhibit homogeneous colour throughout the item. The hot needle test [57] was also applied for suspected cases where we were unable to distinguish between plastic and organic matter. In the presence of a hot needle, plastic pieces will melt or curl, while biological and other nonplastic materials will not. Although the aforementioned rules were applied to identify plastic materials, the whole identified fragments were not classified as totally plastic substances until they were verified by FT-IR spectrophotometer. Thus, the so-called plastic-like debris was employed for the identification of anthropogenic debris in this study.

Food types were photographed with a microscope (NIKON Eclipse E200, Nikon instruments Inc., Melville, NY, USA) attached to a digital camera (NIKON DS Fi2, Nikon instruments Inc., Melville, NY, USA). The anthropogenic debris in this study was grouped according to colour as blue, black, red, green, and white. The observed anthropogenic debris items were regarded as microplastic-like items (<5 mm), mesoplastic-like items (5–25 mm), or macroplastic-like items (>25 mm) with reference to the relevant literature [58].

2.4. Experimental Control

To avoid contamination, only laboratory glassware was used during laboratory work. To prevent sample contamination during laboratory work and visual identification, specific care was applied. To prevent contamination, an 8 cm petri dish with a few millilitres of distilled water (blank) was placed next to the working zone beside the microscope to

prevent any atmospheric contamination. The results from the blank control showed no microplastic-like debris contamination.

2.5. Data Analysis

Raw diet data were analysed to determine the feeding features of fish in terms of (i) the average number of food types (AF) and (ii) percentage of index of relative importance (%IRI).

AF refers to the average number of food types observed in each stomach. Prior to estimating the %IRI of the fish, the index of relative importance (IRI) was calculated to determine the food preference of fish by the following formula [5]:

$$IRI_i = \%F(\%N + \%V)$$

where %V refers to the percentage contribution of all food items in nonempty stomachs that were estimated by visual inspection and calculated based on the area covered by each prey type on a scaled Petri dish by the Hyslop formula [59]. %N and %F represent percentages of number and frequency of occurrence of prey "$_i$", respectively. Finally, %IRI was determined by the following formula [3]:

$$\%IRI = 100\ IRI_i / \sum IRI_i$$

2.6. Statistical Analysis

Analysis of variance (ANOVA) was used to test for the ingestion of anthropogenic debris related to their feeding features, while the ingestion of debris colour in the stomach content of fish was tested. In addition, the differences of AF in fish collected from different depths and in different seasons were tested. To reduce non-normality, raw data were transformed to log (X + 1) before testing. If statistically significant, Tukey's HSD post hoc test was then applied for the factors depth, season, feeding features, and debris colour using the R program [60].

3. Results

3.1. Food and Dominant Food Items

For data elaboration, 3236 samples of nonempty stomachs from 5478 samples of 34 fish species consisted of 22 different prey categories, which could be designated into seven main feeding guilds based on their index of relative importance (%IRI): planktivore, *Lucifer* feeder, fish feeder, *Acetes* feeder, shrimp feeder, piscivore, and zoobenthivore fishes (Table 1). Out of 34 species, only three polychaete feeders (*Nuchequula gerreoides*, *Johnius belangerii*, and *J. borneensis*) were recognised as zoobenthivorous fish. In this study, fish (24.1%), *Lucifer* (14.7%), and penaeid shrimp (13.4%) were the most important groups and the largest contributors for fish inhabiting the vicinity of the natural bay environment, followed by *Coscinodiscus* sp. (8.4%), copepods (8.3%), diatoms (7.6%), *Acetes* sp. (5.6%), polychaetes (5.1%), and other prey items (<3.0) (Table 2). Among planktivores, *Eubleekeria splendens* and *Photopectoralis bindus* mainly feed on diatoms with an average of 60.0% and 48.1% by %IRI, respectively. Examples of the stomach content of fish are shown in Figure 2.

Table 1. Feeding habit and occurrence of anthropogenic debris by fish species off Pattani Bay, in the lower Gulf of Thailand (AF = number of food types; bold indicating statistically significant *p* value). Parentheses enclose the average number of fish sampled (N) per month. * Statistical analysis applied with month factor when seasonal factor was not available; ** anthropogenic debris present.

Family	Species	Sample (N)	Nonempty Stomach	Total Length (Mean ± SD)	AF (Mean ± SD)	Depth	Season	Feeding Features
						p Value		
Clupeidae	*Anodontostoma chacunda*	625 (48.1)	193	12.9 ± 1.3	13 (1.7 ± 1.3)	0.779	0.611	Planktivore **
	Hilsa kelee	136 (22.7)	110	15.3 ± 1.7	13 (3.3 ± 1.3)	**0.0001**	**<0.0001**	Planktivore **
	Sardinella fimbriata	211 (30.1)	181	13.7 ± 0.8	14 (2.3 ± 1.2)	**0.036**	0.071	Planktivore **
	S. gibbosa	231 (30.4)	218	13.5 ± 1.6	13 (1.7 ± 1.5)	**0.007**	**<0.0001**	*Lucifer* feeder **
Engraulidae	*Setipinna taty*	185 (20.6)	49	13.9 ± 1.5	10 (1 ± 0.8)	**0.0008**	**0.032**	*Lucifer* feeder
	Stolephorus commersonnii *	18 (4.5)	8	9.6 ± 1.9	5 (0.9 ± 0.4)	**<0.0001**	0.588	Fish feeder
	S. waitei	19 (4.8)	6	8.5 ± 2.0	7 (1.5 ± 1.9)	0.586	0.358	*Acetes* feeder
	Thryssa hamiltonii	325 (25)	168	17.9 ± 1.8	9 (1.0 ± 0.4)	0.791	0.075	Shrimp feeder
	T. kammalensis	148 (21.1)	43	9.9 ± 0.9	10 (0.9 ± 0.7)	**0.036**	0.059	*Acetes* feeder **
	T. setirostris	59 (14.7)	10	14.6 ± 0.9	4 (0.9 ± 0.3)	**<0.0001**	**<0.0001**	Shrimp feeder
Chirocentridae	*Chirocentrus nudus*	29 (5.8)	27	29.9 ± 4.2	6 (1.0 ± 0.3)	**0.019**	**0.0001**	Piscivore
Pristigasteridae	*Opisthopterus tardoore*	293 (22.5)	100	14.8 ± 1.6	8 (0.8 ± 0.4)	**0.002**	**0.046**	*Lucifer* feeder
Synodontidae	*Harpadon nehereus* *	116 (38.7)	22	21.1 ± 1.6	3 (0.9 ± 0.4)	0.755	0.763	Piscivore
Carangidae	*Alepes kleinii*	243 (20.3)	121	12.0 ± 1.9	8 (0.7 ± 0.5)	0.295	0.239	*Lucifer* feeder
	A. vari	14 (2.3)	10	13.1 ± 1.6	3 (0.8 ± 0.4)	0.072	1.000	*Lucifer* feeder
	Megalaspis cordyla	184 (15.3)	159	15.6 ± 2.3	7 (0.9 ± 0.5)	0.559	0.113	Piscivore **
	Scomberoides tol	23 (3.8)	18	15.4 ± 2.4	5 (0.9 ± 0.4)	0.664	0.891	Piscivore
Leiognathidae	*Deveximentum insidiator*	102 (7.8)	71	9.8 ± 2.5	10 (1.0 ± 1.2)	0.312	**<0.001**	*Lucifer* feeder **
	Eubleekeria jonesi *	40 (20)	34	7.3 ± 0.8	9 (1.0 ± 1.7)	**<0.001**	**<0.001**	Planktivore
	E. splendens	272 (20.9)	146	7.5 ± 1.0	16 (1.7 ± 1.4)	**0.003**	**0.023**	Planktivore **
	Leiognathus equula	76 (5.8)	61	9.2 ± 0.8	11 (0.6 ± 1.0)	**0.002**	0.098	Planktivore **
	Nuchequula gerreoides	41 (20.5)	31	8.9 ± 1.2	6 (0.7 ± 0.9)	**0.002**	0.098	Zoobenthivore
	Photopectoralis bindus	319 (26.6)	191	9.4 ± 1.1	12 (0.5 ± 0.9)	0.100	**0.009**	Planktivore **
Sciaenidae	*Dendrophysa russelii*	65 (6.5)	32	12.3 ± 1.6	8 (0.7 ± 0.6)	**0.048**	0.371	Shrimp feeder
	Johnius belangerii	68 (13.6)	35	14.7 ± 1.1	6 (0.7 ± 0.6)	0.335	0.076	Zoobenthivore
	J. borneensis	167 (18.6)	89	15.1 ± 1.3	9 (0.7 ± 0.6)	**0.011**	**0.001**	Zoobenthivore
	Otolithes ruber	131 (13.1)	36	18.3 ± 2.1	6 (0.9 ± 0.5)	0.064	0.284	Piscivore
	Panna microdon	62 (8.9)	39	20.6 ± 3.3	5 (0.8 ± 0.5)	0.926	0.299	Shrimp feeder
	Pennahia anea	76 (15.2)	12	14.0 ± 1.6	3 (0.9 ± 0.5)	0.611	0.426	Piscivore
Polynemidae	*Eleutheronema tetradactylum*	72 (6)	53	21.9 ± 2.6	5 (1.1 ± 0.4)	0.997	0.433	Shrimp feeder
Mugilidae	*Planiliza subviridis*	132 (14.7)	39	17.7 ± 2.0	10 (0.8 ± 1.2)	0.542	0.096	Planktivore **
Trichiuridae	*Trichiurus lepturus*	62 (8.9)	28	44.1 ± 5.3	2 (1.0 ± 0.0)	0.427	0.431	Piscivore
Scombridae	*Rastrelliger brachysoma*	554 (42.6)	552	16.4 ± 1.9	15 (3.8 ± 1.2)	**0.031**	**0.003**	Planktivore **
	Scomberomorus commerson	380 (29.2)	344	21.1 ± 4.3	7 (1.0 ± 0.2)	**0.033**	0.799	Piscivore

Table 2. Index of relative importance (%IRI) contribution of anthropogenic debris and other dietary types of fishes inhabiting the natural bay environment (Deb = anthropogenic debris; Fish = fish; Luc = *Lucifer* sp.; Shri = shrimp; Ace = *Acetes* sp.; Cop = copepods; other = other zooplankton; Poly = polychaetes; Cosc = *Coscinodiscus* sp.; Diat = other diatoms, Dino = dinoflagellates; Stom = stomatopods; Squid = squid; Cru = other crustaceans excluding copepods; Crab = crabs; Sagi = *Sagitta* sp.; Brit = brittle stars; Sea = sea urchins; Moll = molluscs; Amp = amphipods; Nem = nematodes; Mis = miscellaneous). In bold: main food contribution.

Species	Deb	Fish	Luc	Shri	Acet	Cop	other	Poly	Nem	Cosc	Diat	Dino	Stom	Squid	Crus	Crab	Sagi	Bri	Sea	Moll	Amp	Mis
A. chacunda	1.7	0.0	0.0	0.0	0.0	8.3	**38.0**	1.2	7.5	25.5	11.9	3.4	0.0	0.0	0.4	0.0	0.0	0.0	0.0	1.4	0.0	0.6
H. kelee	0.1	0.0	0.0	1.0	0.0	**22.7**	2.1	0.3	0.1	37.0	23.3	12.6	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.4	0.0	0.1
S. fimbriata	2.5	0.0	11.6	2.4	0.0	**45.5**	0.1	1.4	0.7	16.8	15.8	1.1	0.0	0.0	0.0	0.5	0.0	0.0	0.0	1.4	0.0	0.1
S. gibbosa	0.0	0.0	**35.5**	0.3	0.0	16.8	1.1	3.4	0.0	23.3	15.2	3.4	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.6	0.0	0.1
S. taty	0.0	0.0	**61.4**	21.9	7.1	0.0	0.0	3.6	0.0	1.7	0.0	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0	0.9	0.0	0.0
S. commersonnii	0.0	**57.1**	14.3	0.0	0.0	14.3	0.0	0.0	0.0	14.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S. waitei	0.0	0.0	17.5	0.0	**36.8**	20.2	0.0	0.0	0.0	18.4	7.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T. hamiltonii	0.0	33.2	2.0	**33.5**	18.1	0.0	8.6	0.7	0.0	0.0	0.0	0.0	2.8	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.0
T. kammalensis	3.8	0.0	0.0	30.8	**50.0**	0.0	0.0	0.0	0.0	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.5	0.0
T. setirostris	0.0	0.0	22.2	**44.4**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C. nudus	0.0	**79.4**	0.0	15	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
O. tardoore	0.0	2.8	**47.2**	25.5	22.4	0.2	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0
H. nehereus	0.0	**89.5**	0.0	10.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A. kleinii	0.0	0.0	**87.0**	0.8	6.8	0.0	0.0	2.3	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	1.4	0.0	0.0	0.0
A. vari	0.0	0.0	**100.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
M. cordyla	1.2	**80.9**	1.5	0.8	10.3	0.0	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.4	0.0	0.0
S. tol	0.0	**57.7**	20.0	2.3	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D. insidiator	3.0	0.0	**53.0**	0.0	2.8	12.6	0.0	2.8	0.0	7.4	13.7	1.4	0.0	0.0	0.0	3.5	0.0	0.0	0.0	0.0	0.0	0.0
E. jonesi	0.0	0.0	3.5	0.0	0.0	**36.1**	4.6	0.0	2.6	36.9	4.7	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	10.3	0.0	0.0
E. splendens	2.6	0.0	1.6	0.0	0.0	11.2	7.2	1.3	0.7	8.3	**60.0**	2.7	0.0	0.0	0.1	0.2	1.0	0.0	0.8	0.4	0.0	1.8
L. equula	7.9	0.0	11.4	0.0	0.0	**22.5**	1.8	16.4	0.3	4.9	24.1	4.3	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	5.2
N. gerreoides	0.0	0.0	0.0	0.0	0.0	2.6	2.8	**33.8**	0.0	28.4	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	9.9	21.5	0.0
P. bindus	10.1	0.0	8.5	0.0	0.0	10.8	0.9	7.4	0.3	1.9	**48.1**	0.6	0.0	0.0	0.3	0.0	1.7	0.6	0.0	0.4	0.8	7.7
D. russelii	0.0	20.3	0.0	**50.3**	5.9	0.0	0.0	5.9	0.6	0.0	0.0	0.0	0.0	5.9	0.0	8.2	0.0	0.0	0.0	0.0	0.0	2.9
J. belangerii	0.0	7.1	0.0	30.2	0.0	0.0	0.0	**37.7**	0.0	0.0	0.0	0.0	0.0	0.0	17.9	7.1	0.0	0.0	0.0	0.0	0.0	0.0
J. borneensis	0.0	15.7	0.0	15.0	0.0	0.0	0.0	**50.8**	1.4	0.0	0.0	0.0	0.0	0.0	3.5	7.9	0.0	0.5	0.0	3.9	0.0	1.4
O. ruber	0.0	**50.0**	0.0	32.1	5.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.6	3.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.4
P. microdon	0.0	13.5	0.0	**63.6**	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	18.6
P. anea	0.0	**87.3**	0.0	12.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
E. tetradactylum	0.0	25.3	0.0	**71.6**	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P. subviridis	5.0	0.0	0.0	0.0	0.0	10.6	13.8	1.8	1.2	**35.5**	16.2	4.9	0.0	0.0	0.5	0.0	0.0	0.0	0.0	1.5	0.0	8.9
T. lepturus	0.0	**100.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
R. brachysoma	0.4	0.0	1.3	0.1	0.0	**47.3**	0.9	0.3	0.1	20.9	17.0	8.0	0.0	0.0	0.3	0.2	0.0	0.0	0.0	2.9	0.0	0.3
S. commerson	0.0	**99.4**	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Average	1.1	24.1	14.7	13.4	5.6	8.3	2.5	5.1	0.5	8.4	7.6	1.2	1.2	0.4	1.0	0.9	0.1	0.03	0.1	1.0	1.0	1.6

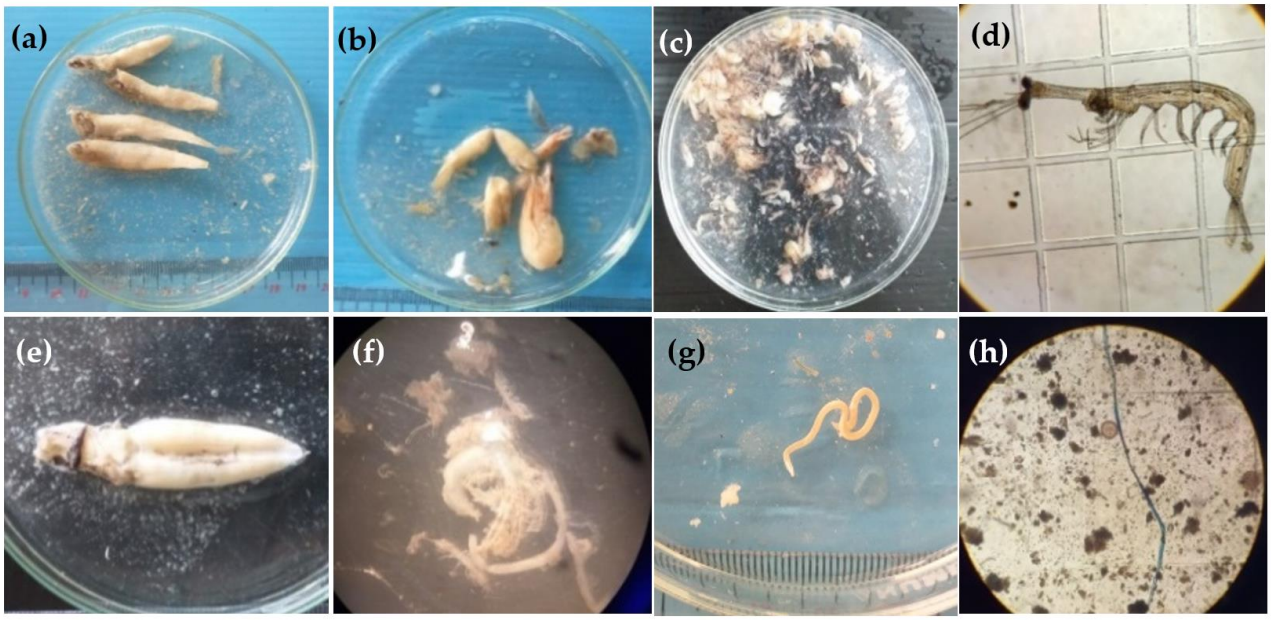

Figure 2. Examples of fish stomach content: (**a**) fish, (**b**) penaeid shrimp, (**c**) *Acetes* sp., (**d**) *Lucifer* sp., (**e**) squid, (**f**) hermit crab, (**g**) nematode, and (**h**) anthropogenic debris.

The average number of food types (AF) ranged from 2 (*T. lepturus*) to 16 (*E. splendens*) types (Table 1). More than 10 food types were found mostly in planktivores, whereas fewer than 8 types were found in piscivorous fishes. The fish that had high AF were considered opportunist feeders. AF of individual fish was influenced significantly by water depth and season ($p < 0.05$), especially for most of the planktivorous fish, some *Lucifer* feeders, and one zoobenthivorous fish (*J. borneensis*) (Table 1). On the contrary, planktivorous fish such as *Anodontostoma chacunda*, *Planiliza subviridis*, some *Lucifer* feeders (*Alepes kleinii* and *A. vari*), *Acetes* feeders (*S. waitei*), shrimp feeders (*Thryssa hamiltonii*, *Panna microdon*, and *E. tetradactylum*), zoobenthivorous fish (*J. belangerii*), and piscivorous fish (*H. nehereus*, *M. cordyla*, *S. tol*, *Otolithes ruber*, *Pennahia anea*, and *T. lepturus*) showed no statistical significance, indicating their feeding was not influenced by the water depth or season.

3.2. Spatial and Temporal Impacts of Depth and Season

The ingestion of anthropogenic debris by fish was influenced only by the season ($p < 0.0001$) and not by the water depth ($p = 0.840$). Tukey's HSD post hoc test showed that high ingestion of debris was observed in the northeast monsoon season. In addition, among the anthropogenic debris ingestion of four feeding features, this was more significant for planktivorous fish ($p = 0.022$) than for the other studied fishes (Table 3). Among the five debris colours, blue was significantly more common than the others ($p < 0.001$).

By the analysis of variance, AF of 34 fish species was influenced significantly by water depth and season ($p < 0.001$) (Table 3). Tukey's HSD test indicated that AF significantly differed between 2 and 4 m depths. Based on the season, AF significantly differed between the dry and northeast monsoon seasons.

Table 3. Results of analysis of variance on the number of food types and ingestion of anthropogenic debris in 34 fish species by depth and season in addition to feeding features (df = degrees of freedom, MS = mean sum of squares).

	Factor	df	MS	F Value	*p* Value
Ingestion of anthropogenic debris in fish	Depth (d)	2	0.02	0.17	0.840
	Season (s)	2	1.25	12.97	<0.0001
	d × s	4	0.17	1.76	0.135
Ingestion of anthropogenic debris in four feeding features	Feeder (f)	3	1.21	3.23	<0.022
Colour of anthropogenic debris in fish stomach	Debris colour (c)	4	4.05	5.38	<0.001
Food items (AF)	Depth (d)	2	1.13	3.98	0.019
	Season (m)	2	10.99	38.67	<0.0001
	d × s	4	1.49	5.23	<0.001

3.3. Ingestion of Anthropogenic Debris in Fish

From the 5478 fish samples of 34 species, 3236 nonempty stomachs were assessed, and anthropogenic debris was observed in the guts of 12 fish species. A total of 2477 debris items were observed in the 67 guts of those 12 fish species, which accounted for 3.4% of a total of 1964 fish samples (Table 4). More debris was ingested by planktivorous fish than by piscivorous fish. Among planktivorous fish, *R. brachysoma* had the highest ingestion (2.6 ± 16.4 items/fish), whereas *Deveximentum insidiator* had the lowest (0.4 ± 2.5 items/fish). Compared with planktivores, *Acetes* feeder fish *Thryssa kammalensis* and piscivorous fish *M. cordyla* had low ingestion of debris at 0.02 ± 0.2 items/fish and 0.01 ± 0.1 items/fish, respectively. Examples of anthropogenic debris found in the stomachs of fish are shown in Figure 3.

Including plastic fibres and plastic bags found in the stomach content of fish, the average numbers of anthropogenic debris items are shown in Table 4. The most consumed colour of debris with a length of less than 3 mm in different species was blue, followed by green, red, black, and white, while *M. cordyla* had 3 cm of degraded plastic bag. Blue-coloured debris was dominant in *S. gibbosa*, *D. insidiator*, *E. splendens*, *P. bindus*, *P. subviridis*, and *R. brachysoma*. The green colour was dominant in *A. chacunda* and *Sardinella fimbriata*; red colour, in *H. kelee*; and black colour, in *Leiognathus equula*.

Table 4. Abundance of ingested anthropogenic debris by number and colour in the 12 marine fish species of non-empty-stomach fish. Parentheses enclose the number of fish with ingested debris.

Species	Examined Fish	% of Debris in Fish	No. of Items	Items/Fish	Length of Debris (mm)	Blue	Green	Red	Black	White
A. chacunda	193 (9)	4.7	195	1.00 ± 4.9	1–3	0	170	25	0	0
H. kelee	110 (3)	2.7	60	0.50 ± 3.5	<1.0	10	20	30	0	0
S. fimbriata	181 (4)	2.2	120	0.70 ± 5.9	1–3	0	120	0	0	0
S. gibbosa	218 (4)	1.8	135	0.60 ± 5.6	1–3	75	0	60	0	0
T. kammalensis	43 (1)	2.3	1	0.02 ± 0.2	1–2	1	0	0	0	0
M. cordyla	159 (1)	0.6	1	0.01 ± 0.1	3 cm	0	0	0	0	1
D. insidiator	71 (2)	2.8	30	0.40 ± 2.5	1–2	20	10	0	0	0
E. splendens	146 (12)	8.2	210	1.40 ± 5.1	<1.0–2.0	80	35	25	70	0
L. equula	61 (3)	4.9	45	0.70 ± 3.3	1–2	10	0	15	20	0
P. bindus	191 (9)	4.7	180	0.90 ± 4.5	<1.0–2.0	120	0	60	0	0
P. subviridis	39 (2)	5.1	45	1.20 ± 5.3	<1.0	45	0	0	0	0
R. brachysoma	552 (17)	3.1	1455	2.60 ± 16.4	0.5–3	955	260	240	0	0
Total	1964 (67)	3.4	2477	1.30 ± 9.5	-	1316	615	455	90	1

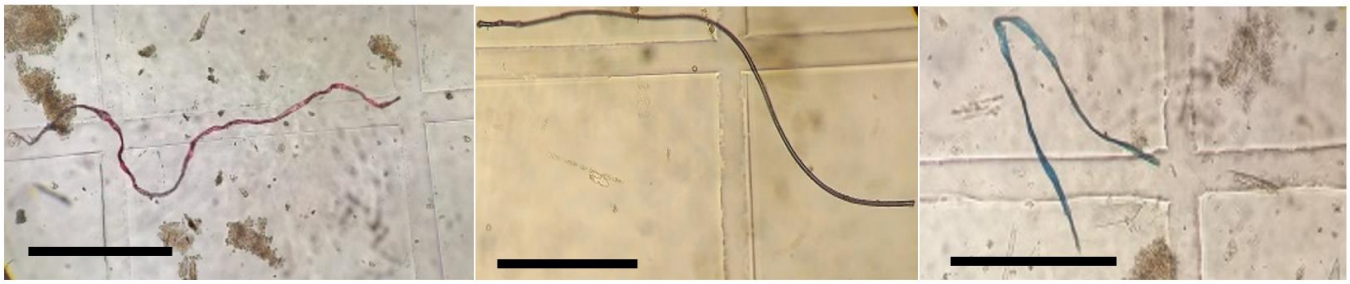

Figure 3. Examples of anthropogenic debris observed in the stomachs of fish. Scale bar 1 mm.

4. Discussion

The present study investigated the feeding habits and anthropogenic debris ingestion of fish collected by mackerel gill nets from the natural environment off Pattani Bay, located in the southern region of the Gulf of Thailand. All fish inhabited the vicinity of the natural bay and competed for food resources, as shown by the occurrence of more than one dietary item in their stomach content. In general, most fish are opportunistic feeders and their diets may shift according to their food habitat and environmental conditions [1]. Most fish species are omnivorous in tropical estuarine environments [61], and young individual omnivorous fish of several taxa may serve as food for carnivorous fish [9]. In the nonestuarine habitat, more carnivorous or predatory fish and fewer contributions of herbivorous species were mainly observed in mangrove habitats, whilst omnivorous species dominated along the edge of mangrove and in the seagrass bed [62]. This agreed with the present study, in which most of the fish species were assigned as omnivorous, including planktivorous, *Lucifer* feeders, and *Acetes* feeders, but herbivorous fish were not observed. Pattani Bay supports a rich diverse fauna community and is an important fishing ground for local fisherfolk due to the presence of seagrass and seaweed meadows, mangrove forests, and sand–mud beds [63–65].

Out of 34 fish species, 19 species exhibited influences of water depth and seasonal factors on their AF by means of spatial and temporal variation of their dietary preference. In comparison within feeding features, both water depth and season were significant influence factors for most planktivorous fish, shrimp feeders, and *Lucifer* feeders, though some piscivorous fish were not affected by those factors. Particularly, fish inhabiting 2 and 4 m depths had more available prey items. It is postulated that shallow water provides more prey types for small fish, while deeper water supports larger fish (piscivorous fish).

The higher AF detected during the northeast monsoon season may be related to high rainfall and rivers (Pattani and Yamu) carrying a lot of nutrients from the land. The potential reasons for this pattern may include a recognisable seasonal trend of food availability that manifests in prey types. Consequently, this area may support the food chain of various fish feeding features. Therefore, Pattani Bay provides an important feeding ground for fish resources that should be sustained for future recruitment. The highest value of dietary items of fish might be related to the concurrence of the high abundance of prey during a specific period [18,66] during which plenty of food is derived from the land, river, and tidal mixing [61]. Some nemipterid fish showed that the AF of fish was influenced significantly by fish size classes in the lower part of the South China Sea [67].

Greater ingestion of anthropogenic debris was detected in sardines (*A. chacunda*, *H. kelee*, *S. fimbriata*, and *S. gibbosa*) than in anchovy fish (*T. kammalensis*). Bakun [68] stated that sardine fish are opportunist feeders whereas anchovy fish are specialists. In addition, the ingestion of anthropogenic debris was related to the filtration apparatus, as debris was ejected into the surrounding waters by the brachial system of adult fish [69]. However, Pennino et al. [70] reported that the highest microplastic ingestion was found in the lower body conditions of anchovies compared to sardines, which was in contrast to our study. In addition, de Moura and Vianna [41] reported that the ingestion of microplastics in the teleost fish was commonly fibres (20.2%) and fragments (22%). In the southern region of Thailand, some studies have been conducted on plastic debris in commercial fish and

shrimp species [25,35,36,39,71]. Compared with the present study, there were different results depending on the study focus by species. For instance, there was no presence of anthropogenic debris in some sciaenid fish in this study, in contrast to the report by Azad et al. [35]. Moreover, the ingestion of plastic debris in *S. gibbosa* (0.3 items/fish), *E. splendens* (1 item/fish), and *R. brachysoma* (1 item/fish) from Azad et al. [36] was lower than that in the present study, while the ingestion of plastic debris in *M. cordyla* (1.6 items/fish) was lower than that in the aforementioned publication. The differences likely depend on feeding habitat, fishing ground, and seasonally available food. Season is a significant factor in this habitat, with especially high ingestion of plastic debris during the northeast monsoon season, but not irrespective of water depth. This may be related to the seasonal river inflow that carries plastic contaminants during the rainy season [20,72]. In addition, Barletta et al. [20] reported that the concentration of microplastic debris was higher in upstream locations during the dry season, while seaward areas had higher concentrations during the rainy season.

Hajisamae et al. [2] concluded that carnivorous fish depend mostly on their visual ability for prey detection. This was in agreement with the present study; the occurrence of anthropogenic debris ingestion was high among planktivorous fish, and *R. brachysoma* had especially high amounts of ingested anthropogenic debris. Therefore, it may be assumed that the ingestion of plastic debris may depend on the feeding behaviour of an individual species. In addition, Lima et al. [72] reported that planktivorous fish might ingest microplastics along with their food and then transfer them to larger predators. Klangnurak and Chunniyom [73] reported that microplastic accumulation in the gastrointestinal tracts of pelagic and demersal fishes showed no significant differences indicating the potential threats of microplastics throughout the water columns. On the contrary, Jabeen et al. [74] stated that the ingestion of plastic items in fish was closely related to the habitat and the gastrointestinal tract structure (such as intestine and stomach). However, Borges-Ramírez [22] reported that high ingestion of microplastics was detected in demersal fish species compared to pelagic fish. Meanwhile, omnivorous fish showed higher ingestion of MPs compared to herbivorous and carnivorous fish [32]. Therefore, future work on microplastic ingestion by fish should include the entire gastrointestinal tract and digestion process and then be extended to compare surface water with substrata. Among microplastic types including fragment, foam, fibre, film pellet, and others, the first was dominant and accounted for 42% with an average size of 3.72 $\pm$ 4.70 mm in the Yellow Sea [75]. In Taiwan, 91% of the microplastics found in common seafood species (shrimp, crab, oyster, and clam) were plastic fragments [31]. For comparison of plastic debris size, Núñez et al. [76] examined the distribution of microplastics across Galápagos Island. It was found that the size range of 0.15–0.5 mm was dominant in 100% of the water samples and marine organisms [76], and this is smaller than our result of mostly 1–3 mm in length with the exception of degraded plastic bag in *M. cordyla* that was 3 cm size in the stomach content of fish.

Most of the anthropogenic debris found in the present study was blue in colour, and the contributions differed significantly by debris type. According to a report by Pradit et al. [25], there was more blue-coloured debris in the mudflats than at beach sites. This finding corresponds with the bottom characteristics of Pattani Bay (sandy–muddy), which is a semienclosed bay located in the lower Gulf of Thailand, facing the South China Sea. Blue-coloured anthropogenic debris was ingested at the highest rate by *R. brachysoma*. This could be related to the utilisation of fishing gear and fishery activities where this species is intentionally caught by local fisherfolk in this fishing ground. Moreover, de Sa et al. [77] reported that the presence of microplastics in natural waters moves with water movement; therefore, it seems similar to natural prey, which leads to fish facing food selection difficulties. In addition, the size of plastic particles varied according to their colour, including white, tan, and yellow plastics; in particular, white colour plastic, reduced in size, was similar to prey for some planktivorous fishes [78]. Teleosts and elasmobranch

fish mostly ingested blue microplastics [41], which was similar to the findings of the present study of teleost fish.

5. Conclusions

AF varied according to water depth and season; in particular, there were more available prey types at 2 and 4 m depths for fish. Along with food consumption by fish, anthropogenic debris ingestion differed by feeding features, though it was especially high in planktivorous fish. The ingestion of plastic debris by colour also differed by fish species, with especially high ingestion of blue-coloured plastics. Our study provides evidence of plastic pollutant ingestion by fish inhabiting the vicinity of Pattani Bay and alerts for the potential effect of these pollutants on the trophic web. Further studies are urgently needed to verify plastic debris using FT-IR spectrophotometry and investigate the contamination of fish from different water columns and substrates, and the investigation of the stomach content of fish should be extended to pursue a better understanding of the effects of plastic debris contamination on the marine trophic web.

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ARTICLES FOR FACULTY MEMBERS

INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

Impacts of habitat quality on the physiology, ecology, and economical value of mud crab Scylla sp.: A comprehensive review / Pati, S. G., Paital, B., Panda, F., Jena, S., & Sahoo, D. K.

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Review

Impacts of Habitat Quality on the Physiology, Ecology, and Economical Value of Mud Crab *Scylla* sp.: A Comprehensive Review

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Abstract: The water of the mangrove ecosystem and surrounding coastal areas are gradually shrinking due to the intense destruction. Therefore, the effects of the physicochemical properties of the habitat water on the in-habitant species must be studied. *Scylla* sp. is involved in the food chain and bioturbation structure formation in mangrove forests. Five major electronic databases, such as PubMed, Scopus, Web of Science, AGRICOLA, and Google Scholar, were systematically searched to review the cause and effects of influencing abiotic factors, mainly physicochemical properties of habitat water, including water pollution on *Scylla* sp. Responses of mud crabs at biochemical, molecular, physiological, growth, reproduction, and production level were independently reviewed or in relation to physicochemical properties of habitat water, pathogens, heavy metals, and harmful chemicals present in their habitat water. Review results suggest that these crabs are mostly under threats of overfishing, varied physicochemical properties of habitat water, pathogens, heavy metals, and chemical toxicants in water, etc. At low temperatures, the expression of calreticulin and heat shock protein-70 mRNA expression is elevated. Like melatonin, the hormone serotonin in mud crabs controls ecdysteroids and methyl farnesoate at 24 °C, 26 ppt salinity, and pH 7.2 of habitat water, facilitating their reproduction physiology. Xenobiotics in habitat water induce toxicity and oxidative stress in mud crabs. These crabs are prone to infection by white spot and rust spot diseases during the winter and spring seasons with varied water temperatures of 10–30 °C. However, elevated (65%) weight gain with higher molting at the juvenile stage can be achieved if crabs are cultured in water and kept in the dark. Their larvae grow better at 30 ± 2 °C with salinity 35 ppt and 12 hL/12 hD day length. So, monitoring habitat water quality is important for crab culture.

Keywords: abiotic factors; chemical pollutants; water-induced physiology; water physicochemical properties; mangrove habitat water; *Scylla* sp.

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1. Introduction

Saline water areas of mangrove ecosystems in coastal zones provide huge habitats worldwide for several aquatic and semiaquatic organisms. As per the latest update in the year 2023 by the Food and Agriculture Organization of the United Nations, 17,075,600 hectares of mangrove areas in the world give sustenance to various life forms in 112 countries [1]. The water quality of mangrove ecosystems can seasonally vary with 5–35 ppt salinity, 8–8.5 water pH, 3.5–8.3 sediemnt pH, 25–35 °C temperature, 3.37–3.89 mg/L dissolved oxygen, 2.65–4.46 biochemical oxygen demands, and 5.44–10.67 NTU turbidity, while the values are always specific and flexible to a specific water body [2]. Estuarine water bodies also vary seasonally in a similar way affecting their water pH, salinity, turbidity, hardness, temperature, dissolved oxygen,

etc., and the variation is mainly due to tidal flows and day cycle [3,4]. Such a variable ecosystem imposes several cellular insults and physiological disturbances leading to hampering the growth, production, reproduction, immunity, etc., of the inhabitants [5,6]. The above responses or stress to the inhabitants by a change in their habitat water quality is magnified when pollutants and other chemicals or toxicants are added to the ecosystem mainly by anthropogenic activities followed by surface runoff [6–9]. Therefore, a detailed study on the impacts of the changing water quality on the life cycle, metabolism, growth, stress management strategies, production, and reproduction, including the economic values of any particular mangrove species, is warranted [10]. Results of such research on the water qualities and associated biotic and abiotic stressors/markers will lead to the draw appropriate policies to protect the mangrove/estuarine habitats and their inhabitants [11–13].

Mud crabs genus *Scylla* (*S. serrata*, *S. tranquebarica*, *S. olivacea*, and *S. paramamosain*) is an important member of mangrove/estuarine saline water ecosystems than other crustaceans due to its major activities (biological burrowing and bioturbation creation) in protecting and spreading mangrove forests [14]. Mud crabs are generally found in estuaries, especially in mangrove forests of India, Taiwan, Japan, China, South Africa, Indonesia, and the Philippines of Indo-Pacific places. Similarly, Malaysia, Singapore, Western Samoa, Salmon Island, Fiji, and New Caledonia are big mud crabs habitats. Generally, mud crabs have high demand worldwide for their nutritious and delicious taste of meat, which leads to overfishing at commercial bases across the coastal sites. The production of mud crabs surged from about 4000 to 175,000 t between 1990 and 2012. This compares with an estimated 10,000 t harvest worldwide in 1990 to 40,000 t in 2012 [15]. The world's fisheries produced 1,523,000 t of crabs and sea spiders from catch fisheries and 447,000 t from aquaculture in 2019, with an international export of a total of 588,110 t [1]. As a result of rising demand from consumers in recent years, the crab industry has flourished, and the market cost of mud crabs has increased. Wholesale prices average roughly USD 30 per kilogram, even in the high-yielding seasons, with the highest demand in the United States, China, South Korea, Thailand, and Japan [16]. In addition to overfishing, the availability of mud crabs is impacted by the fact that they depend on numerous abiotic factors at various stages of their lifecycle. Mud crabs spend their entire lifespan in water or water sediments of the intertidal and subtidal zones of estuaries and mangrove systems. This shallow water system is characterized by 150 days of the warm, wet season and 210 days of the cool, dry season, which result in the fluctuation of abiotic factors [17]. Therefore, study on the morphology, life cycle, growth, production, reproduction, and cellular physiology need to be studied with respect to the water qualities of these crabs for their better exploitation (Figure 1).

Figure 1 illustrates the evolution of mud crab research from biochemical to physiological to molecular studies from 1967 to 2022 [18]. It enables us to comprehend the transition of research on them from early biochemical to middle physiological to the contemporary molecular level. *S. serrata* has some distinguishable features, such as a convex body and broad carapace, which is present in a triangular shape at its abdominal side. There are 21 serrations prominently found at the line of fusion between the upper and lower body surface. One pair of antennae, antennules, and eyestalk is distinctly visible at the line of fusion at the anterior side in mud crab *S. serrata* [19]. In India, *S. serrata* has a carapace of 80–181 mm in width and body weight ranging from 0.68–0.83 kg [20]. Additionally, the mud crab habitat is quite more complex than other marine crabs because of its wide range of tolerance towards environmental factors, which makes mud crabs a significant organism for ecological studies.

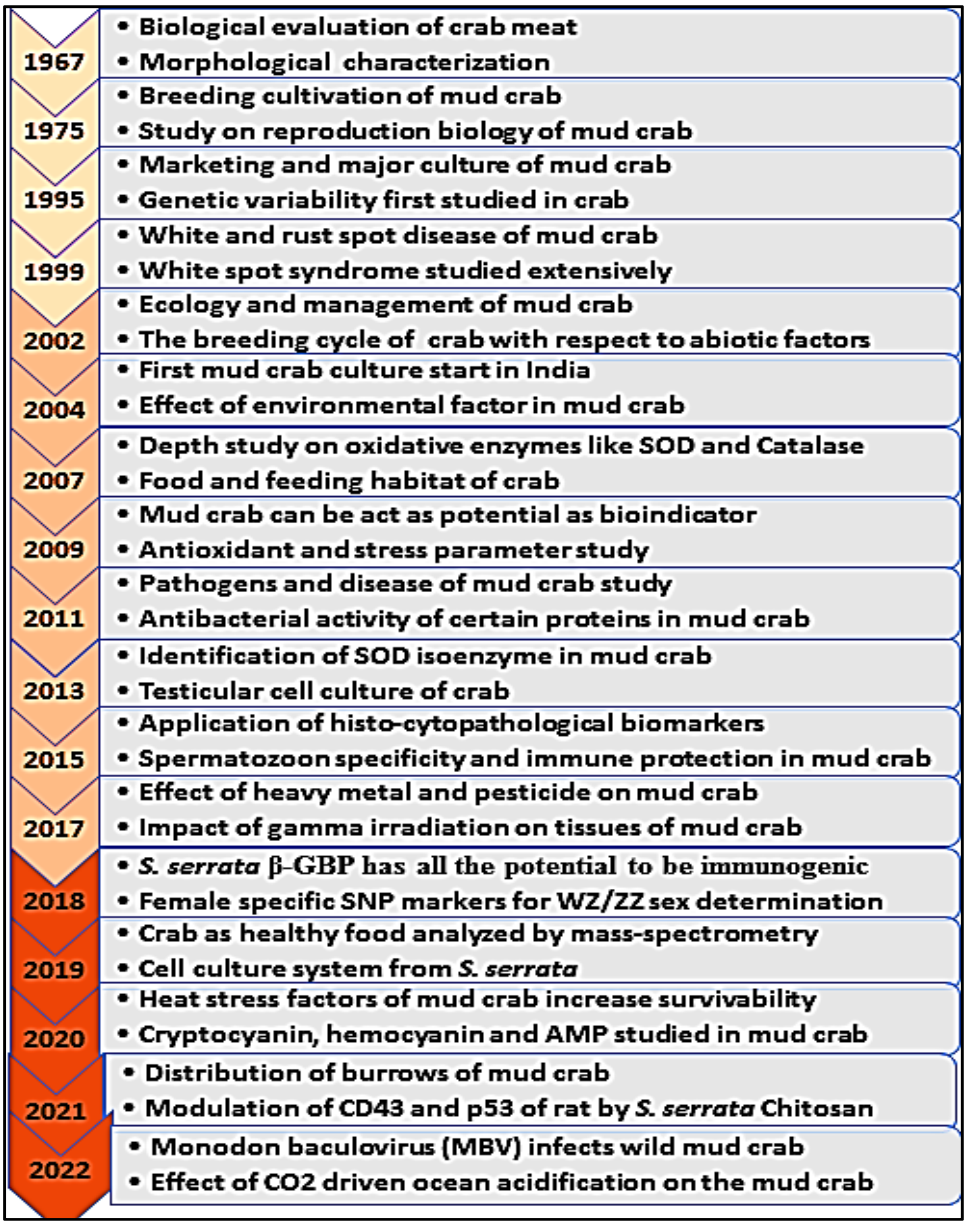

Figure 1. Major research findings on mud crabs in chronological order. Major studies have been conducted on mud crabs from the beginning to till date, and the sequence is as follows. Most studies focus on ecology, morphology, and reproduction in the early decade. The middle studies largely focus on pathogen and biochemical studies, while molecular and biotechnological-related studies are mostly conducted in this current decade.

Previous studies have proven that this animal model poses a high-stress resistance ability in addition to a well-defended immunological response to combat physiological stress under the changing habitat water qualities [21–26]. The innate immune system is well-observed in mud crabs, which also protects them from most pathogens [24]. However, water physicochemical factors, including temperature, salinity, pH, day length, tide height, and food availability, determine the growth and physiology of most crustaceans, including mud crabs [17]. For instance, variation in water temperature leads to stress response in aquatic organisms due to changes in their cellular metabolism. Mostly, it induces a stress response, irregular gene expression, pathogen spreading, and immune resistance against pathogens by down-regulating or up-regulating the synthesis of antimicrobial peptides and reproduction by hormonal changes in some cases [27]. Apart from this, high temperature

for a prolonged period induces drought, which results in a rise of metal and toxicants in the water of a natural habitat that leads to bioaccumulation and bio-transfer of such toxicants in mud crabs and organisms depending on them [28].

Similarly, salinity or salt concentration in habitat water plays a significant role in several physiological processes of mud crabs, including the oxidative stress (OS) response. In turn, it influences the larval stages due to an increase in demand for oxygen during rapid growth and, to a lesser extent, immunity [29]. The growth, maturation, survival, and fecundity of mud crabs are well-maintained by abiotic factors such as natural diet, day length, and wind speed [30].

It is pertinent that abiotic factors, especially water physicochemical factors, directly affect an organism's body physiology, stress resistance, immunity, gene expression, reproduction, distribution, and bioaccumulation of toxic products [17]. Because water physicochemical factors have such a significant impact on the biochemical, molecular, and physiological processes of the mud crab, a comprehensive review is needed to explore the mechanisms from both its environmental water perspective and also for the aquaculture of the genus *Scylla*. Therefore, the scope of this research review compiles various sources to present their economic importance, unified environmental physiology, and environmental biotechnology, focusing more on the function of abiotic variables, especially water physicochemical factors (Figure 1). This systematic review provides the context necessary to evaluate the impact of key water physicochemical factors on mud crabs at the biochemical and molecular levels and information on their biotechnology that can be related to the environmental cause and effects in crabs in general and in mud crabs in particular. The review may be useful for both environmental water monitoring using *Scylla* sp. as indicator animals and also for the betterment of their aquaculture.

2. Materials and Methods

For a comprehensive review of the genus *Scylla* in relation to environmental and pollutant effects, key terms such as "*Scylla*", *S. serrata*", *S. tranquebarica*, *S. olivacea*, *S. paramamosain*, and "mud crabs" alone or along with "physiology", "stress", "biochemical", "molecular", biotechnological, medical, etc., were searched in PubMed, AGRICOLA, Scopus, Web of Science, and Google Scholar databases and only the peer-reviewed published literature was selected using traditional methods. The electronic databases were used to comprehensively search the literature on this topic related to *Scylla* sp., focusing on the literature written in English only.

2.1. Data Sources and Search Strategy

PubMed/MEDLINE is a large database in biomedicine, and the Google search engine eventually stores the entire published peer-reviewed and other articles, while AGRICOLA contains about 6 million records associated with agriculture and allied sciences. It is to be noted that *Scylla* sp. is exclusively available in cultivated as well as in natural brackish water, saline, or mangrove forest lands, so it is included in the AGRICOLA database. Similarly, Scopus and Web of Science also provide a large scope for peer-reviewed high-quality articles. The aforementioned factors played a role in our decision to use the above five databases. Of the papers available in all the above databases on mud crabs, only peer-reviewed literature published in English on "*Scylla* sp." was screened in relation to various additional search terms. Search terms such as "abiotic and biotic stressors, heavy metals, antibacterial proteins, antioxidant enzymes, behavior, bioindicator, breeding, biotechnology studies, chromosomes, culture, distribution, ecology, ecosystem importance, ecotoxic, endocrine system, environment, fisheries, food and feeding, genes, genomics, harvest, heavy metals, immunity, life cycle, migration, mitochondria, morphology, neurotransmitter, organic and inorganic waste, organophosphorus, pathogens, physiological responses, physiology, pollutants, polychlorinated biphenyls, poly-halogenated compounds, production, regulatory proteins, reproduction, respiration, salinity, temperature, and toxic chemical, along with "*Scylla* sp." or "mud crabs" were electronically searched in the above databases.

2.2. Study Selection

The entire published peer-reviewed articles in the English language in books, journals, periodicals, and various authentic reputed webpages till September of 2022 on "*Scylla*" in relation to the search terms in the above-mentioned electronic search engines were included. The word was searched with search terms as mentioned in Section 2.1 were screened in this review article. Articles not containing any of the above terms were not included in the review. Articles matching within the subject area of the *Scylla* sp. were included in the review, whereas any axillary article in which the above term(s) is merely used was excluded from the review. Because 895 and 17 articles were published on the crab *Scylla* in PubMed and AGRICOLA electronic engines, respectively, all the relevant articles were included in the screening while the articles were hugely filtered in the Google search engine that hit s about 10,800,000 numbers with the term "*Scylla*". Similarly, articles from Scopus and Web of Science were also screened as per the need of the topic. All the data from the published papers were independently extracted unbiasedly, irrespective of the geographical region. The published literature from all geographical areas was selected for screening. The filtering was performed by increasing the specific keywords or manually selecting the most relevant and recent articles published in peer-reviewed journals. For example, out of 895 articles present on *Scylla*, articles that describe the tissue culture from pancreatic cells of *Scylla*, the morphology of *Scylla*, etc., were excluded from the study, whereas articles that were relevant to the change in the species as a function of their environmental factors were included in the study. About 220 articles were included in this review article.

2.3. Data Extraction

In order to streamline the entire review process securely and to ensure the reliability and consistency of this arthropod species, we have covered various aspects, such as the economic importance, species origin, morpho-anatomical adaptive features, adaptations, biochemical and molecular responses against stress, cellular, organ-specific, metabolic, and behavioral adaptations against the biotic and abiotic stressors of the species *Scylla* species. In addition, we have extensively reviewed the effects of the specific chemicals and environmental parameters, such as water salinity, temperature, and pollutants, on its studied biochemical and molecular pathways. The role of this organism in its ecosystem as a disease carrier and ecologic relation with other co-habitants has also been reviewed for its better exploitation. It will extend the existing understanding of the link between enzymatic and pathophysiological activities or responses in lower animals and *Scylla* sp., in particular. The objective of this article was to systematically review information to elucidate for the first time the ecotoxic responses, such as redox regulatory activities, neurotransmitter enzyme levels, and responses to heavy metal exposure of this mud crab in their habitat water. As a result, it can be helpful for its conservation in general and environmental ecotoxic and environmental chemistry studies in particular.

2.4. Synthesis and Analyses

Once the authors were convinced of the peer-reviewed articles on these species, these were analyzed and filtered to a <200 number, and some of the repetition articles among all databases were considered only once. The subject area of each article was extracted and categorized to describe various aspects of *Scylla* sp. in relation to various environmental factors, which could be natural or anthropogenic in nature. However, the number of peer-reviewed articles on *Scylla* sp. indicates vast studies on these species, starting from physiological responses to life history trade-offs, which were emphatically included in the present review.

3. Economic and Biotechnological Values of *Scylla* sp. under Varied Water Physicochemical Factors

Apart from the economic aspect, mud crab is a key species for restoring the coastal water ecosystem; indeed, its activity help in spreading mangrove forest that indirectly

contributes to the ecosystem as a whole for the survival of other species [31] and, in turn, it contributes to the artisanal culture and fishing in mangrove areas. The need to study a species is more dependent on its economic values, and *Scylla* sp. has tremendous environmental, food, and biotechnological values worldwide [15,16].

3.1. Nutritional Values of Mud Crab Meat

The mud crabs are well known for their nutritional value, and their delicious meat comprises 83.64% moisture, 22.77% protein, 1.35% lipid, and 2.09% ash when they are cultures in optimal saline water, which could be about 17 ppt salinity [32]. The nutritionally valuable element concentration was analyzed in pre-molt, hard-shelled, and newly molted (soft-shelled) crabs. Preferably, elements such as K, Ca, Mn, Cu, etc., were analyzed and present in the crab muscle tissue. Additionally, the exuvium of soft-shelled and the carapace of pre-molted hard-shelled crabs were collected for nutritional analysis. The analysis concluded that newly molted crabs are more valuable for consumption than hard-shelled ones due to the higher absorption efficacy of essential elements in soft-shelled crabs. Nonetheless, toxic elements such as Pb are excreted during exuviation, and elements such as Zinc are found in a balanced concentration in soft-shelled crabs [33]. Some facts, such as a high level of protein, lower fat content, balanced essential elements such as Fe, Zn, Mg, and Cu, and availability of free amino acids make crab meat nutritionally much valuable [34,35]. Thus, the demand for adopting advanced cultural techniques to optimize their complex environmental condition is the need of time.

3.2. Value and Environmental Water Management of Crab Shell Bio-Waste

Mud crab outer shell waste generated from fisheries markets and processing industries creates intense pollution in coastal areas. However, this waste is highly valuable as it primarily contains chitin, and according to studies, it can be converted into useful compounds [36,37]. *S. serrata* carapace management revealed that economical carapace could be used in industries as a catalyst for the transesterification of palm oil. Additionally, chitin plays a significant part in biodiesel production at a commercial level [38]. Apart from this, bioceramics have a high market value because of their use in the medical and dental industry as implants. Crab shell is enriched with calcium and other components, making it a potential material for bio-ceramics. The analysis of crab shells revealed the presence of 19.78% carbon, 24.53% oxide, 4.81% MgO, 3.98% P_2O_3, and 71.42% CaO. However, after calcination at 1000 °C, the above composition changes as 6.27% carbon, 28.96% oxide, 5.87% MgO, 5.65% P_2O_5, and 82.3% CaO [37]. Thus, the above composition can effectively employ the outer shell of mangrove crabs for bio-ceramic production.

In everyday life, the use of plastic leads to huge environmental pollution, especially in water bodies, and researchers try to resolve this problem by conducting studies to generate (micro)-bioplastics [39]. Huge quantities of biowaste (shell, scale, and carapace) are generated from aquatic animals like mud crabs. These waste materials are a high source of chitin and chitosan, which are well-known for their ability as natural biodegradable and biocompatible polymers. The yielding ability of chitin from waste can be enhanced by a mild extraction method, but it still needs some improvement to extract pure chitin [40].

3.3. Antimicrobial Proteins in Mud Crabs under Varied Water Temperature

Most aquatic organisms, including mud crabs, maintain their body fitness with an active immune system that indirectly depends upon water physicochemical factors, such as water precipitation, atmospheric humidity, water salinity, ultraviolet radiation, food availability, and wind speed. Infectious diseases significantly affect crabs by slowing down their growth and survivability rate. Despite the availability of various drugs and antibiotics against microbial diseases, emerging threats are also noticed against the health of organisms due to the rise in multiple drug-resistant pathogens. In order to reduce this infectious disease, the identification of immune molecules is indeed essential in crabs. In mud crabs, an insect-like innate immune system is recognized, which is confirmed by the presence of

antimicrobial peptides (AMPs), antilipo polysaccharide factor (ALF), hemocyanin, cryptocyanin along with crustin-like immune proteins (Figure 2). These antimicrobial agents' expression and activity vary with abiotic factors, especially water physicochemical factors, as discussed in the following sections and shown in Table 1. Therefore, the exact role and their regulation under variable habitat water physicochemical conditions will provide a new window to protect such organisms with natural neutraceuticals rather than using any artificial medicines in aquaculture farms.

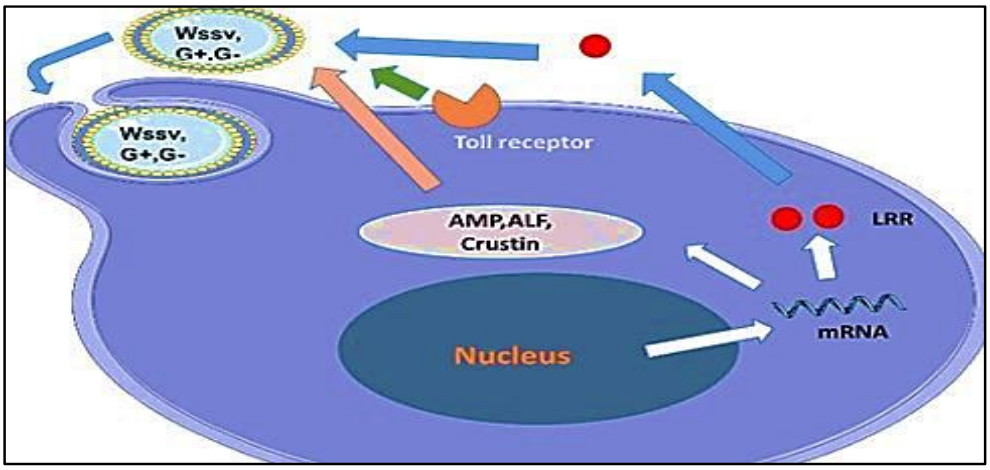

Figure 2. Phagocytosis is mediated by various immune proteins identified in mud crabs. The mud crab's innate immune system comprises both humoral and cellular responses. In cellular response, the peptidoglycan of bacteria and the protein coat of the virus trigger expression of AMPs. Antimicrobial peptides, anti-lipopolysaccharide factor (ALF), crustin-like factors, toll-like receptors, and leucine-rich repeat (LRR) work separately to phagocytose microbial pathogens. Phagocytosis occurs when these proteins bind on the surface of the pathogen leading to the engulfment of viruses and bacteria. The arrows indicate the direction of the movement or action of the molecules in cell.

The β-glucan binding protein (GBP) is an immunogenic agent present in the hemolymph of mud crabs. The antibiofilm efficacy of Ss-β-GBP was measured at different concentrations using light microscopy and confocal laser scanning microscopy, confirming that Ss-β-GBP is immunogenic to most pathogenic bacteria [41]. Multidrug resistance bacteria are generally treated through antibiotics, but amp-like arasin is also effective against infective disease-causing organisms. This peptide has 65 amino acids, molecular weight of 7 kDa, and an isoelectric point of 10.68. The N-terminal of this protein has a Gly/Arg-rich domain, and the C-terminal contains a cysteine-rich domain. It was observed that when the crab was subjected to lipopolysaccharide, Ss arasin mRNA of hemocyte expression increased, indicating its antimicrobial activity [42].

The gut bacteria of certain animals are novel sources of antibiotics as they can be used against multidrug-resistant pathogenic bacteria. The gut bacteria isolated from *S. serrata* were prepared and tested against gram-positive, gram-negative, and human cells (HaCaT) on bacterial-conditioned media, which showed significant antibacterial activities. Additionally, the antibacterial activity after heat inactivation suggests that the antibacterial property is maintained through bacterial metabolites or peptides [43]. However, human cells, on the other hand, recognize very little cytotoxic effect compared with gram-positive and negative bacterial cells, suggesting that gut bacteria are potential antibiotics for humans. Apart from this, carcinogenic agents are increasing, which also show a significant effect on crab health. The variations of the antimicrobial proteins, such as β-glucan binding protein in mud crabs, need to be studied as a function of water's physicochemical factors.

Marine invertebrates mostly depend upon AMPs to prevent pathogen invasion under various saline conditions (Table 1) [44,45]. The antimicrobial activity of these immune proteins mainly depends upon optimal temperature. The granular hemocyte of mud crabs

contains an 11 kDa antimicrobial protein known as *S. serrata* antimicrobial protein, which resembles a protein scygonadin found in the ejaculatory duct of *S. serrata* and shows optimum activity at 35 °C. Techniques such as RT-PCR and Northern and Western blot analyses were employed to confirm that *S. serrata* antimicrobial protein is expressed in various tissues across the body, unlike scygonadin [46]. The hormonally active form of vitamin D3 is the only inducer of the AMPs family in higher-order animals, and no role of other biological, hormonal, microbial, and water physicochemical factors is recognized [46].

Table 1. Effects of temperature on AMP, crustin, and ALF-like innate immune molecule of *S. serrata*.

Innate Immune Molecules	Expression Upregulation at Temperature	M.W.	Highly Expressing Tissue	Response to Bacteria or Antigens	Response to WSSV	Antibacterial Activity	References
SSAPs	35 °C	11 kDa	Ejaculatory duct	UP: Bacteria	ND	G+, G-	[44]
Sc-ALF	25 and 35 °C	11.17 kDa	Hemolymph	UP: Bacteria, LPS	ND	ND	[47]
Ss Toll	ND	NA	NA	Up: Peptidoglycan, LPS	ND	ND	[48]
LRR	ND	NA	Various tissues	Up: Bacteria	Up-regulation	G-	[49]
Vg-2	25 °C	NA	Testicular spermatozoa	ND	Up-regulation	ND	[50,51]
Ss ALF	25 and 35 °C	NA	Hemocyte, heart and, muscle	Up: Bacteria and LPS	ND	G+, G-	[52]
B-GBP	ND	100 kDa	Hemolymph	Up: Bacteria	ND	G+, G-	[41]

Note(s): Immune proteins found in different parts of mud crabs significantly affect bacteria, viruses, and other antigenic molecules. Antibacterial activity on both gram-positive and gram-negative is found in most cases. (G+: gram-positive, G-: gram-negative, MW—molecular weight, ND—not detected, NA—not available, Up—upregulated, SSAPs—stage-specific activator protein, ScALF—anti-lipopolysaccharide factor in *Scylla*, LLR—leucine-rich repeat, GBP—glucan-binding protein, WSSV—white spot syndrome virus, LPS—lipopolysaccharide, and AMP—antimicrobial peptides).

Mud crab's heart, hemocytes, and muscle tissues are the sites of SsALF and crustin mRNA expression. mRNA expression of this immune protein regulation depends on factors such as time and temperature [53]. Antimicrobial activity was noticed when the purified SsALF protein was incubated against both bacterial and cell cultures [45]. A complete characterization of Sc-ALF (MW-11.17 kDa) was carried out, suggesting that ALF expression increases from 13 to 49 fold at 25 and 35 °C water temperature, while its expression remains normal at 30 °C [47,53]. This protein also exhibits lipopolysaccharide (LPS) binding ability that might indirectly regulate the human vaginal epithelial cell immune responses through modulation of LPS-TLR4 binding in the NF-kB pathway [52]. However, specific studies on Sc-ALF expression in relation to temperature are still missing. There are crustins characterized in different crab species that show a high similarity with mud crab crustin, a cationic antimicrobial protein with a molecular weight of 7–14 kDa present in the hemocyte of mud crabs [54]. Various studies confirm that this protein expression also varies with time and temperature. A significant up-regulation in crustin expression was seen in crabs acclimated to temperatures close to the extremes of winter (5 °C water temperature) and summer (20 °C water temperature) [55]. Therefore, during winter and summer, crabs are less susceptible to pathogen infections (Table 1).

It has been noticed that the mud crab, like all arthropods and mollusks, possesses hemocyanin, a component of the innate immune system found in the hemolymph [56]. Hemocyanin, a metalloprotein commonly found in crustaceans, is well-known as an oxygen-carrying protein [57]. This oxygen-carrying protein is a potent antimicrobial and anti-cancerous protein. Additionally, purified hemocyanin from crab hemolymph has agglutinative properties confirmed by affinity proteomic studies [24,58]. Cryptocyanin is another innate immune agent having a molecular weight of 79.11 kDa, observed in all stages from oocytes, embryos, and zoeas to adult mud crabs, and structurally similar to hemocyanin [59,60]. The hemocyanin expression in crab was found to be similar in conditions such as warm water (22 °C)/high food, warm water/low food, and cold water/high food

fixed at pH of 7.4 and salinity 30–33 ppt. On the other hand, the expression of hemocyanin was found to be down-regulated in cold water (14° C)/low food conditions [61].

Similarly to metalloproteins, phenoloxidase resembles the hemocyanin of 76 kDa with two homologous peptide chains. These enzymes exhibit higher activities during microbial pathogen-associated molecular pattern formation, where the metal center acts as an activator. However, a complete characterization of properties and the effect of water physicochemical factors on this protein can be revealed only after conducting in silico and in vitro experiments [62].

A toll-like receptor that recognizes pathogen-associated molecular patterns is constitutively expressed in all tissues and found in all life stages of mud crabs. The life of mud crabs is spent in various water habitats, especially salinities. Ligands like peptidoglycan and lipopolysaccharide modulate the expression pattern of SsToll. However, an up-regulation in SsToll was recognized when exposed to a virus [48]. Vg protein generally found in the female crab is responsible for vitellogenesis, but a separate variant detected in testicular spermatozoa, which is involved in immune protection during the spermatozoon maturation in mud crabs under normal water habitat (Figure 3.3, [50]). Leucine-rich repeat (LRR), like SsToll, is also involved in defense mechanisms and signal transduction in humans and mosquitoes [49]. The effects of temperature or any other factor on the expression of these proteins are still missing. Thus, a study on the differential expression of these proteins and mRNA will be helpful in determining the infection status of mud crabs under varied water physicochemical factors, such as temperature and salinity (Section 3.3 and Figure 4).

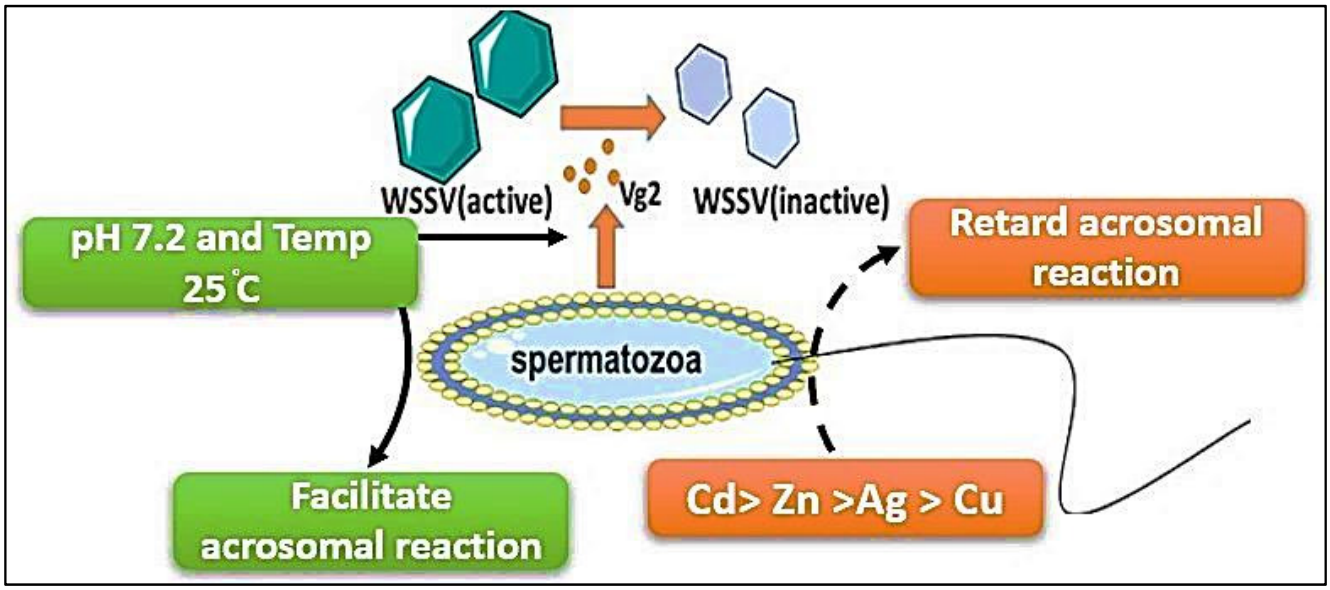

Figure 3. Impact of water temperature, salinity, and heavy metals on spermatozoa and role of Vitellogenin 2. The toxic effect of heavy metals on acrosomal reaction during the process of fertilization while optimum pH and temperature facilitate the acrosomal reaction. Vitellogenin is generally found in female crabs, which helps in egg maturation and production; however, male mud crabs also synthesize this protein in their spermatozoa. Vitellogenin 2, a separate variant detected in testicular spermatozoa, is involved in immune protection during spermatozoon maturation in mud crabs. Active WSSV in the presence of Vg2 becomes inactive.

An antimicrobial peptide, "rSparanegtin" was found to have an immuno-protective role in *S. paramamosain*, which is correlated to its increase in survival rate [63]. Similarly, Yang et al. (2020) [64] noticed that the antimicrobial peptide Scyreprocin present in *S. paramamosain* has a promising antifungal and anti-biofilm activity that could be used in aquaculture, including in mud crab culture. Similarly, many toxic chemicals, including Chlorpyrifos and Cypermethrin, and heavy metals such as Cd can induce stress in the

above crab that leads to hampering the growth and production of the crabs [65,66]. Therefore, the water quality of mud crabs must be carefully monitored for their optimal growth and production.

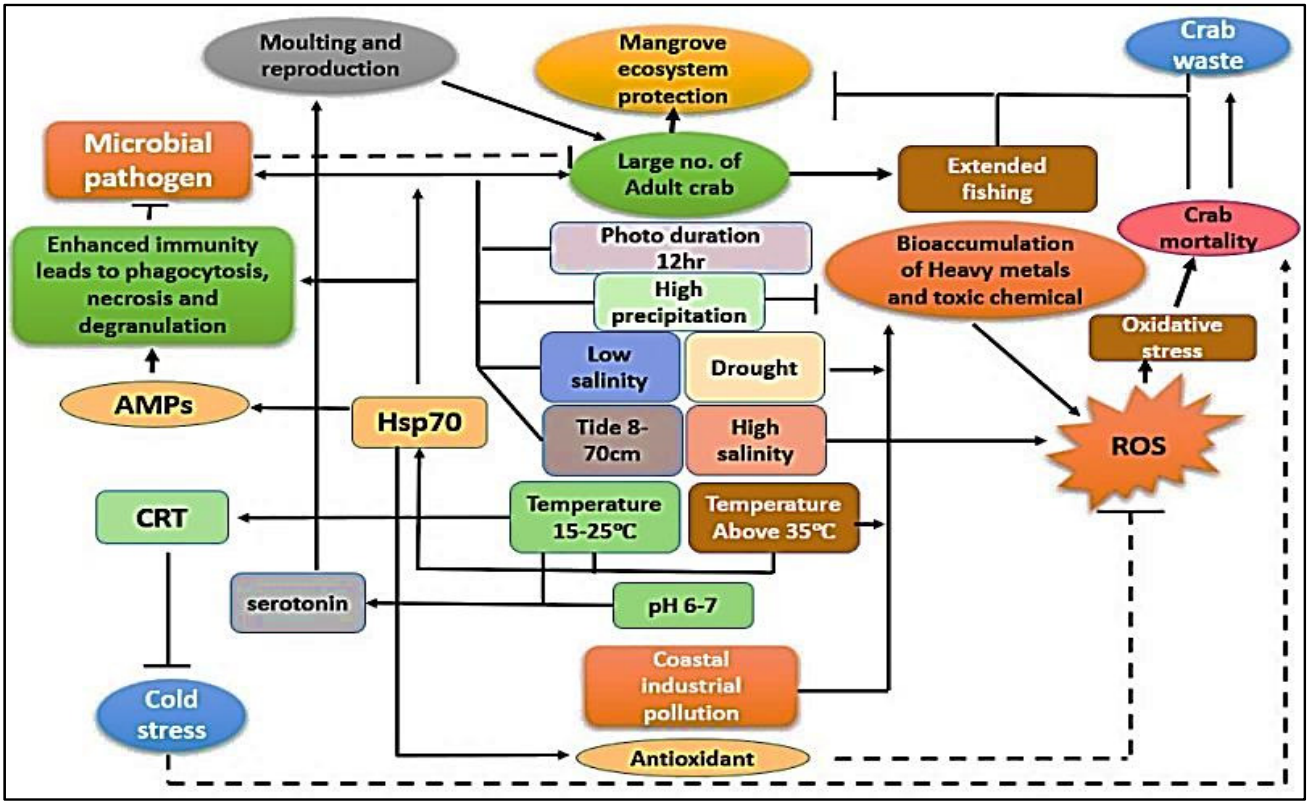

Figure 4. Biochemical and molecular responses of mud crab *Scylla serrata* in its natural environment. *S. serrata* is a mud crab and highly demanding worldwide. Many eco-physiological and molecular works have been done to enhance aquaculture in relation to its natural environment with highly fluctuating water physicochemical factors. For example, the involvement of molecules such as antioxidants, Hsp 70, is modulated under fluctuating environmental factors, including salinity, temperature, drought, photoperiod, etc. Such information and their pattern of involvement in cellular physiology, as shown in the figure, is extrapolated for its aquaculture.

3.4. Allergens in Mud Crab Meat and Their Modulation under Abiotic Factors

Most crustaceans are well-known for their delicious meat but also come with allergic reactions. Despite its delicious taste as food, it has some life-threatening effects; for example, an incident of death after consumption of crab meat was observed in China. The samples collected from the victim's different parts showed a high concentration of histamine in the intestine and a very high concentration of histamine in the crab sample [67].

Crustacean meat is mostly responsible for allergic reactions conducted through IgE. Recently, mud crab meat was identified with an allergen, arginine kinase. Mud crab arginine kinase also resembles other crustaceans, confirmed by sequence alignment studies. The native arginine kinase has a molecular weight of 40 kDa, and an isoelectric point of 6.5 was confirmed by a two-dimensional electrophoresis study, suggesting its similarity with other arginine kinases. It has been proven that serum from people with crustacean allergy positively reacted with the arginine kinase of mud crab, confirmed by immunoblotting analysis and colloidal gold immuno-chromatographic assay [68]. However, a complete high throughput method still needs to be included to identify crab meat allergens properly. Therefore, liquid chromatography-tandem mass spectrometry plays a major role in quantitatively detecting multiple allergens in crab meat and its product. Additionally, allergen proteins and peptides can be identified by analyzing data collected from the ion

spectrum of polypeptide fragments through ultra-performance liquid chromatography-quadrupole/electrostatic. Thus, using such modern tools and techniques identification of allergens in crab meat will be helpful for health issues [69]. Nevertheless, the expressions of such allergens with respect to several water physicochemical factors need to be studied in mud crabs.

3.5. Anti-Cancer Molecules in Mud Crab and Their Variation under Habitat Water

The β-glucan binding protein in mud crabs was considered to make Ss-βGBP-ZnONPs and then tested against gram-positive *Enterococcus faecalis*, gram-negative *Pseudomonas aeruginosa*, and human MCF7 breast cancer cells. Both bacterial and cancer cells show a significant decrease in growth in the presence of this nanoparticle in the habitat water [70]. As described earlier in Section 3.5, amp-like arasin of mud crab is known for its effectiveness against multidrug-resistant pathogens and plays a great role in the inhibition of human cervical carcinoma (HeLa) and colon carcinoma (HT-29) cell growth [42]. However, cancer-related studies on mud crabs have not been investigated much so far.

Chitinase enzymes responsible for chitin digestion in mud crabs have an altered behavior in the presence of a dioxane solution. This solution is well-known for its carcinogenic effect and is mostly found in water bodies due to the discharge of toiletries, such as shampoo, body wash, bubble baths, foaming hand soap, cosmetics, deodorant, and skin lotion. Beta-N-acetyl-D-glucosaminidase cleaves N-acetylglucosamine polymers to simplify chitin into N-acetylglucosamine (NAG). It reversibly inactivates the enzyme NAGase, and the enzyme kinetics reaction is recorded with the help of the enzyme-substrate method. This enzyme is more prone to inactivation if it is not bound to its substrate, which means the substrate gives protection against inactivation by dioxane solution [71]. Therefore, environmental factors, especially habitat water parameters and pollution caused by humans, play a significant role in mud crab disease, reproduction, physiology, and gene expression, which motivates the need for research into strategies for protecting *Scylla* sp. Owing to the importance of the species, the crab industry reached a high level in most Asian countries that act as the hub for mud crabs.

4. Status of Mud Crab Industries in Asian Countries

The total coastal area of southern, southeastern, and eastern Asia is about 169,131 km, which includes several estuaries and mangrove areas that provide a huge potential area for large scalability of mud crab farming. Countries such as India, China, Japan, Vietnam, Cambodia, and the Philippines are examples where mud crab farming has been growing at a large scale [72]. Several Southeast Asian countries and wild-catching sectors source from the artisanal crab industries and export and meet 50% of mud crabs for the entire world's consumption. In 2021–2022, the mud crab landing all over India was 3291 mt from a total land area of 2998 ha. Additionally, the total crab export has increased from 5509 to 6938 mt in recent years, indicating a 25% growth from the previous year [72]. In a state-wise comparison, Andhra Pradesh and West Bengal are the major contributors to crab production in India. According to the World Bank report of 2019, Myanmar exported 15,649 mt mud crabs globally from 2016–2017, about 30% higher than the 2011–2012 [73]. Similarly, in the Philippines, the export value of mud crabs during 2017–2018 was 16,326 mt, which was found to be 33% higher than in 2009–2010 [74]. These hikes in mud crab export in recent decades suggest an overall increase in the rate of mud crab demand worldwide. However, the mud crab culture is still limited in most East, South, and Southeast Asian countries due to a lack of technical expertise, limiting the landing values mostly from wild catches from natural habitats [72].

Some countries, such as China, hovered over crab culture despite technical difficulties. The annual mud crab landing in mainland China was 60,000–70,000 mt, and the highest annual production from farming was 120,000 mt [75]. It indicates an exponential growth in mud crab production that could result from large-scale crab farming. Similarly, Thailand and Bangladesh have also started crab farming to enhance their export. The Thai Depart-

ment of Fisheries estimated that the total mud crab production in 2010 was 2130 mt, and in the last decade, the production from coastal aquaculture was 6921 mt which showed about 200% elevated value than previous years [76]. Similarly, Bangladesh earns about USD 6 million annually by exporting 1500 metric tons of live mud crabs to Singapore, Hong Kong, China, Taiwan, and Japan. However, 95% of mud crab exported from Bangladesh is still collected from wild habitats because of limited technical difficulties for artisanal culture [77]. Great technical difficulties exist in crab seed supply to farmers in countries such as India. Though high-end technical advancement is still wanted in crab industries, mud crab production has risen from 134,000 to 407,000 t globally after including large-scale crab farming in coastal areas of East and Southeast Asia in the last decade [1]. Alternatively, it imparts a large-scale contribution to the mangrove ecosystems.

5. Mud Crab and Its Importance in the Brackish Water Aquatic Environment

5.1. Direct Contribution of Mud Crabs into Habitat

With the above economic values, mud crabs also contribute to the mangrove or estuarine water management indirectly. They form a bioturbation structure in sediment soil that helps in trapping the seeds of mangrove plants. It increases the chance of a mangrove forest area, and this has a positive impact on the management of water quality in the area as it leads to a green ecosystem in the area. Mud crabs play a significant role in changing nutrients, increasing mineralization, the oxygen-carrying capacity of the soil, and providing support for other aquatic organisms [31]. Extended fishing and dependency on natural sites gradually damage the number of crabs and natural habitats for other organisms. The purposive sampling method is generally used to analyze abundance, the frequency distribution of carapace, and the growth parameter of crabs by using FISAT 111 and Bengen statistics. Additionally, the carapace takes 4 and 6 months to mature in males and females, respectively [78]. Thus, extended fishing of mud crabs on a commercial basis should be avoided in their natural habitat. The exact role in protecting the mangrove ecosystem is quite interesting.

Mud crab plays a key role in balancing ecosystems by using their biological burrowing activity on the soil, making soil porous, laid to aeration, and nutrient flow in soil. They make burrows where the water level is below 100 cm, and the percentage of burrows increases by more than 40% with a lack of shade [79]. In the natural habitat, the porous soil makes mangrove forest conservation as the soil holds the seeds of the plant (bioturbation), which greatly impacts forest making and coastline protection [80]. Another dimension of facilitating aquatic life by mud crab is that they produce a large number of pelagic larvae that provides a great source of food for planktophagus aquatic organisms. Thus, from the above data and observation, it has been clear that mud crab plays a vital role in the food web by directly controlling the complex mangrove ecosystems.

The mangrove mud crabs that contribute to world fisheries are under-threat in many places due to varied water physicochemical factors, overfishing, pathogens, heavy metals, and chemical toxicants in water. Along with environmental factors, such as temperature and salinity, the effects of xenobiotics, heavy metals, and other toxicants must be checked in their habitat water and soil for their better growth, production, and reproduction [81]. Their omnivorous food habits have been experimentally proved, so the larval and adult care of these species under a suitable environment is suggested for their health management. Different behaviors of mud crabs, such as migration, reproduction, and breeding, are exclusively hormonally and environmentally regulated as a function of age [81]. Finally, mud crabs and their bio-waste are also used for various purposes, such as environmental monitoring, analyzing toxic loads, and in clinical and pharmaceutical sciences, indicating their demands. Therefore, the ecological interaction of these species during their life stages is environmentally important [81].

5.2. Role of Habitat Water on Ecology and Life Cycle of Mud Crabs

The lifecycle of mud crabs such as *S. serrata* comprises three primary stages: the dispersing larvae phase, the benthic juvenile stage, and the adult stage. In order to mature into adults, mud crabs generally migrate from the seawater to estuaries during their benthic juvenile stage [82]. Usually, in these stages, they inhabit a muddy mangrove forest with changing temperatures and salinities [15,83]. *S. serrata* in Okinawa inhabits marshy mangroves, and in Taiwan and the Philippines, it prefers sandy, muddy bottoms of seaward water [84]. According to some studies, they prefer varied habitats at various stages of their life cycle, from larvae to adults. Its larvae prefer stenohaline water and structurally complex habitats, which contain both refuge and food, but the seagrass habitat is preferred by crablets of *S. serrata* [15]. Extensive studies in this field proved that water physicochemical factors play a huge role in maintaining the variation among these habitats (Table 2).

Specifically, in India, it is noticed that mud crabs inhabit a variable benthic coastal region of different estuaries with fluctuating several abiotic and biological factors in the water of coastal sites. They can sustain in a varied range of soil sedimental and physio-chemical water parameters, such as pH, organic carbon, turbidity, temperature, and salinity affecting their growth and survivability (Table 2). *Scylla* sp. can thrive well in water temperatures ranging from 18–31 °C, 1–33 ppt of salinity range, alkalinity range from 70 to 119 mg $L^{-1,}$ and the dissolved oxygen concentration in water fluctuating between 4–10 mg L^{-1} [85]. Tidal heights ranging from 8.60 to 72.52 cm are optimum for crab survivability and growth. Additionally, organic matter content in water between 1.91% to 3.25% and a slightly basic pH with an average pH of 7.04 is optimum for *Scylla* sp. [86]. Food availability also plays a major role in their survivability in varied environmental factors and habitats depending on their life cycle.

Table 2. Effect of pH, temperature, and salinity on the physiology of mud crabs.

Water Physicochemical Factors	Location	Ranges	Duration (days)	Effects on Crab	Reference
pH	Coimbatore, Tamil Nadu, India	8.2 7.8	60 days	Normal growth, feed intake, and survival rate	[87]
		7.6 7.2 7.0		Decrease in growth rate, survival rate, and feed intake	
	Chantaburi, Thailand			Hemolymph osmolality (%)	[88]
		4–6 6–12	10 days 10 days	11% decrease 15% increase	
Temperature	Terengganu, Malaysia			Growth rate (%)	[89]
		24 °C 28 °C 32 °C 27–30 °C	45 days 45 days 45 days 45 days	7.28 ± 1.31 9.69 ± 0.75 7.83 ± 0.56 9.48 ± 1.02	
	Northern Territory of Australia	20 °C/20 ppt 25 °C/20 ppt 30 °C/20 ppt 35 °C/20 ppt	1 day 1 day 1 day 1 day	7.75 ± 1.28 12.68 ± 0.77 15.98 ± 0.36 12.59 ± 0.60	[90]

Table 2. *Cont.*

Water Physicochemical Factors	Location	Ranges	Duration (days)	Effects on Crab	Reference
Salinity	Queensland, Australia			Hemolymph osmolality (mOsm kg^{-1})	[91]
		4 ppt 12 ppt 20 ppt 28 ppt	NA	415 ± 12 (hyperregulated) 312 ± 8 (hyperregulated) 194 ± 15 (hyperregulated) 122 ± 12 (hyperregulated)	
	Iilan, Taiwan	14 ppt 24 ppt 34 ppt 44 ppt	1 day 3 days 0 day 1 day	772.38 (stabilized) 803.50 (stabilized) 1034.50 (stabilized) 1274 (stabilized)	[21]
	Queensland, Australia	30 ppt	4 days	968.73 ± 8.85 (stabilized)	[92]
	Odisha, India			Mitochondrial respiration rate complex I and II (nmol)	[93]
		10 ppt 17 ppt 35 ppt	21 days 21 day 21 day	4.42 ± 0.88 and 6.41 ± 1.69 1.69 ± 0.41 and 4.04 ± 0.58 2.19 ± 0.55 and 4.42 ± 0.88	

Note(s): Mud crabs need 27–30 °C temperature and salinity of 34 ppt for better growth and acclimatization. In addition, the optimum pH of water is 7.8–8.2 for normal growth and other physiological activities of mud crabs, as concluded from different local studies.

5.3. Predatory Contribution to Food Chain under Varied Water Habitats

The gut analysis and presence of material remnants like 51% mollusks, 10% crustaceans, 22% fishes, and 4% plant products in adult mud crabs suggest that the crabs are predatory in nature [35,94]. The feeding pattern varies with each larval stage of mud crabs, but they prefer rotifers and *Artemia nauplii* (decapsulated cysts) as their food due to their non-motile nature [95]. Nutrients rich in essential fatty acids are beneficial for the growth and survivability of larval stages of crabs [96]. *Scylla* species tend to feed at night, making it difficult to spot during the day [94,97]. Reports on the dietary preferences of mud crabs indicate that it has both an animal and plant-feeding nature. However, seasonal and environmental changes in water quality have a major impact on the way the mud crab feeds and interact with other organisms and the ecosystem in which it lives [15].

5.4. Behavioural Contribution to Ecosystem

The nocturnal feeding habit of mud crabs' juveniles and their burrowing behavior helps them to escape from predators in deep water as well as marshy areas. Generally, hiding behavior is noticed in mud crabs' juveniles (e.g., they are found under the leaf and aquatic plants in order to avoid direct sunlight). Habitats of most aquatic animals are simple and have little interference with others, but in the case of mud crabs, *S. serrata* habitat is quite complex in structure [98] as mud crabs are not static to a particular zone, so they can change their habitat according to their favorable condition by covering a long distance of 219 m to 910 m in water per night. Male shows great care towards female mud crab protection during molting and shell casting in the mating season. Molting and food scarcity induce autobalism and cannibalism nature in *S. serrata.* Besides this behavior, mud crab shows abnormal development and physiology under varied environmental conditions.

5.5. Contribution as Biomarkers and Bio-Indicators

Biomarkers are essential to assess the health status of an aquatic animal with respect to varied water environmental conditions and for their monitoring. Polychlorinated biphenyls (PCB) and poly-halogenated compounds (PHC) are particles that gradually increase in water bodies and are consequently consumed by aquatic organisms like mud crabs. Enzymatic and non-enzymatic antioxidant assays in *S. serrata* show a considerable downregulation of

the defense genes in summer with respect to the winter season when the PCB and PHC are at their peak concentration in water [99]. Additionally, ulcerative skin disease and parasitism epidemics in *S. serrata* were reported to coincide temporally and spatially with changes in water quality [100]. Another biomarker on mud crab was reported by Van Oosterom et al. (2010) [101], and it is an enzyme called Glutathione-S-transferase (GST) that can be used to study pollution impact assessment in saline water bodies.

Mud crab larvae are proposed to be used as an effective bioindicator for measuring the effects of sewage loads in saline water because they show a slower rate of larval development from stage I to stage II larval forms under pollution loads in habitat water. Secondary treated sewage has a significant role in toxicity in the zoea larva development of mud crabs, and it was observed when the progress of larval stages from stages I to II was examined [99]. Under a condition of constant photoperiod (12 hL/12 hD), a salinity of 35 ppt, and a temperature of 30 ± 2 °C, the growth of larval stages is found to be high when the habitat has sewage loads. Based on the aforementioned data, it is evident that mud crabs have a high potential to serve as bioindicator species. However, an extensive study of environmental factors and pollutants is essential to evaluate their impact on crab physiology.

6. Adaptive Responses of *Scylla* sp. to Water Physicochemical Factors

Major water physicochemical factors that are crucial to the survival of the coastal ecosystem and the organisms that rely on it include precipitation, atmospheric humidity, ultraviolet radiation (UVB), mineral nutrients, wind speed, salinity level, temperature, and the tides of the sea [102,103]. Out of all these factors mostly, temperature, salinity, and pH of water and soil affect ecosystems and organisms specifically [104].

Distribution at the population level and physiology, morphology, ecology, behavior, and life cycle at the individual level is highly influenced by water physicochemical factors such as strong katabatic winds, levels of salinity, tides of the sea, water temperature, dissolved oxygen levels [105], and nutrient availability in most invertebrate of the coastal aquatic ecosystem [106]. As invertebrates are poikilothermic species, temperature, salinity changes, and decreasing O_2 directly affect them by increasing their heart rate, lowering their respiration, and disrupting their osmotic balance [107]. The diurnal variation in sea tides maintains the aerial exposure timing of invertebrates at the coastline, by which respiration, morphology, and behavior are mostly affected [108]. Most invertebrates in the mangrove ecosystem are susceptible to oxidative stress (OS) exacerbated by water chemical factors. Factors such as temperature, O_2 level, and salinity induce free radical generation at the cellular level, resulting in physiological stress for organisms. In addition, gene expression and immunity of most invertebrates are also influenced by temperature, salinity, and solar radiation. Crustaceans, a major group of invertebrates common to coastal water bodies, are also affected by such factors [109]. Most crustacean mud crabs, especially *S. serrata*, have a special position in the whole mangrove ecosystem because of their special activities [110]. Before analyzing the effect of water physicochemical factors, it is essential to gather some general findings on these species of mud crabs.

6.1. Migration in Saline Water Bodies

The migration of mud crabs is generally environmentally specific, and the habitat water is changed where the crabs migrated. Mud crabs usually migrate to the open sea between November to March [111]. They are euryhaline in nature, facilitating their migration from marine water to estuaries, where they develop into the juvenile and return to marine water during the breeding season [19,82]. Thus, the crab is adaptive to both marine and estuary ecosystems. However, this dual property of the crab to cope with both environments may affect the lifecycle and development due to variations in water physicochemical and other habitat factors [19,95,112].

6.2. Reproduction Maturity in Natural Saline Water

The water physicochemical factors like temperature ranging from 30–35 °C, day length minimum of 12 h, and salinity 28 ppt play major roles in reproduction, with a distinct peak spawning season in the summer. Mating in mud crabs is species-specific, especially during the reproductive stage, with a sex ratio of male to female 3:1 [15]. Depending on specific geographical locations, the size of sex organs and carapace width in crabs are considered major indices through which reproductive maturity can be studied [83]. They attain reproductive maturity depending upon the size of carapace width, which varies across the different locations of the world, for example, 9 to 11 cm of carapace width in Australia and 9.2 cm carapace width in South Africa [113]. However, in the Indian subcontinent, a carapace width of 12.1 cm for *S. tranquebarica* and 7.9 cm for *S. serrata* is considered the mature reproductive stage in mud crabs [20]. Thus, in reference to the carapace width index, which decides the anatomical size of the sex organ, it will be easier to analyze the maturity of the sex organ in mud crabs.

Mud crab, *S. serrata*, is found to be exclusively intraspecific in mating and polygamy, generally in male species. The duration for copulation usually lasts for 2–3 days in their breeding season. The molting of the female starts during copulation when the male crab dorsally grasps the female for 3 to 4 days, leading to the shedding of the shell in the female crab. The male crab now moves for real mating by ventrally moving on the body of the female crab. The sperm transfer to a seminal female receptacle requires a minimum of 6 to 7 h during the mating season. However, it has been observed that the process of copulation lasts for 12 h or more than 24 h in some cases [114]. In addition, female crabs are capable of receiving sperm from two separate males during the process of copulation, and they are able to retain the spermatozoa of the second male crab for future use [82]. Inside the female seminal receptacle, sperm is stored in viable condition for 9 to 10 months, which is indeed helpful for the fertilization of 2 to 3 batches of eggs [82].

Fertilization is internal in mud crabs as it takes place inside the female body, and female mud crabs eject around 5 million eggs at one time. They usually come to the shoreline to lay eggs during spawning [82,115]. Environmental conditions and physical factors play a big role in the deposition of eggs, i.e., spawning [115]. The developmental life of a mud crab comprises five zoea stages and one megalopa stage (after hatching successfully from the egg). However, a clear distinction between sex and species is tough through compound microscopy in their larval stage [82].

In natural and/or artificial conditions, a formulated healthy diet is essential to attain the stage of reproductive maturity in mud crabs. The growth rate in mud crabs can be enhanced by supplementing adequate amounts of protein (45%) and essential fatty acids in the diet [96,116]. However, scarcity of food in natural conditions and lack of proper healthy food leads to stunted growth in most crabs [83]. However, age is also a relevant factor for the growth of crabs; as it grows older, the growth rate decreases. In *S. tranquebarica,* the size increases from 8 to 12 cm in one year, and in the second year, it increases from 14 to 15 cm. Similarly, in one year, the carapace of other *Scylla* sp. may grow from 2 to 9 cm. However, in the case of *S. serrata*, maximum growth occurs within a year after hatching in its natural habitat.

6.3. Breeding and Induced Breeding in Mud Crab and the Role of Habitat Water

Mud crab shows a perennial breeding activity, and their spawning takes place all around the year, but the spawning rate seasonally varies. For example, during the rainy season in the Rasimi River of Kenya, a slight increase in the spawning of mud crabs was observed [117]. On the contrary, in South Africa, an increase in spawning was reordered in the early spring season [118]. In certain cases, such as in the Kabira Bay of Ishigaki Island, the summer season is commonly considered favorable, while early winter is unfavorable for spawning eggs by mud crabs. However, in India, specifically in the Chilika lagoon, the spawning season for *S. serrata* spreads all around the year, and for *S. tranquebarica*, it ranges between May to September. Similarly, the variation in breading period is recognized

in *S. serrata* and *S. tranquebarica* at the Chilika Lagoon of India, ranging from August to November and March to June, respectively [20]. The above seasons are verified with specific habitat water conditions, such as high temperature and salinity in the summer seasons, then lower temperatures in the winter, and lower water salinity in the rainy season.

Induced breeding is commonly used in fishery culture to increase egg production for commercial use; this technique must be done under ambient water conditions and is essential to grow mud crab culture. Broadly two types of methods are used to perform induced breeding in mud crabs, i.e., unilateral and bilateral eyestalk ablation. In the case of mud crabs, the unilateral eyestalk ablation technique is used to perform induced breeding [119]. However, in both unilateral and bilateral eyestalk ablation, hyperglycaemic hormone physiologically stopped to achieve metabolic dysfunction. Simultaneously, eyestalk ablation also affects the secretion of the molting inhibition hormone, which facilitates the molting process in mud crabs during copulation and the subsequent breeding period [120]. Additionally, this eyestalk ablation directly acts on ovary maturation and hunger induction, facilitating faster growth in mud crabs at 28 °C and pH 7.6 [119]. This increases the oocyte number and causes great weight gain in the whole body, giving the proper female strength for the breeding process [121]. The induced breeding technique increases the production of eggs up to 4 million in *S. serrata*. However, water physicochemical factors, such as temperature, salinity, pH, etc., affect the survivability of eggs to some extent in culture conditions [93,102].

The risk of survivability is always high from the higher to the lower invertebrate group due to an unstable environment, especially varied water quality in aquatic animals. In order to overcome this kind of situation, mud crabs are adapted to breed in large no at a time so that a few juveniles may survive in the end. However, about 60% of mud crab attains a natural death in their natural habitat [122]. Although mud crab mortality is not observed up to the pre-zoeal stage, i.e., around 90% successfully viable till this stage, the next stage larvae become vulnerable to death. After the pre-zoeal stage, it enters the zoea stage and becomes a photo-tactic movement in the water, making these larvae vulnerable to predation by surface water predators and thus leading to a sharp increase in mortality. However, if they survive to the pre-adult stage, they strongly fight predators with their chelipeds and easily survive till natural death [82]. Meanwhile, specific proteins play a key role in countering variation in water physicochemical factor-induced stress during the entire life cycle of mud crabs.

6.4. Regulatory Proteins and Their Regulation in Reproduction

Many specific proteins play an effective role in growth, immunity, reproduction, molting, and development in crustaceans. In mud crabs, a special endoplasmic protein known as calreticulin (CRT) is involved in Ca^{2+} homeostasis and works through Ca^{2+} -dependent signal pathways in growth, immunity, reproduction, molting, and development. Additionally, CRT protein has multiple roles in homeostasis as it fights against low temperature and salinity stress. The expression of CRT is found to be higher in the hepatopancreas tissue of crabs [123]. At lower temperatures, i.e., below 10 °C, the expression of CRT mRNA increases, which supports the adaptability of mud crabs towards cold stress through the expression of CRT protein. Similarly, CRT mRNA expression is found to be higher at low salinity conditions. The male reproduction-related (Mrr) protein synthesized from the sex-specific gene in *Macrobrachium rosenbergii* (Mr-Mrr) is also recognized in mud crabs. This protein helps in sperm maturation processes in mud crabs and facilitates acrosome activation during fertilization [124]. The survival of mud crabs in hot climates against heat stress is determined by certain heat stress factors [125]. Thus, temperature has a big role in the expression and reproduction of regulatory proteins. However, endocrine physiology also plays an important role in regulating growth and reproduction in most crustaceans. It is because the harvesting or final production value is also dependent on the reproductive rate. Besides temperature, the effects of other water physicochemical parameters on the CRT-like proteins need to be studied in mud crabs.

6.5. Harvesting of Crabs as a Function of Seasonal Variation of Habitat Water

In eastern coastal sites, crab culture from April to October is suitable because different environmental factors are favorable in this period, as studied in Bangladesh. Collected data from various levels and discussions, as well as interviews with locals, suggested that a water salinity range of 2 to 10 ppt, a pH range of 7.8 to 8.6, and the silt-loam soil from April to October favors crab culture [126]. Thus, this survey suggests that harvesting should be done between April and October for those areas to produce better quality and landing quantity of crabs. In India and other coastal countries, similar studies need to be conducted in coastal areas to get the maximum harvesting from the crab culture.

Effectively managed crab culture can sustain a better livelihood for interested fish farmers as well as commercially dependent people in this field. Survey studies also confirmed that rearing crablets from <1.0 to 4.0 cm for 42 days or by phases is viable under a medium saline water state. Moreover, issues related to crab marketing, area ownership, and distance from the household need to be technically enhanced. Thus, a friendly environment needs to be established between farmers and intellectuals from the respective research institute to use technology to improve crab cultivation under the best water physicochemical conditions [127].

7. Mud Crab Physiology under Fluctuating Water and Pollution Stress

Mud crabs can tolerate a wide range of stressors, but when a threshold level is surpassed, then these species may become susceptible to respond to the effects generated by environmental factors, such as water temperature and salinity, etcetera [128,129]. Despite the high fluctuation in environmental water salinity and temperature, mud crabs are able to maintain physiological homeostasis, indicating their euryhaline and eurythermal nature [21,22,130,131].

7.1. Effects of Water Salinity

Out of all the environmental factors, water salinity has a significant role in the regulation of physiological activities in *S. serrata* [129]. For instance, small antioxidant levels and enzymatic antioxidant activities are found to be varied above 35 ppt of water salinity. Moreover, high water salinity also causes greater oxidative damage to lipid and protein molecules and deregulates the redox regulatory system in animals in general and in the mud crab in general [8,28]. However, no role in manipulation at the genetic level is observed under higher water salinity, such as 35 ppt [23].

A rare incident of shifting ammonotelism to ureotelism in excretion is well noticed in *S. serrata* when the salinity of water increases from lower to higher concentration [21,130]. However, an increase in salinity from 10 to 35 ppt causes an elevation in the excretion of ammonia and depletion in oxygen uptake and carbon dioxide release in the mud crab *S. serrata* [23]. The excretion of ammonia and associated studies always play a major role in aquatic body homeostasis maintenance [23]. Therefore, this field needs to be studied more to resolve the above ambiguities on the impact of salinity.

7.2. Effects of Water Temperature

The effect of water temperature and inorganic salt on the metabolism of mud crabs was studied by Ruscoe et al. [90], and they suggest that its larvae, zoea depends upon the rotifers as food, which is found in the maximum number in water salinity ranges, from ppt of 10 to 35 and temperature at 30 °C. Cold water stress data provide ideas for generating OS [132,133] and varied mitochondrial counts in mud crab tissue [60]. Factors such as temperature significantly affect mud crab physiology as it can increase or decrease the absorption capacity of drugs by the hemolymph [134].

The temperature of habitat water also affects the drug absorption rate, confirmed by enrofloxacin drug absorption in *S. serrata* at water temperatures of 19 and 26 °C. It has been observed that the absorption rate of drugs in the hemolymph is higher at 26 °C that decreases when the temperature is lowered down to 19 °C, but the rate of removal/excretion

of drugs from the hemolymph of this crab is higher at 26 °C as compared with a lower temperature such as 19 °C [134]. The study suggested that the temperature of habitat water directly relates to the pharmacokinetics of enrofloxacin-like drugs.

The OS stress parameters and antioxidant enzyme systems are also greatly affected by variations in water temperature and other factors. To assess the environment, the OS parameters of *S. serrata* are most helpful as these are good ecotoxicological indices [102]. The result of OS analysis suggests that summer OS indices are higher in comparison with winter and rainy season OS indices. Thus, such findings indicate that temperature has a major role in maintaining the body's physiology, metabolism, and drug uptake. However, increasing industrialization and the discharge of untreated sewage into coastal water bodies cause major health hazards for aquatic and terrestrial organisms, including humans.

The salinity of water plays a major role in the physiology of aquatic animals in general [135]. The combined effects of both water temperature and salinity indicate that these environmental factors substantially affect its development, but water temperature induces more impacts on its growth than water salinity. It was identified in a study on the juvenile stone crabs *Menippe mercenaria* and *M. adina* that temperature (between 5 and 40 °C) has profound effect than salinity (between 10 and 40%) on molting frequency; however, both parameters have synergistic effects on their survivability [136]. The interaction of temperature and salinity on the growth and survival of juvenile mud crabs has been explored to determine optimal ranges for these factors [90]. Ruscoe et al. [90] grew two instar crablets separately at several temperatures (from 20 to 35 °C) and salinities (from 0 to 40 ppt) for 2.5 weeks. They observed that temperature had a major impact on the survival of the crablets than salinity because the rise in salinity from 5 and 40 ppt did not affect their growth and survivability. However, regardless of the temperature, the mortality of crabs was 100% when they were kept in freshwater, i.e., 0 ppt salinity. The authors reported in their study that the optimal conditions for weight-specific growth rate in the crab were 30 °C temperature and 10 to 20 ppt salinity [90].

7.3. Effects of Inorganic and Organic Metals/Pollutants in Habitat Water on Mud Crabs

The accumulation of heavy metals and other toxic chemicals in aquatic organisms occurs due to the direct discharge of industrial pollutants into these coastal water bodies. Heavy metals and toxic chemicals are the most pollutants found in the aquatic ecosystem, which is proved by the analysis of tissues collected from crustaceans, especially crabs, as they are benthic in nature ([137], Table 3).

7.3.1. Effects of Heavy Metal in Habitat Water on Crab Physiology

Mud crabs and other crustaceans exhibit a high rate of metabolism and smaller body size that favors higher metal accumulation (Table 3). The physicochemical nature of water and soil sediment factors, such as pH, temperature, salinity, nutrients, organic carbon, Ca, Mg, and environmental conditions, are the deciding factors of the bioaccumulation rate of metals by mud crabs [138]. Bioaccumulation of heavy metals, such as Mn, Zn, As, Co, Cu, Pb, and Cd, in habitat water lead to creating health hazards in humans and other organisms (Table 3). Several other factors are also responsible, such as sex and season, for the bioaccumulation of heavy metals. It has been observed that during pre-monsoon, Cr 11.4 mg L^{-1}, Pb 3.3 mg L^{-1}, and Cd 0.07 mg L^{-1} were found in the highest available concentration, while the lowest Cr 1.4 mg L^{-1}, Pb 2.4 mg L^{-1}, and Cd 0.04 mg L^{-1} during post-monsoon [139]. A recent study on this bioaccumulation with respect to sex carried out in Sri Lanka by Harris et al. [140] found that a high accumulation of As, Pb, Hg, and Zn with exceeding levels of Pb (0.2 mg kg^{-1}) and Hg (0.5 mg kg^{-1}) occur in mud crab. The metal Zn is commonly found in both sexes, with males having 40.76 mg kg^{-1} and females having 45.10 mg kg^{-1} body weight, while Cd and Sn are found in the lowest concentration.

The variation of Zn and Hg with respect to sex is insignificant, but in the case of Pb and As, the amount in the female is higher than male. Thus, aquatic food derived from contaminated estuaries, consumed at 7.7 kg $person^{-1}$ $year^{-1}$, causes various health

issues [138]. Additionally, if people consume crab collected from those contaminated habitats, then the possibility of health hazards cannot be avoided. However direct effects of metals on sperm physiology described below are quite interesting.

The increasing pollution causes an increase in metal toxicity which subsequently interferes with sperm cell physiology in mud crabs [141]. The effect of metal toxicity was first confirmed in a sperm cell sensitivity test in mud crabs. Additionally, the sensitivity of metal toxicity is more stringent in mud crabs than in sea urchin sperm. The acrosomal reaction is directly affected by metallic ions, such as Cd, Cu, Ag, and Zn, as first studied by Zhang and colleagues [142]. The group observed structural changes in sperm and AR count after exposure to heavy metals. An increased rate of swelling, shape irregularities, and the acrosome filament of some sperm cells was crooked, ruptured, and even dissolved when heavy metal toxicity occurs. The intensity of metal toxicity in sperm cells of mud crabs is in the following order $Cd^{2+} > Zn^{2+} > Cu^{2+} > Ag^{+}$ [142]. Mud crabs can overcome cadmium toxicity with the help of metallothionein, a protein released during the resistance period [143]. Thus, the physiology of mud crab is heavily affected by heavy metals, and their subsequent consumption by human or other organism result in a health hazard. Apart from this, various toxic chemicals also affect crab physiology under various water physicochemical factors.

Table 3. Effects of heavy metals on body parts of mud crabs.

Inorganic Toxicants	Season	Site of Accumulation	Range of Accumulation	Effect	References
As, Cu, Zn	Wet	Muscle	High	Tissue damage	[138]
Cd, Cr, Pb	Wet	Na	Low	NA	[138]
Zn, Hg	Wet	Whole body	Balanced	Muscle damage	[144]
Pb and As	Summer	Whole body	High in female		[139]
Pb	Summer	Highest in hp, lowest in muscle	Too high	Body damage	[139]
Ag^{+2}, Cd^{+2}	Wet	Muscle	High	Structural changes in sperm	[142]

Note(s): Heavy metals listed using *S. serrata* as model organism under varied temperatures and salinity. All metals are more or less toxic to the crab in the summer and rainy seasons. Gill and hepatopancreas are mostly affected by heavy metals. Pb was found to be most toxic to mud crabs. The table indicates that mud crab responds quite well to toxicants and, therefore, may be used as biomarker species to study environmental status, including environmental pollution. HP—hepatopancreas.

7.3.2. Effects of Toxic Chemicals in Habitat Water on Mud Crab's Physiology

In addition to metals, toxic substances such as naphthalene, enrofloxacin, ciprofloxacin poly-, and perfluoroalkyl substances (PFASs) induce OS in mud crabs. The significant effect of naphthalene on the reproductive dysfunction of mud crabs was studied by manipulating the vitellogenesis [145–147]. Pharmacological compounds like enrofloxacin and ciprofloxacin were maximally retained for a longer duration in the hepatopancreas of mud crabs, and their elimination was quite slow [134]. When naphthalene level in habitat was studied in mud crabs, it was observed that levels of cytochrome P450, aryl hydrocarbon hydroxylase, glutathione-S-transferase, and UDP-glucuronyl transferase in hepatopancreas are elevated. DNA damage and cell necrosis are significant when exposed to naphthalene in the ovary, hepatopancreas, and hemolymph. This, in turn, suggests that naphthalene metabolism produces lethal oxidants [146,148].

Aquatic organisms (crustaceans) generally store lethal substances in their tissues in the freshwater system but generally depurate them when moving to decontaminated water. The migratory nature of mud crabs exposes them to several toxic products, such as perfluorooctyl sulfonate (PFOS), perfluorohexanesulfonic acid (PFHxS), and perfluorooctanoic acid (PFOA). These products can be depurated from mud crab if transferred into uncontaminated water, but each toxicant has a different depuration rate. PFOA is depurated from

crab within 72 h of exposure to decontaminated water. However, depuration from PFHxS and PFOS were not recognized in mud crab, indicating its harmful effect on organisms connected in that food chain [149]. These health risks can be slowed down if mud crabs facilitate to migrate away from contaminated water.

The larvae of mosquitoes are considered to be one of the major food sources of mud crabs; however, the nano-formulated larvicides have a harmful effect on its physiology by inhibiting the action of antioxidant enzymes like acetylcholinesterase (AChE) and GST [150]. Thus, like heavy metals, these toxic compounds have similar harmful effects on crab physiology. The effects of these chemicals on biomolecule degradation and biomarker level in water have been summarized under varied temperatures and salinity conditions in Table 4. However, the effect of water physicochemical factors on mud crab physiology will be incomplete without going through the mechanism of its reproductive cycle.

Table 4. Effect of toxicants on biomarker molecules of mud crabs under different abiotic setups in habitat water.

Pollutants Level in the Water	Temperature and Salinity	Effect on Biomolecule	Levels of Biomarker	Mortality %	Reference
Naphthalene (10 μg mL^{-1})	28 °C, 30 ppt	DNA-16%, RNA-20%	ADP-5.38% ↓, ACP-30% ↓, ALP-38% ↓, AST-35% ↓, and ALT-13% ↓	50%	[145]
Perfluorooctyl sulfonate (30 μg mL^{-1})	21 °C, 30 ppt	NA	SOD-73%, CAT-71%, and Gpx-50%	66%	[151]
Carbon dots (8 μg mL^{-1})	25 °C, 30 ppt	NA	AChE-12% and GST-50%	55%	[150]

Note(s): Toxicants listed using *S. serrata* as a model organism under varied temperatures and salinity. All pollutants are more or less toxic to crabs at high temperatures and low salinity. DNA and RNA are mostly affected by these toxicants, and crab mortality is higher in the case of PFOS. Naphthalene was found to be the most toxic to mud crabs. The table indicates that mud crab responds quite well to toxicants and, therefore, may be used as biomarker species to study environmental status, including environmental pollution. SOD—superoxide dismutase, CAT-catalase, GST—glutathione-S-transferase, GPx—glutathione peroxidase, AChE—acetylcholinesterase, ACP—acid phosphatase, ALP—alkaline phosphatase, AST—aspartate transaminase, ALT—alanine transaminase, and lactate dehydrogenase, ACP—acetylcholine, NA—not available, and ↓—decreased.

7.4. Endocrine Systems under Habitat Water Fluctuation

According to studies, mud crab has a similar kind of endocrine system as other crustaceans. However, a difference is observed in mud crab heart growth from the rest of the crustaceans. The molting hormone is solely responsible for the growth of crustaceans, but in mud crabs, heart growth is regulated by both the molting hormone of Y-organ and eyestalk factors, confirmed by analyzing eyestalk extractions and bilateral eyestalk ablations (Figure 5). It also confirms that the rate of heart growth is 1.78% faster in males than in female mud crabs [120].

In crustaceans, melatonin activity significantly modulates physiological functions, such as reproduction, molting, and glucose homeostasis [152]. Melatonin causes hyperglycemia in mud crabs and other crustaceans, hence considered a hyperglycemic hormone [153]. Additionally, in mud crabs, melatonin works as a reproductive hormone as it regulates the levels of methyl farnesoate and ecdysteroid, and in the presence of melatonin, secretion of juvenile hormone and ecdysteroid increases in the mandibular organ and Y-organ, respectively. Thus, melatonin induces the secretion of methyl farnesoate and ecdysteroid, which subsequently induce reproduction in mud crabs [154].

In addition to melatonin, the hormone serotonin also regulates the levels of juvenile hormone and ecdysteroids in the mud crab at a temperature of 24 °C, salinity of 26 ppt, and pH of 7.2 and thus considered a reproductive hormone. Like melatonin, serotonin also induces Y-organs to increase the secretion of ecdysteroids up to 132%, but mandibular organs have no direct effect on methyl farnesoate synthesis. However, due to the presence of serotonin, an increased level of methyl farnesoate was detected during circulation. Serotonin induces the release of methyl farnesoate from the mandibular organ, which is triggered

by the inhibiting hormone secreted from eyestalk, further leading to a rise in the level of methyl farnesoate up to 86.5% [155]. Apart from this, the effect of neurotransmitter levels on the cerebral physiology of mud crabs was observed by administering pentylenetetrazole (PTZ) drugs.

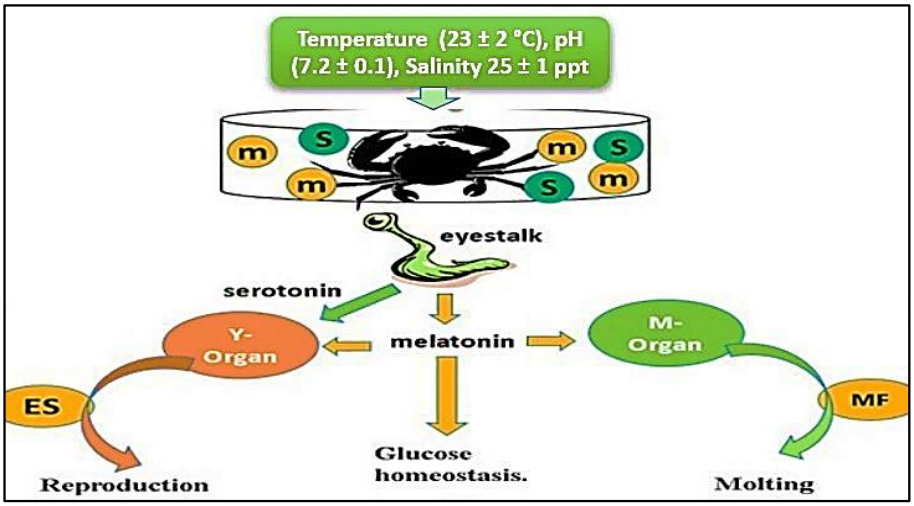

Figure 5. Effect of water physicochemical factors on melatonin and serotonin on the endocrine system of mud crab. The endocrine system of mud crab comprises eyestalk that releases melatonin and serotonin, which induce mandibular organ (MO) and Y-organ (YO) to produce methyl farnesoate and ecdysteroid, respectively together, they help in reproduction, glucose homeostasis, and molting. Physicochemical parameters such as temperature, pH, and salinity play a critical role during such induction. The Y-organ is influenced by serotonin and melatonin, while the M-organ only depends on melatonin. When left in a medium containing melatonin and serotonin, the crab shows an increase in farnesoate and ecdysteroid secretion (ES).

7.5. Neurotransmitter Changes under Habitat Water Fluctuation

The mud crab neural network is quite similar to other crab neural systems, but a complete structural and functional analysis still needs to be included. Convulsant drugs like PTZ directly affect cerebral ganglion by inducing epileptiform activities in mud crabs. The presence of an antiepileptic drug, i.e., sodium valproate in habitat water, induces sedative action in cerebral ganglion and prevents PTZ-mediated epileptiform discharges. Additionally, drugs like PTZ and sodium valproate significantly affect glutamate and gamma-aminobutyric acid (GABA) discharge in the cerebral ganglion of mud crabs. Administration of PTZ decreases GABA concentration; on the other hand, it increases the level of glutamate. Similarly, sodium valproate decreases GABA concentration and does not affect glutamate levels. Thus, epileptic seizures are inducible by administrating convulsant drugs in cerebral ganglion in *S. serrata* [156]. The possibility of bioaccumulation of these drugs rises during drought conditions. However, the direct role of water physicochemical factors still needs to be investigated in the endocrine regulation of mud crabs through genetic analysis.

7.6. Immunity and Disease Aspects under Habitat Water Fluctuation

Immunity can also be affected by water physicochemical factors and inorganic contaminants such as Ni and Hg in aquatic organisms (Figures 6 and 7). The immune-associated impact in crabs exposed to environmentally relevant concentrations of Hg can be detected by analyzing the effect on hemocyte, lysosomal membrane stability, phenoloxidase, superoxide generation, and phagocytosis. Additionally, OS resulting from Hg exposure increased lipid peroxidation levels and decreased the activity of the antioxidant enzymes, including SOD, CAT, and glutathione-mediated enzymes in serum. After observing and analyzing the above parameters, it can be concluded that Hg significantly reduces the immune-associated

factors in hemolymph and reduces antioxidants [157]. The detrimental effect of metals on aquatic life has been discussed in the previous sections. In coastal water bodies, xenobiotic contaminants, especially metals such as Ni, exhibit immuno-toxic effects in mud crabs. Superoxide anion generation and phagocytosis activity in the hemolymph were considerably higher when exposed to Ni than the normal one. Additionally, the accumulation pattern of xenobiotic contaminants was shown to be high in gills compared w the hepatopancreas and ovary [148]. Apart from these, other proteins also show antimicrobial activities where the effects of water physicochemical factors on its expression pattern have not been studied yet.

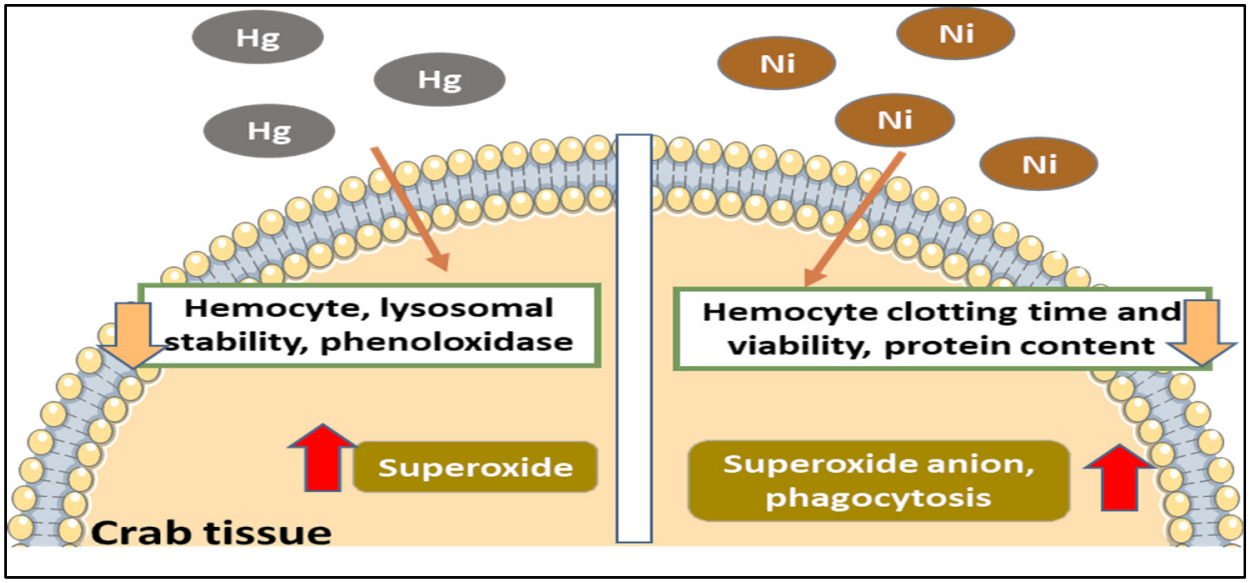

Figure 6. Effect of heavy metal on the immunity of mud crab. Bioaccumulation of heavy metals is highly toxic for mud crabs, of which mercury and Nickel cause serious health issues in mud crabs. Mercury and Nickel induce oxidative stress by producing superoxide anion, and meanwhile, Hg decreases hemocyte and membrane stability, and Ni reduces hemocyte clotting time and viability. The arrows indicate the direction of movement or action of the respective molecules.

The majority of aquatic organisms, including mud crabs, are susceptible to a wide range of diseases. The infection rate of mud crabs by microbial pathogens is highly dependent upon water physicochemical factors, such as precipitation, atmospheric humidity, host availability, levels of salinity, solar radiation (UVB), strong katabatic winds, seasonal and diurnal variations in temperature, etcetera [158]. Some common diseases of mud crabs include bitter crab disease, white spot syndrome, rust spots, and algal diseases. The "bitter crab" disease, caused by dinoflagellates, is commonly found in Alaska, characterized by a bitter flavor in the flesh of crabs [1,74]. Similarly, dinoflagellate *Hematodinium* sp. infects tissue and hemolymph, which leads to the clotting of hemolymph and eventually results in the death of mud crabs in Australia. The dominancy of different pathogens in Alaska and Australia suggests the role of varied water physicochemical factors in facilitating diseases [1,74].

The first viral disease detected in mud crabs is the white spot syndrome virus (WSSV), characterized by white patches on its shell surface. The viral disease was confirmed by injecting the respective viral strain, and the subsequent appearance of the symptoms in laboratory conditions was maintained at 30–32 °C and a salinity range of 25–30 ppt [159]. Some common symptoms of this syndrome include inactiveness, shoreward movement, and the appearance of white patches. However, the crab may overcome the deadly effect of this syndrome with time but continue as a carrier of the white spot syndrome virus for other crabs or crustaceans [160].

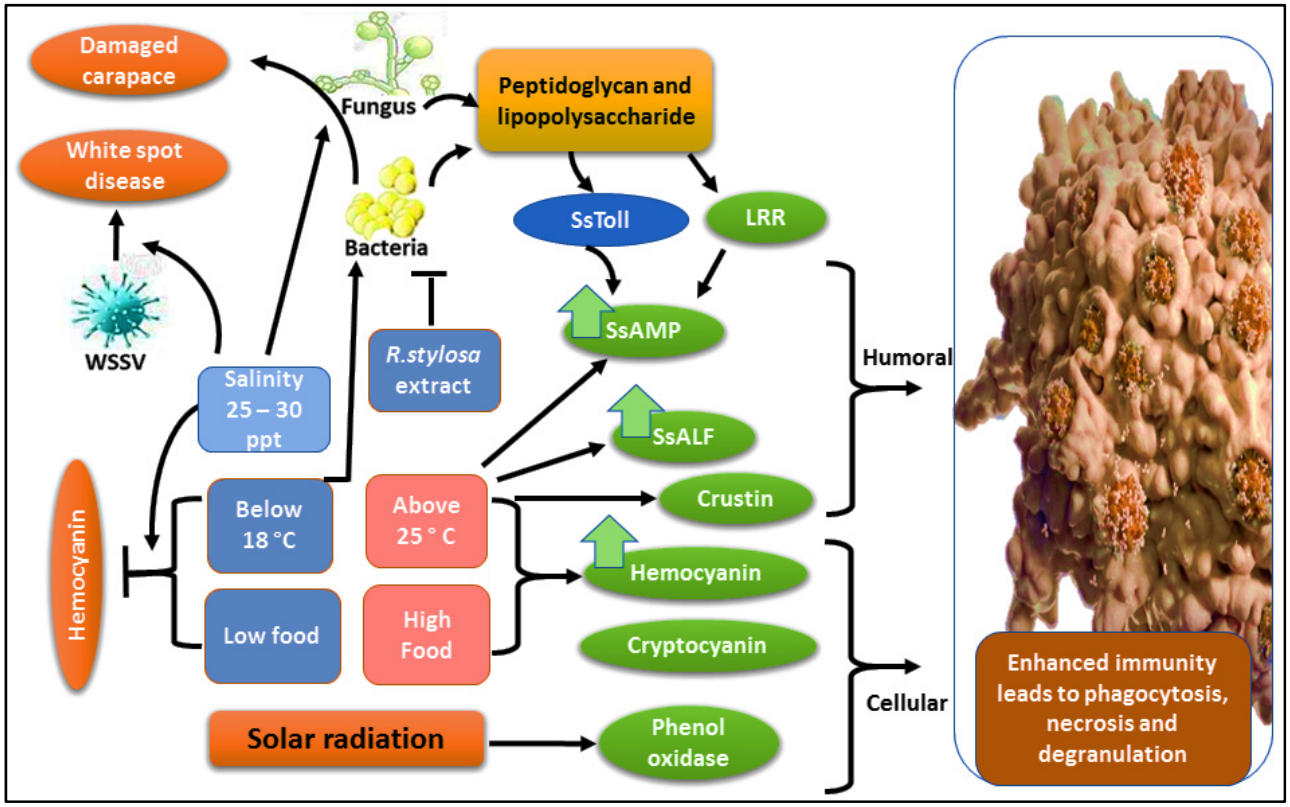

Figure 7. Role of water physicochemical factors and radiation in regulating innate immunity of mud crabs. White spot syndrome virus (WSSV), bacteria, and fungi depend upon abiotic variations. For example, temperature below 18 °C and salinity below 25 ppt help in propagating disease in mud crabs. Above 25 °C temperature facilitates antimicrobial agents and strengthens humoral immunity of innate systems such as SsAMP, SsALF, and crustin. Both 25 °C and high food enhance hemocyanin level, while below 18 °C and low food reduce hemocyanin level, solar radiation help in activating phenol oxidase activity in mud crabs, making cellular immunity of the innate system. Peptidoglycan from bacteria and lipopolysaccharide from fungal colonies induce SsToll and LRR proteins that lead to an increase in SsAMP synthesis. These altogether lead to phagocytosis, necrosis, and degranulation-like activity in mud crabs. *Rhizophora stylosa* extract from mangrove fruits works as a natural antibacterial agent. The arrows indicate the direction of movement or action of the respective molecules.

Next to the white spot, rust spot disease is also common in mud crabs during the winter and spring seasons (10–30 °C) and is represented by rust spots over the body surface of the crab. It is a bacterial-born disease with a significant effect on crab health and growth as it causes physical damage to the carapace by weakening the chitin with the help of a specific fungus. This exposes the crab's internal organs to the outer oxidizing environment as well as other harmful bacterial agents where salinity and high nutrients in the medium play a damaging role for the crab [161]. However, in this condition, neither below 20 ppt nor above 30 ppt salinity is favorable for mud crabs, as in both cases, bacterial and dinoflagellate infection significantly rises [162]. Studies on disease related to crabs led to the discovery of mud crab reovirus (MCRV), which causes great damage in crab culture and harvesting and propagate largely in 20 ppt salinity and temperature at 28 °C [112]. Thus, water physicochemical factors like temperature and salinity have a major role in viral, bacterial, fungal, and algal diseases of mud crabs. However, little information is available on other water physicochemical factors' role in crab microbial diseases, which need to be explored.

Highly pathogenic viruses, such as *S. serrata* reovirus (SsRV) and mud crab-specific virus McRV genome, are completely sequenced using molecular characterization methods. The virus has 12 dsRNA, out of which seven are sequenced earlier and have sequence

similarities with other members of the family, while all the remaining segments contain an open reading frame on the positive strand, and the terminal sequence is conserved [163]. Out of all segments, S4 is bicistronic, and comparisons between S4–S6 and S8–S12 and other reovirus genes show a very low homology. Moreover, all remaining segments of SsRV have higher sequence similarity with McRV, indicating that these two viruses belong to the same species. SsRV virus has a total of eight structural proteins encoded in S1, S3, S6, S9, S11, and S12 segments. However, a non-structural protein p35 (viroporin) is encoded by the S10 segment of SsRV. The role of the p35 protein is found to be crucial in the SsRV replication cycle, which is confirmed by screening a cDNA library derived from *S. serrata* [164]. Thus, several proteins that play a key role in these viruses can be targeted for future drug design.

Gram-negative, rod-shaped, yellow colony-forming *Aquimarina hainanensis* bacterium is highly pathogenic to mud crabs as it degrades chitin and gelatin, which are structural and integral parts of tissue and carapace. The homology of the gram-negative bacterium with the *Aquimarina* genus has been confirmed by analyzing the 16 s rRNA gene [165]. Additionally, mud crabs are prone to *Vibrio* bacterial diseases, but fruits are available in mangrove forests with antimicrobial activities. The minimum inhibitory concentration test of methanol and chloroform extract from *R. stylosa* and *Acaryochloris marina* suggests that these antimicrobials are safe to apply to mud crabs [166]. However, studies on antimicrobial and other pathogen-related drugs under varied water physicochemical factors are still not clearly observed.

8. Molecular Response under Physicochemical Variation of Water Quality

The total number of haploid chromosomes in *S. serrata* was found to be 47–53(n) [167]. Initially, the haploid chromosome numbers in *S. serrata* were estimated to be 53 by Niiyama [168] and 47 by Vishnoi [169]. Although a complete genome analysis is missing in mud crab, only some studies on specific genes have been carried out in the last decade. Complete sequence analysis of the mitochondrial genome has been conducted to measure the length of the genome, types of protein-coding genes, number of ribosomal genes, and percentage of adenine and thiamine contents. The above data can be used to analyze population and phylogenetic studies of other crab species [170]. Apart from this, sex-specific markers are identified in mud crabs, which are crucial for sex determination in organisms. A genomic study performed by Shi et al. [171] on *Scylla* sp. considering (female-specific) SNP markers (from 335.6 million raw reads, out of which 204.7 million reads were observed in 10 females and 130.9 million reads were observed from 10 males) indicates that WZ/ZZ sex determination system for mud crabs *S. paramamosain, S. tranquebarica,* and *S. serrata* can also be useful for rapid genetic sex identification in mud crabs. Twenty sex determination markers were identified as sex-specific through sequence assembly and female-male comparison to date. Half of these markers are heterozygous in females while homozygous in males [171]. A complete study on these sex-specific markers confirmed that mud crabs could have WZ/ZZ sex-determination system. So, chromosomal differences accompanying sex determination in mud crabs seem to be through XY and ZW chromosomal arrangements [171,172]. However, the latter system needs a wide range of studies to be confirmed.

Several genes play crucial roles in the analysis of phylogenetic, evolutionary, and biomarker studies, and their expression directly or indirectly vary with temperature, salinity, and nitrate concentration. The mud crab Hsp70 gene is a key gene for stress resistance, disease resistance, and phylogenetic as well as evolutionary analysis [173–176]. The immuno-defense activity of cytosolic Hsp70 cDNA of mud crab is confirmed by reverse transcriptase-polymerase chain reaction coupled with cDNA amplification. The level of Hsp mRNA expression was analyzed in various tissues at different temperatures, salinity, and nitrite concentration, suggesting that the expression of mRNA is 85% higher at 4 °C and 78% higher at 40 °C than 25 °C ([174], Figure 8). Similarly, mRNA expression was found to be 85% higher at 0 ppt and 75% higher at 45 ppt than at 25 ppt. However, mRNA

expression rises to 88% with rising nitrate concentration [174]. Additionally, genes can be used as tools to understand genetic diversity as well as enzyme functionality.

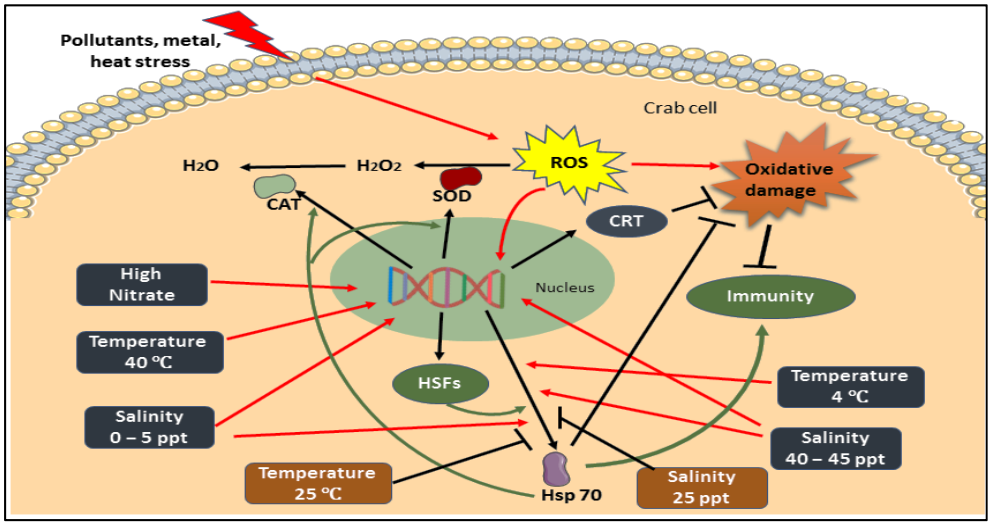

Figure 8. Expression of the gene under the influence of different salinity and temperature. Low salinity, high temperature, and high nitrate are the inducing factors for heat stress. Hsp 70 induced by these factors and HSFs also increase antioxidants like SOD and CAT mRNA expression. Below 4 °C, cold stress response induces CRT mRNA expression that neutralizes oxidative damage also. Hsp 70 also induces immunity in mud crabs. Temperature below 25 °C and salinity below 25 ppt inhibit Hsp 70 expression.

Overexploitation of historical bottleneck of an organism's population can be analyzed based on DNA sequences, which will help in understanding genetic diversity and connectivity among the population of different zones. Microsatellites and cytochrome oxidase genes are considered for analyzing population connectivity. On analyzing these genes, it was confirmed that the mud crab population is under overexploitation or historical bottlenecks [177]. In order to design a marine protected area observing the pattern of connectivity and measuring genetic diversity is indeed essential [178,179].

In order to counter superoxide activity, an enzyme like superoxide dismutase plays a major role, without which OS cannot be regulated, which leads to big damage to the crab body. Toxicants, temperature above 35 °C, salinity above 35 ppt, and below 5 ppt are common factors of reactive oxygen species (ROS) production at the cellular level leading to damage of biomolecules like proteins, lipids, and nucleic acids [180]. A complex antioxidant system is activated to neutralize the effect to counter ROS. This antioxidant system comprises several enzymes and proteins, out of which superoxide dismutase (SOD) and catalase (CAT) play a major role in ROS neutralization [181]. Thus, it is essential to know the physiological effect of SOD and CAT, such as major antioxidant enzyme inhibition by various kinds of inhibitors, as well as the role of water physicochemical factors involved in enzyme expression. Molecular docking was conducted in order to know the mode of binding of different inhibitors of SOD, such as hydrogen peroxide, potassium cyanide, and sodium dodecyl sulfate. The result suggested that potassium cyanide was not bound to the predicted structure of MnSOD, but hydrogen peroxide and sodium dodecyl sulfate showed a significant interaction. These data give an idea about the presence of some specific amino acids in the active site of the enzyme, which leads to the prediction of the binding modes of the proteins [182].

In silico study on this enzyme gives an idea about its structural character and also provides data on amino acids in the active site of the enzyme. Several inhibitors exist for this enzyme, but information on their binding with the enzyme on a structural basis is still unclear. However, using inhibitors of SOD such as hydrogen peroxide, potassium cyanide,

sodium dodecyl sulfate (SDS), β-mercaptoethanol, and dithiocarbamate, the cleavage sites on this enzyme and blocking the activity of inhibitors were established. SOD-SDS complex interactions reveal that residues such as Pro72 and Asp102 of the predicted crab extracellular-SOD as common targets of inhibitors [26]. Thus, this study will give an idea about the inhibitors which can interact on these sites, and subsequently, this information can be used to perform other enzymatic studies in crabs.

Similarly, another important antioxidant enzyme, CAT, has been structurally analyzed. It plays a significant role in keeping an organism healthy by protecting it from oxidation and peroxidation. A predicted three-dimensional structure of catalase in the mud crab is revealed by using a comparative modeling approach. The template PDBID: 7CAT of beef liver catalase of *Bos taurus* having NADPH binding site was used to construct this prediction. In order to know the binding properties of catalase with hydrogen peroxide, they use molecular docking. With the help of molecular dynamics, the structure of the receptor for docking and from which it is revealed that Arg 68, Val 70, and Arg 108 in catalase are responsible for binding with H_2O_2 [183]. Thus, the structural detail of specific enzymes will be helpful in monitoring the effect of organic and inorganic compounds and drug design. As important as it is to design drugs to protect the mud crab from various diseases, it is necessary to first address common diseases and the immune system that leads to its production.

9. Habitat Water Quality Management and Techniques to Enhance Mud Crab Production

Now crab culture is rapidly growing in coastal areas due to the rise of global demand, but this is still challenging as it has a long period of fattening and complex water physico-chemical factors variation throughout the culture process [184]. A nutritious, rich protein diet plays an important role in the rapid growth of the mud crab. Studies conducted to optimize the nutritional composition of the diet of mud crabs suggest that 55–79% protein, 6% lipid, 1% cholesterol, and 3–4% phospholipid are highly effective for the growth of its larval (megalopa) stage. Similarly, for optimal growth during the juvenile stage, 32–40% protein, 6–12% lipid, 0.51–1% cholesterol, and 13.5–27% carbohydrate are effective [185]. In addition, a micro-bound probiotic diet is useful for growth in different stages of mud crabs [185]. Besides the nutritional approach, water physicochemical factors significantly influence the growth rate of mud crabs. During the culture process, the zoeal larvae must be maintained between a salinity of 30–35 ppt and a temperature of 28–32 °C. However, megalopa larvae survive well in 25 °C/35 ppt conditions, and the mean larval development time is found to be increased from 16 days to 25 days when the temperature decreases from 34 to 20 °C (Table 5, [186]). In captive conditions, the time taken during the hardening of the shell of mud crabs is 2 to 3 weeks which needs a high cost for its maintenance during the rearing of the crab [187].

Table 5. Optimum temperature, salinity, pH, and light in habitat water on survivability at different stages of mud crab.

Water Physicochemical Factors	Zoea-II	Zoea-III	Zoea-IV	Zoea-V	Megalopa	First Crab Stage	Adult Crab	Reference
Temperature	25–28 °C	25–28 °C	25–28 °C	25–28 °C	28–34 °C	22–25 °C	22–25 °C	[186]
Salinity (ppt)	30	35	30–34	30–34	34	24	20–21	[186]
pH	8.1–7.8	8.1–7.8	8.1–7.8	8.1–7.8	8.1–7.8	7.8–7.5	7.3–6.4	[188]
Light condition	Low light	Low light	Low light	Low light	Full light	Full light	Full light	[189]
Survivability	86%	85%	82%	78%	60%	85%	90%	[186]

Note(s): Larval stages of zoea need high temperature and salinity for better survivability, while low temperature and salinity are required for the subsequent stages of the lifecycle. The pH of water and light duration also affect the larval and adult stages of mud crabs. High pH and low light induce larval stages, and low pH and full light induce later stages of the lifecycle of mud crabs.

As crab absorbs water during the molting process, incorporating minerals in water lowers the hardening period of the crab's soft shell. However, minerals through diets, drinking, and direct absorption via gills and epidermis play an important role in hardening the soft shell [190]. The fattening period varies according to the condition of the crab habitat and food quality. It has been shown that brackish water is most favorable for shortening the fattening period of around 30 days [191]. This information explains how minerals, temperature, and salinity play a key role in the shell hardening and survival of larval stages (Figure 9). Additionally, if crabs are reared in high salinity and in the presence of significant minerals, such as $CaCO_3$, $MgSo_4$, and KCl, the hardening of the shell occurs within nine days [187]. In order to forecast fishing industries about the standing stocks of eggs and larvae, DNA barcoding of different life stages of mud crabs is essential, which is possible by using specific genes such as mitochondrial cytochrome oxidase subunit 1 [192]. Based on these findings, although advanced culture systems are developed, the major obstacle to culturing these crabs is cannibalism during larviculture from megalopa to crablet stages during the nursery. It leaves this potential crab industry with challenges, especially those dependent on capturing wild crabs, which has serious sustainability issues [89]. Therefore, challenges still exist in studying food and feeding behavior, especially cannibalism during the above early stages in the life of mud crabs [89]. Special attention must be paid to their cultural techniques under the control of environmental factors for better production of mud crabs [89].

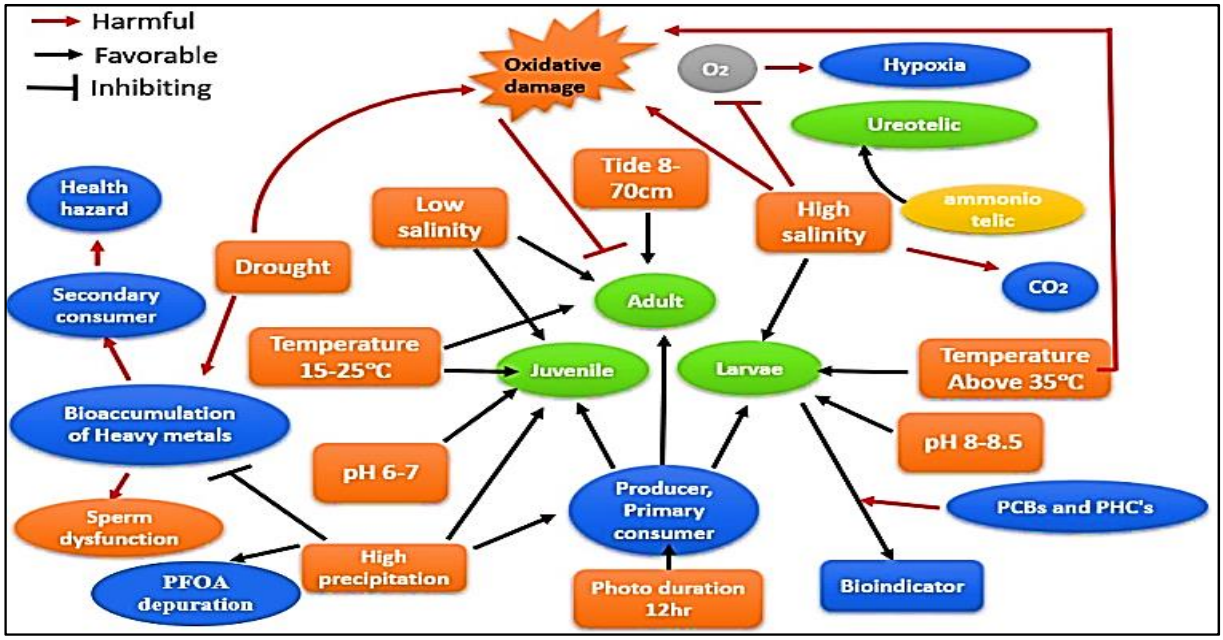

Figure 9. Effect of water physicochemical factors on life stages and physiology of mud crab. Temperature range between 15–25 °C, low salinity, and pH range from 6–7 favors both juvenile and adult stages of mud crab. High precipitation and photo-duration increase the producer and primary consumer of the aquatic food chain, which are essential for the growth of mud crabs. Bioaccumulation of heavy metals and toxicants increases in drought conditions, while high precipitation reduces the bioaccumulation rate in mud crabs. Bioaccumulation of heavy metals leads to sperm dysfunction and health hazard. PCBs and PHCs retard larval development from stages 1 to 2, which makes larvae a bioindicator. Temperature 30–34 °C, a high pH, and high salinity favor larval stages. High salinity and temperature cause oxidative stress, hypoxia, and carbon dioxide content. High salinity also changes excretion of mud crabs from ammoniotelic to ureotelic. Arrows indicate the direction of action while $-|$ symbol indicates the inhibitory action of the respective molecules.

The box culture system for mud crabs is one of the most reliable culture systems. The weight gain rate was 65.0 ± 26.3%, and the molting of the juvenile is significantly

higher when crabs are grown in a light-proof environment. However, it has been shown that the adult crab growth rate of 37.6 ± 20.6% fastens in the presence of light inside the culture box provided by the solar power generator. Growing mud crabs in highly dense conditions will raise the probability of cannibalism. Shelters with specific density, i.e., recirculation aquaculture systems, can increase the growth and production rate of mud crabs [189]. Because metabolic waste, fecal matter, and wasted food management are major issues during the crab culture period, a physical filtration system has been employed using Malang sand to overcome the above problems. This system enables the clearance of bottoms in the culture area from waste products and increases the availability of nitrifying and denitrifying bacteria in the medium [193]. The major components of a healthy crab diet include seaweeds, poultry waste, earthworms, and fish meal as food products such as seaweeds provide 57.18% of carbohydrates, while fish meal contains 61% protein and poultry waste provides 25% lipid content [97]. Adopting all these techniques shall be helpful to improve the culture system and production even under deadly pathogen attacks.

Although crab culture in artificial conditions is rarely affected by infectious diseases, still information on pathogens needs to be investigated. The milky disease of crab has been reported since 2005 when the mud crab was cultured along the coast of southeastern China. The disease mainly occurs between September and November [162]. Similar kinds of symptoms are noticed in the case of bitter crab disease or pink crab disease caused by members of the genus *Hematodinium* are common in cultural sectors. Thus, lowering the fattening period, implementing the box culture system, and maintaining proper environmental conditions will enhance crab culture.

10. Future Prospective of *Scylla* sp. with Respect to Mangrove/Estuarine Habitat Water

Owing to the importance of the crab industries, more research in different aspects has been done recently on *Scylla* sp. The comparative evaluation of proximate compositions in mud crabs suggests that females are more nutritionally rich, i.e., 17.07 ± 1.52% protein content, while males are rich in mineral content, especially Ca (1199.71 ± 343.43 mg/100 g) and Fe (14.21 ± 1.28 mg/100 g). So, a combined intake of male and female mud crabs will be more beneficial in terms of nutrition [194]. It has been studied that the mud crab can be edible till 240 min after death at normal room temperature. The crab postmortem investigation on ATP catabolism and succession of the bacterial community suggests that the muscle K value could be used as an optimal nucleotide freshness indicator for the freshness of mud crabs, with a proposed threshold of 20%. The muscle K value can be influenced by *Photobacterium*, *Peptostreptococcaceae*, average path distance, OTU richness, and Shannon index of bacterial muscle community [195].

Recent toxicological studies indicate that the effect of marine diesel oil on mud crabs under ocean acidification and warming conditions has been investigated, suggesting a decreased ingestion and absorption rate whereas a significant increase in the rate of respiration and ammonia excretion [196]. Similarly, human activities and natural sources, such as the weathering of Uranium-bearing rock, lead to contamination of marine ecosystems. Uranium exposure can lead to hemocyte reduction, mitochondrial anomaly, lamellar disruption of the gill, necrosis of hepatopancreas, and disruption and rupture of muscle bundles mud crab tissues [197].

Studies on the disease and immunity of mud crabs have been done recently. The SpBAG3, a Bcl2-associated athanogene 3, plays a key role in regulating apoptosis, development, cell movement, etc., and has been characterized in *Scylla* species. Specifically, it has a crucial role in assisting WSSV by inhibiting hemocyte apoptosis in mud crabs [198]. The WSSV infection in mud crab downregulates SpBNIP3, a BCL2 and adenovirus E1B 19-kDa-interacting protein 3. This leads to an increase in the apoptosis rate and Caspase 3 activity but decreases the mitochondrial membrane potential and hemocytes autophagy levels [199]. Mud crab reovirus (MCRV) infection in mud crabs induces phagocytosis, apoptosis, and unsaturated fatty acid biosynthesis, as well as other metabolic enzymes that give novel cellular mechanisms in crustaceans with respect to MCRV infection [200].

Recently, investigations on growth and reproduction have been done to elevate their production value. The ovarian development of mud crabs can be regulated through Vitellogenin (Vtg) and Vitellogenin receptor (VtgR) genes, which are involved in oocyte maturation. Both genes can be targeted with the help of agomiR-34, which binds at 3′-UTR and leads to inhibiting the expression of the genes [201]. The female reproductive output in mud crabs has been investigated by Fazhan and colleagues [202]. It indicates that sand type can influence the weight of the egg clutch, total egg number, fecundity, and clutch size. They observed that fine sand (<70 µm) substrate could maximize female reproductive output. Photoperiod plays a critical role in mud crab growth, survival, and metabolism. In constant darkness, lipogenesis-related genes are found to be up-regulated, while lipolysis-related genes are down-regulated. Thus, in order to utilize the lipid as an energy source, mud crab needs an optimum photoperiod [203].

The mangrove mud crab *Scylla* sp. plays a pivotal role in maintaining mangrove ecosystems in coastal areas. Being an ectothermic animal, these crabs exhibit altered physiological responses, including metabolic depression under the changing climate, global warming, and associated changes that affect the physicochemical properties of their habitat water. The mangrove ecosystems are also vulnerable to insults from various biotic and abiotic factors, including water physicochemical factors that may come out under climatic and anthropogenic activities. The contribution of *Scylla* sp. to the food chain and bioturbation activity in mangrove areas is commendable. Therefore, it is reviewed to compare biochemical, molecular, and physiological responses, growth, reproduction, and production of *Scylla* sp. independently or in relation to water physicochemical factors, pathogens, heavy metals, and harmful chemicals. Fluctuation of water physicochemical factors greatly impacts on physiology, reproduction, immunity, and other vital processes of mud crabs. So, necessary steps for conserving these species and their habitat, especially the mangrove ecosystems, are needed. As these crabs are under frequent overfishing threats, the current review may be useful to improve the production and management of mud crabs through a detailed analysis of several biomarkers to set up possible climatic resilient strategies.

Nevertheless, crab seed supply is a major challenge in Asian countries like India. On the one hand, importance on the physicochemical factors of water, such as salinity and pH, and hormonal regulation in broodstock along with infectious diseases must be carefully regulated for mud crab culture; on the other hand, this crab can be used as a bioindicator species as physicochemical properties of habitat water including temperature, salinity, pH, heavy metals, and chemical toxicants regulate their specific proteins expression. Therefore, environmental factors, especially habitat water parameters and pollution caused by humans, play a significant role in mud crab disease, reproduction, physiology, and gene expression, which motivates the need for research into strategies for protecting *Scylla* species. Owing to the importance of the species, the crab industry reached a high level in most Asian countries, which needs to be expanded in other crab-consuming countries.

11. Conclusions

The current review extensively discusses mud crab physiology, reproduction, immune system, and ecotoxicology in response to water physicochemical factors, which could be useful for its management in aquaculture and fisheries. Induced breeding, culture technique, bio-waste management, and the protection of gravid females are needed for the enhanced production of mud crabs under abiotic environmental insults. Environmental factors, such as temperature, salinity, pH, and infectious diseases, affect mud crab growth and survivability in the natural and culture system (Figure 9). Due to its ectothermic nature, the role of the endocrine system, namely eyestalk factors, M-organs, and Y-organs, for induced breeding of mud crabs under low temperatures is important, but maintaining a balanced titer for induced serotonin and melatonin is required. Drought and high temperature are the deciding factors of bioaccumulation of heavy metals, such as As, Cd, Co, Cu, Mn, Pb, and Zn, and toxic products, such as PFASs, PFOS, PFHxS, and PFOA, lead to health hazards. The toxic effects of organic and inorganic pollutants create health hazards not

only in mud crabs but also in other predator organisms by entering the food chain system, which can be overcome by lowering pollution levels in coastal water bodies. The pathogen of mud crab ranges from virus to algae, but there are several innate immune proteins and natural drugs that boost the immune system to combat harmful pathogens of mud crab under varied water physicochemical factors. Certain key genes under the influence of temperature and salinity play a significant role in immuno-defense activity, sex determination, and phylogenetic analysis, leading to a better understanding of its distribution and survivability. Components of crab shells can be used as biodiesel, bio-ceramic, naturally biodegradable, and biocompatible polymer production, leading to biowaste management in coastal areas. Crab culture can be commercially enhanced by adopting techniques, such as box culture, recirculation aquaculture system, and providing a healthy composite diet. In their natural habitat, they play a crucial role using their biological burrowing activity, making soil porous like earthworms, leading to the sustenance of mangrove forests and the whole ecosystem. Additionally, the expression pattern of biomolecules under the influence of water physicochemical factors makes mud crabs a potential organism for biomarker studies. Thus, being an environmentally and economically significant species, the mud crab draws special attention to its stress physiology for its aquaculture management.

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Abbreviations

ALF—antilipo polysaccharide factor, AMPs—antimicrobial peptides, CRT—calreticulin, CAT—catalase, Hsp—heat shock protein, H_2O_2—hydrogen peroxide, LPS—lipopolysaccharide LPx—lipid peroxidation, MCRV—mud crab reovirus, OS—oxidative stress, PFOA—perfluorooctanoic acid, PFOS—perfluorooctyl sulfonate, PPT—parts per thousand, PTZ—pentylenetetrazol, ROS—reactive oxygen species, SOD—superoxide dismutase and WSSV—white spot syndrome virus.

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ARTICLES FOR FACULTY MEMBERS

INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

Integrated physiological, transcriptome and metabolome analyses of the hepatopancreas of the female swimming crab portunus trituberculatus under ammonia exposure / Meng, X., Jayasundara, N., Zhang, J., Ren, X., Gao, B., Li, J., & Liu, P.

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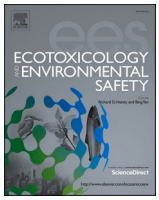

Integrated physiological, transcriptome and metabolome analyses of the hepatopancreas of the female swimming crab *Portunus trituberculatus* under ammonia exposure

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ABSTRACT

Ammonia is a common environmental pollutant in aquatic ecosystem and is also a significant concern in closed aquaculture systems. The threat of ammonia has been increasing with rising anthropogenic activities including intensified aquaculture. In this study, we aimed to investigate ammonia toxicity and metabolism mechanisms in the hepatopancreas, a major organ for Vitellogenin (Vtg) synthesis and defending against ammonia stress, of female swimming crab *Portunus trituberculatus* which is an important fishery and aquaculture species, by integrating physiological, transcriptome and metabolome analyses. The results revealed that ammonia exposure (10 mg/L, an environmentally relevant concentration) resulted in a remarkable reduction in *vtg* expression and depression of multiple signaling pathways for reproductive regulators including methyl farnesoate, ecdysone and neuroparsin, demonstrating for the first time that ammonia impairs swimming crab female reproduction. In addition, a number of important genes and metabolites in glycolysis, the Krebs cycle, fatty acid β-oxidation and synthesis were significantly downregulated, indicating that changes in ammonia levels lead to a general depression of energy metabolism in hepatopancreas. After ammonia exposure, an increased level of urea and a reduction of amino acid catabolism were observed in hepatopancreas, suggesting that urea cycle was utilized to biotransform ammonia, and amino acid catabolism was decreased to reduce endogenous ammonia generation. Furthermore, antioxidant systems were altered following ammonia exposure, which was accompanied by proteins and lipid oxidations, as well as cellular apoptosis. These results indicate that ammonia leads to metabolic suppression, oxidative stress and apoptosis in *P. trituberculatus* hepatopancreas. The findings improve the understanding for the mechanisms of ammonia toxicity and metabolism in *P. trituberculatus*, and provide valuable information for assessing potential ecological risk of environmental ammonia and improving aquaculture management.

1. Introduction

Ammonia is a common environmental pollutant in aquatic ecosystem and is toxic to aquatic animals (Egnew et al., 2019). Two forms of ammonia occur in water, the ionized ammonia (NH_4^+) and the unionized ammonia (NH_3), and the latter is the more toxic because it can diffuse easily through the gill epithelium and into the body fluids (Weihrauch et al., 2004) (in this study, the term "ammonia" refers to the sum of NH_3 and NH_4^+). Generally, ammonia nitrogen can remain very low in marine ecosystem (Romano and Zeng, 2013), and the unionized

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ammonia concentration should be maintained below 0.02 mg/L according to Seawater Quality Standards of China (GB 3097–1997, China). However, ammonia nitrogen in ocean sediments, the habitats of benthic animals, can exceed 39 mg/L in some areas due to elevated anthropogenic activities including discharge of municipal effluents, industrial wastes, and agricultural fertilizer run-off (Romano and Zeng, 2013). The harmful effects of elevated ammonia on marine animals with increasing global anthropogenic activities is a significant concern. Ammonia is also a major consideration in the aquaculture industry. In closed aquaculture systems, ammonia may accumulate over time, mostly from the decomposition of residual feeds and from the excretion of the cultured animals, causing massive mortality (Romano and Zeng, 2007; Zhao et al., 2020). Ammonia levels have become a ubiquitous concern in aquaculture, especially since the increasing trend in aquaculture is for higher feeding strategies and stocking density (Bouwman et al., 2011; FAO, 2011; Romano and Zeng, 2013).

Due to mounting concerns associated with ammonia toxicity, a number of studies have been focused on ammonia metabolism strategies and toxic effects on aquatic animals, including decapod crustaceans (Romano and Zeng, 2010b; Liu et al., 2014, 2020; Cheng et al., 2019; Duan et al., 2020). In their natural habitats, decapod crustaceans may frequently encounter increased levels of ammonia because they inhabit mostly benthic or near benthic dwellings, where the ammonia levels are higher than those in surface waters (Cutrofello and Durant, 2007; Fanjul et al., 2008). Therefore, they have evolved excretion or metabolism mechanisms to avoid ammonia accumulation in hemolymph under high environmental ammonia. Many studies in decapods have shown that ammonia can be actively excreted against an inwardly directed gradient across the gills by a coordinated action of several transporters, such as rhesus protein, Na^+/K^+-ATPase, and K^+-channels (Weihrauch et al., 2002; Pinto et al., 2016; Leone et al., 2016; Si et al., 2018). In addition, internal ammonia can be converted into free amino acids, particularly glutamine, and stored in tissues and then used as an oxidative substrate when returned to normal (Weihrauch et al., 1999; Hong et al., 2007). Furthermore, ammonia can be converted to less-toxic urea via the ornithine-urea cycle (Weihrauch et al., 1999; Ren et al., 2015).

Although decapod crustaceans possess mechanisms to counter ambient ammonia, beyond a certain threshold, ammonia disturbs osmotic homeostasis (Romano and Zeng, 2007; Weihrauch and O'Donnell, 2015), disrupts gas exchange (Chen and Cheng, 1993; Jensen, 1995), perturbs energy metabolism (Shan et al., 2019; Xiao et al., 2019), alters neurohormone regulation (Zhang et al., 2018; Si et al., 2019), increases pathogens susceptibility (Y.Y. Chen et al., 2012; B.P. Chen et al., 2012; Li et al., 2018), reduces growth (Romano and Zeng, 2009; Liao et al., 2010), damages gills (Rebelo et al., 2000; Romano and Zeng, 2010a), and causes mortality (De Lourdes Cobo et al., 2014; Sung et al., 2018). Nevertheless, the underlying mechanisms of the ammonia-induced toxicity in crustaceans are not yet fully understood.

The swimming crab *Portunus trituberculatus* is a typical benthic species, extensively distributed in the estuary and coastal waters of Korea, Japan, China, and Southeast Asia (Dai et al., 1986). It is dominant in portunid crab fisheries worldwide, with a production of 493,000 tons in 2018 (FAO, 2020), and supports a large crab aquaculture industry in China with a production of 116,251 tons in 2018 (China Fishery Statistical Yearbook, 2020). In their natural habitat, swimming crabs may encounter elevated ammonia due to anthropogenic discharges, and in culture ponds, the potential of exposure to increased ammonia is even higher because ammonia may accumulate rapidly due to excessive feeding with nonactive food and high stocking density. As such, understanding mechanisms and consequences of ammonia toxicity in this species is critical. Our recent study implied that ammonia exposure may impair vitellogenesis, a central event of female crustacean reproduction, in *P. trituberculatus*. For this species, vitellogenin (Vtg) is mainly synthesized in the hepatopancreas. Besides Vtg production, this organ is also responsible for many other critical physiological processes, including digestion of nutrients, storage of energy reserves, and detoxification of toxicants (Yang et al., 2005; Chang and Thiel, 2015; Vogt, 2019). Previous studies indicated that the hepatopancreas of decapods plays vital roles in defending against ammonia toxicity through conversion of ammonia and glutamate to glutamine, metabolism of ammonia to urea, and mobilization of energy reserves, meeting the increased demand for energy in organisms due to ammonia stress (Racotta and Hernández-Herrera, 2000; Qiu et al., 2018; Shan et al., 2019). On the other hand, ammonia accumulation may cause severe damage in hepatopancreas (Liang et al., 2016; Li et al., 2018). Although there has been a number of studies on ammonia toxicity and metabolism mechanism in hepatopancreas of decapods, most of them were conducted using the individuals at grow-out stage. Little information is available regarding ammonia toxicity and underlying mechanisms in adult female individuals at reproductive-development stage when hepatopancreas function as the major site of Vtg production.

In this study, we investigated ammonia stress response in the hepatopancreas of adult female swimming crab, with a combined physiological, transcriptomic, and metabolomic analysis. The results provide a better understanding of ammonia toxicity and metabolism mechanisms in the swimming crab, and valuable information for assessing the potential ecological risk of environmental ammonia and improving aquaculture management.

2. Materials and methods

2.1. Animals and sample collection

Healthy female swimming crab *P. trituberculatus* (average body weight is 206.8 g ± 7.5 g) after reproductive molting, which usually will not molt again before spawning, were obtained from Haifeng Company (Weifang, China), where the ammonia nitrogen concentration in rearing seawater was 0.16 mg/L. After transfer to the lab, the crabs were maintained in 3000-L holding tanks and acclimated to the experimental conditions for two weeks. During the period, the water temperature was maintained at 15.2 ℃ ± 0.6 ℃, aeration was provided continuously, the pH was 7.8 ± 0.3, the salinity was 30.8 ± 0.5, ammonia nitrogen concentration was below 0.20 mg/L and the photoperiod was set as 12 h of light: 12 h of dark. The crabs were fed ad libitum with live Manila clam *Ruditapes philippinarum* once a day, and the feces and leftover feed were removed before feeding. Half of the rearing water was exchanged every day using fresh equi-temperature seawater.

Following acclimation, twenty four crabs were evenly and randomly allocated into eight 80-L tanks, four tanks for the control group and the other four for the ammonia exposure group. To prevent cannibalism, all the tanks were separated into three cubicles with perforated plastic plates, with one crab for each cubicle. Based on our preliminary experiment on these crabs, the ammonia concentrations resulting in 50% mortality (LC50 at 96 h) was ~40 mg/L. In this study, a sublethal concentration (10 mg/L, 1/4 of the LC50 at 96 h) was chosen for ammonia nitrogen exposure experiment, and this concentration occurs in the natural habitat of the swimming crab (Dai et al., 2008). The ammonia nitrogen concentration for treatment group was realized by infusion of calculated amount ammonium chloride (NH_4Cl) stock solutions which was prepared with filtered seawater (pH was 7.8, salinity was 30.8). The control group were cultured in the same condition without ammonia addition. Ammonia nitrogen concentration in the treatment groups was monitored using salicylic acid method with spectrophotometer, and it was adjusted with freshly prepared stock solution daily to ensure consistency (Romano and Zeng, 2007). During the experiment, culture conditions were the same as those in acclimation period (ammonia nitrogen concentration for the control group was below 0.20 mg/L).

After the 96-h experiment, ten individuals from each group were placed in an ice bath until anesthetized, and dissected for hepatopancreas collection. One portion of the hepatopancreas was fixed for the TUNEL assay, and the other portion was snap frozen in liquid nitrogen

and stored at −80 °C for determination of physiological, transcriptomic and metabolomic analysis. The animal experiment was approved by the Institutional Animal Care and Use Committee of Yellow Sea Fisheries Research Institute.

2.2. Physiological parameters determination

To study the effects of ammonia on energy reserves and metabolism in hepatopancreas, the levels of glycogen, triglyceride, adenosine triphosphate (ATP) and hemolymph glucose, and metabolic enzyme activities were analyzed. The levels of glycogen, triglyceride, and ATP, and the activities of hexokinase (HK), pyruvate kinase (PK), lactate dehydrogenase (LDH), succinate dehydrogenase (SDH), and lactate dehydrogenase (LDH) in hepatopancreas samples were measured using commercial diagnostic kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (Glycogen assay kit, A043–1–1; Triglyceride assay kit, A110–2–1; ATP assay kit, A095–1–1; HK assay kit, A077–3–1; PK assay kit, A076–1–1; LDH assay kit, A020–2–2; SDH assay kit, A022–1–1). The Hemolymph glucose level was detected using assay kit from Solarbio Life Science (Beijing, China) (BC2495). The activity of carnitine palmityl transferase-I (CPT-I) and fatty acid synthase (FAS) were determined using assay kit from Qiyi Biological Technology (Shanghai, China) (QYS-239013) and Solarbio Life Science (Beijing, China) (BC0555), respectively.

The activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) were analyzed with assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (SOD assay kit, A001–3–2; CAT assay kit, A007–1–1; GPX assay kit, A005–1–2). Malondialdehyde (MDA) and protein carbonyls (PC) were measured to assess oxidative damage to lipids and proteins, respectively. MDA concentrations were measured using MDA assay kit (Solarbio Life Science, Beijing, China) (MDA, BC0025). PC content in hepatopancreas samples was determined with a protein carbonyl content assay kit (Sigma-Aldrich, St Louis, USA) (PC, MAK094).

The activity of the enzymes involved in ammonia metabolism, including glutamate dehydrogenase (GDH), glutamine synthase (GS), arginase (ARG), and the contents of urea in hepatopancreas were measured. GDH and GS activity, and urea content were determined with assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (GDH assay kit, A125–1–1; GS assay kit, A047–1–1; urea assay kit, C013–2–1). Arginase activity was accessed using a kit from BioAssay Systems (Hayward, USA) (Arginase assay kit, DARG-100). In addition, hemolymph ammonia was determined with hemolymph ammonia assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (A086–1–1).

The data of physiological parameters were analyzed using SPSS Statistics 19. Contrasts between the control and treatment group were made using an independent samples *t*-test. The assumptions of equal variances were tested with Levene's tests.

2.3. Transcriptome analysis

Hepatopancreases of three crabs which were randomly chosen from the ten individuals sampled after 4-day exposure at elevated ammonia (treatment group) and at seawater (control group) were used for RNA sequencing (n = 3). Total RNA of the samples was extracted using TRIzol Reagent (Thermo Fisher Scientific, Waltham, USA), and RNA integrity was assessed with the RNA Nano 6000 Assay Kit using the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, USA). A total amount of 1 μg of RNA per sample was used as input material for RNA sample preparation. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in First Strand Synthesis Reaction Buffer. First strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H^-) (Thermo Fisher Scientific, Waltham, USA). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase treatment. After adenylation of the 3′ ends of DNA fragments, adaptors with hairpin loop structures were ligated to prepare for hybridization. To preferentially select cDNA fragments of 370–420 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then PCR was performed with Phusion High-Fidelity DNA polymerase, and the products were purified with AMPure XP system (Beckman Coulter, Beverly, USA) and library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, USA). Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform at Novogene Company (Beijing, China).

First reads containing adapters, reads containing poly-N and low quality reads were removed from raw data, and mapped to the reference genome using Hisat2. v2.0.5. The read numbers mapped to each gene were calculated with Featurecounts v1.5.0-p3 (Zytnicki, 2017) based on the expected number of Fragments Per Kilobase of transcript sequence per millions base pairs sequenced (FPKM) method. Differential expression analysis between the two groups was performed using the DESeq2 R package (1.20.2) (Love et al., 2014) with adjusted *p*-values. Adjusted *p*-value$<$ 0.05 and absolute log2-fold change$\geq$ 1 were set as the threshold for significant differential expression. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of differentially expressed genes (DEGs) were further implemented by the clusterProfiler R package (Yu et al., 2012), to understand the functional roles of the DEGs.

2.4. Metabolome analysis

Hepatopancreas tissues of six crabs from each group were used for metabolome analysis (n = 6). One hundred milligrams of each sample was transferred to reaction tubes that contained 1 ml of ice cold extraction mixture (methanol: chloroform: water, 67.5:7.5:25, v:v:v), homogenized in a high-throughput tissue grinder, ultrasonicated at room temperature for 30 min, and centrifuged at 12,000 rpm for 10 min. Then, the supernatant was transferred to a new tube, dried in a vacuum, dissolved and filtered through a 0.22 μm membrane. Twenty microliters of filtrate from each sample was mixed to make quality control (QC) sample, and the rest of the filtrate was used for LC-MS analysis. The QC sample was used to monitor deviations of the analysis results from pooled sample mixtures and to compare this deviation with the error caused by the analyzer itself.

Chromatographic separation was performed using Thermo Vanquish system equipped with an ACQUITY UPLC® HSS T3 (150 × 2.1 mm, 1.8 μm, Waters, USA) column. The ESI-MSn experiments were executed on the Thermo Q Exactive HF-X mass spectrometer with the spray voltage of 3.5 kV and −2.5 kV in positive and negative modes, respectively. Data dependent acquisition (DDA) MS/MS experiments were performed with HCD scans. Dynamic exclusion was implemented to remove unnecessary information in the MS/MS spectra.

The raw data obtained were converted into mzXML format using ProteoWizard, and then peaks identification, peak filtration, and peak alignment were performed with the XCMS package (Smith et al., 2006). All data were determined using quality control (QC) and quality assurance (QA). Following standardized treatment with mean-centering and scaling to unit variance (UV), orthogonal projections to latent structures discriminant analysis (OPLS-DA) of the metabolome data was performed to visualize metabolic changes between the control group and the treatment group using the ropls package. The metabolites were identified by searching against the databases, including HMDB, Metlin, Massbank, LipidMaps, and mzClound, and the BioDeep metabolome

database (BioNovoGene, Suzhou, China). In the OPLS-DA analysis, the variable importance in the projection (VIP) was sorted according to the overall contribution of each variable to the OPLS-DA model, and those variables with a VIP≥ 1 and *p*-value < 0.05 were considered significant. The differential metabolites were annotated with KEGG pathway analysis using metaboanalyst software (www. metaboanalyst. ca).

2.5. TUNEL assay

To analyze ammonia-induced apoptosis in the hepatopancreas, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out using a Fluorescein Tunel Cell Apoptosis Detection Kit (Servicebio, Wuhan, China), according to the manufacturer's instructions. Briefly, the fixed hepatopancreas samples were dehydrated in graded concentrations of ethanol, embedded in paraffin wax, divided into sections and mounted on glass slides. The tissue sections were deparaffinized, rehydrated, and treated with protease K solution at 37 ℃ for 25 min, and then washed three times with PBS. After that, the slides were incubated with the TUNEL reagent in humid chamber at 37 °C for 2 h, washed three times with PBS, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and visualized on a fluorescence microscope (Nikon, Tokyo, Japan).

2.6. Quantitative real-time PCR analysis

In order to validate the Illumina sequencing data, ten differentially expressed genes between the control and treatment group were chosen for quantitative real-time PCR analysis with the same RNA samples for transcriptome analysis. The PCR reactions were run in ABI 7500 real-time PCR system (Applied Biosystems, Foster City, USA) using SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China). The PCR was performed in a total volume of 20 μl, containing 10 μl of 2X SYBR®

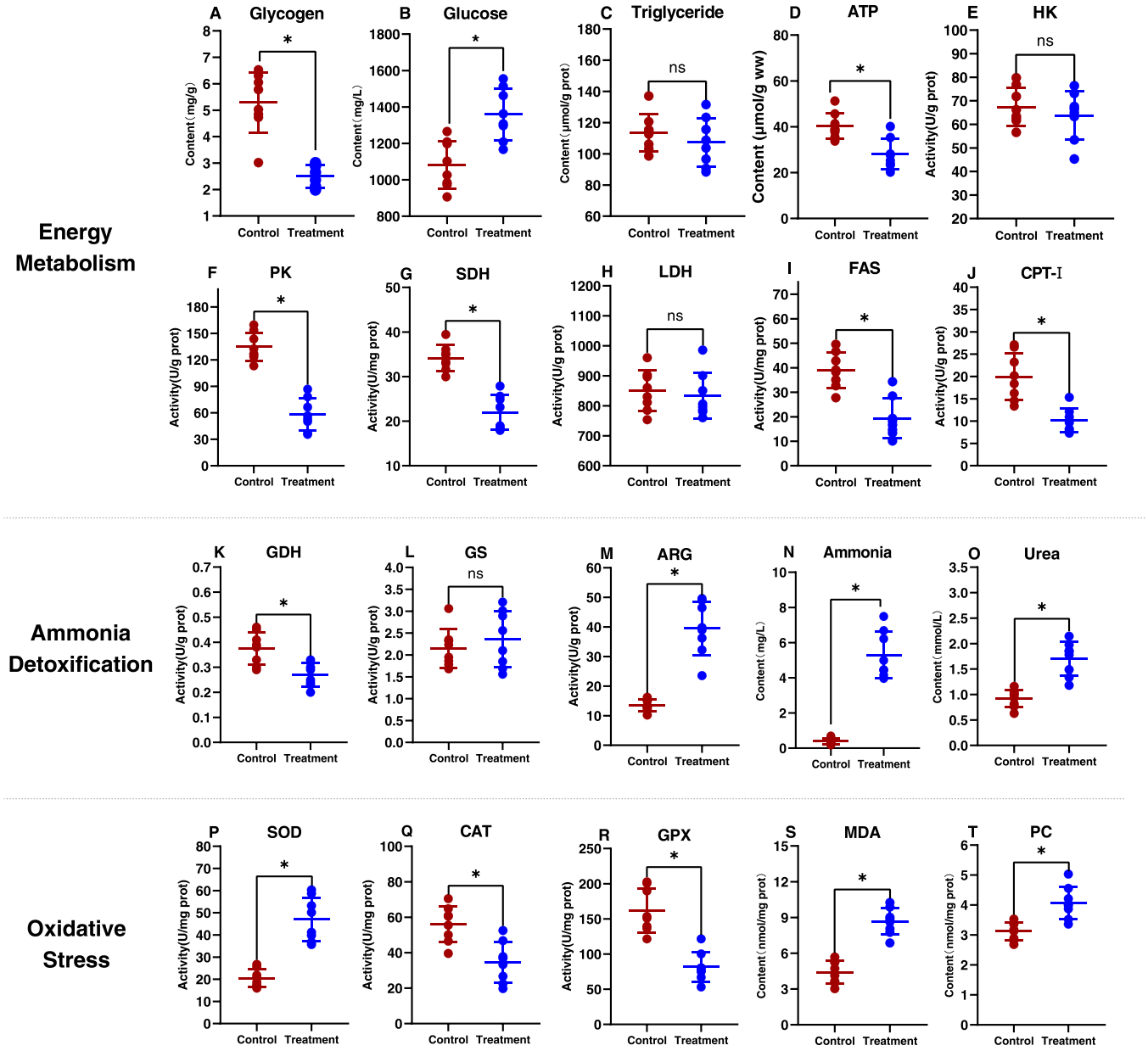

Fig. 1. Results of physiological analysis. Eight replicates for the control group and the HEA group are shown as dots in red and blue, respectively. The long line in the middle represent the average value, and the upper and lower line represent error bars showing SD of mean (n = 8). Asterisks indicate significant difference ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Green Pro Taq HS Premix II, 2 μl of diluted cDNA, 0.8 μl each of 10 mM each primer and 7.2 μl DNase-free water. The PCR program comprised a 95 °C activation step for 30 min, followed by 40 cycles of 95 °C for 5 s (denaturation), 60 °C for 30 s (annealing and extension). The β-actin was used as the reference gene to normalize expression levels of the tested genes. All the specific primers used were listed in Table S2.

3. Results

3.1. Physiological parameters

Overall, metabolic substrate and metabolic enzyme activity levels indicate a suppressed metabolic phenotype in hepatopancreas of the ammonia exposed crabs. The glycogen levels in the hepatopancreas of the treatment group were significantly lower than that in the control group ($p < 0.05$) (Fig. 1A), while there was no significant difference in triglyceride between the two groups ($p > 0.05$) (Fig. 1C). The activities of PK, SDH, FAS, and CPT-I were significantly decreased after ammonia exposure ($p < 0.05$) (Fig. 1F, G, I, J), and HK and LDH activities exhibited no significant difference ($p > 0.05$) (Fig. 1E, H). The contents of ATP in the treatment group were significantly lower compared with that in the control group (Fig. 1D), and the hemolymph glucose in the treatment group was significantly higher ($p < 0.05$) (Fig. 1B).

Data indicate significant oxidative damage in the treatment group as demonstrated by the increase in concentrations of MDA and PC, biomarkers for lipid peroxidation and protein oxidation (Fig. 1S, T). This is likely mediated via increased hydrogen peroxide production and accumulation resulting from increased SOD activity ($p < 0.05$) and decreased CAT and GPX activities ($p < 0.05$) in ammonia exposed animals (Fig. 1P-R). After ammonia exposure, the activities of GDH and ARG in hepatopancreas significantly decreased and increased ($p < 0.05$), respectively, whereas GS activity did not change significantly ($p > 0.05$) (Fig. K-M). Urea contents in hepatopancreas and ammonia in hemolymph were both increased following ammonia exposure ($p < 0.05$) (Fig. 1N,O).

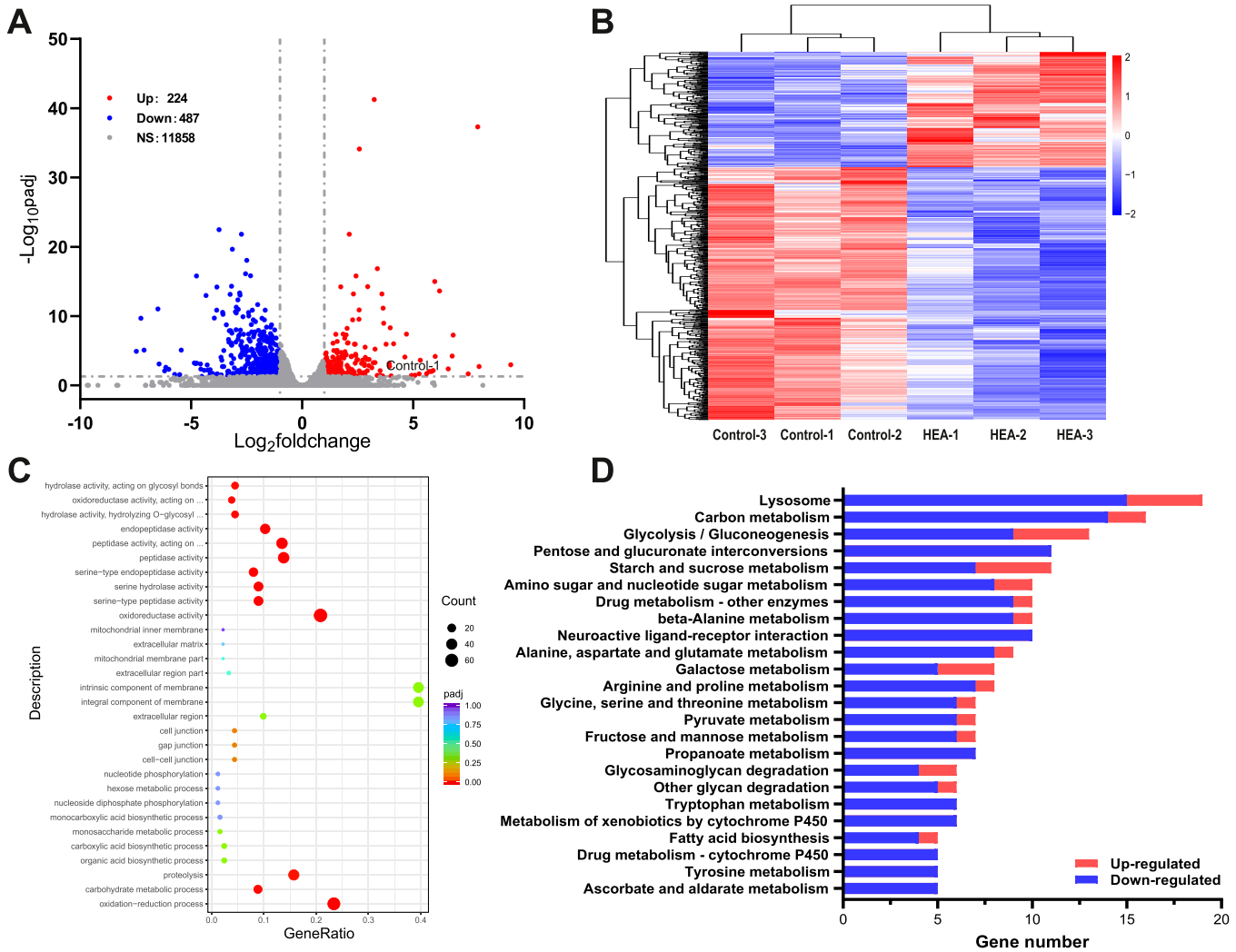

Fig. 2. Transcriptional changes in hepatopancreas of the female swimming crabs under HEA exposure. (A)The volcano plot showing gene expression in hepatopancreas of the female swimming crabs after HEA exposure. Red and blue dots represent significantly upregulated and downregulated genes, grey dots indicate no significant difference (B) Heat map of transcriptome profile for the control group (Left) and the HEA group (Right). Columns represent biological replicates (n = 3 per group) and rows represent individual genes. Level of gene expression is shown as log2-transformed fragments per kilobase of exon per million mapped reads (FPKM) scaled by row (gene). Transcripts with higher expression in a sample appear red and transcripts that has less abundance in a sample appear blue. (C) GO enrichment analysis of the DEGs between the control group and the HEA group. (D) KEGG pathway enrichment analysis of the DEGs between the control group and the HEA group. Red and blue represent upregulated and downregulated genes after HEA exposure in each pathway, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

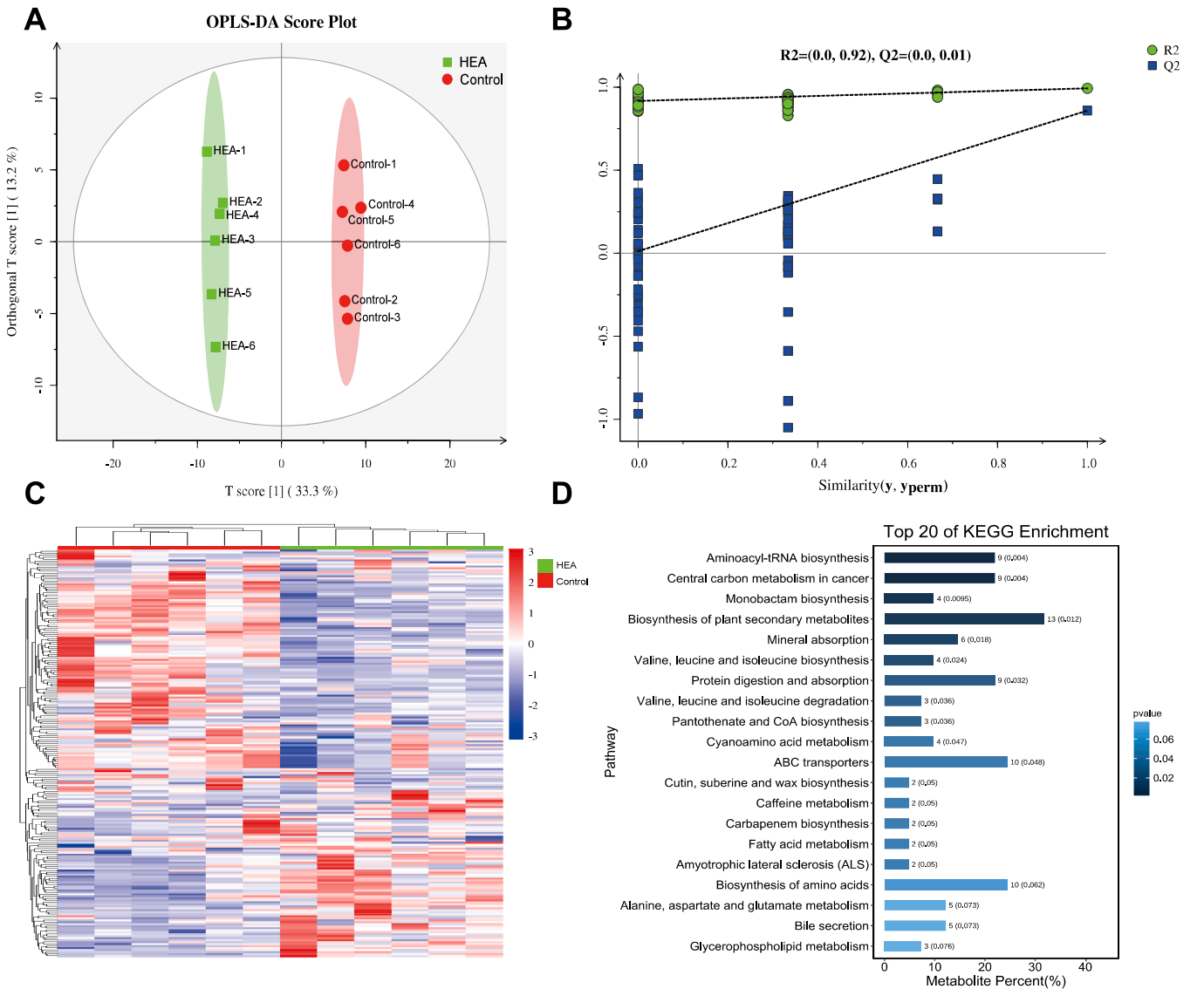

Fig. 3. Metabolome changes in hepatopancreas of the female swimming crabs after HEA exposure. (A) Orthogonal projections to latent structures (OPLS-DA) score plots for the crabs in the control group (red) and the HEA group (green). (B) OPLS-DA permutation test.(C) Heat map of metabolite profile for the control group (Left) and the HEA group (Right). Columns represent biological replicates (n = 6) and rows represent individual metabolite. Metabolites with higher abundance in a sample appear red and transcripts that has less abundance in a sample appear blue. (D) KEGG enrichment analysis of the differential metabolites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Transcriptome alteration under ammonia exposure

RNA-sequencing of the six sample from the control group and the treatment group generated 38.77 Gb of clean data in total. The average clean data for each sample reached 6.46 Gb, with a Q20 base percentage of higher than 97.08% (Table S1). A total of 711 differentially expressed genes (DEGs) were identified between the two groups (adjusted *p*-value<0.05 and absolute log2 fold change≥1), including 224 upregulated genes and 487 downregulated genes after ammonia exposure (Fig. 2A, B). In order to validate expression profiles obtained from Illumina sequencing analysis, ten DEGs were chosen for qRT-PCR analysis using the same RNA samples. All the ten genes showed a similar expression patten, indicating the high reliability of the transcriptome data (Fig. 5). Among the identified DEGs, several important genes related to female reproduction, including *vitellogenin-1* (*vtg-1*), *vtg-2*, *vtg-3*, *farnesyl pyrophosphate synthase* (*FPS*), *JHE-like carboxylesterase-1* (*JHE-like CXE-1*), *ecdysone receptor* (*EcR*), and *neuroparsin* all showed significant down-regulation after ammonia stress (Fig. 6). In addition, many genes associated with energy metabolism (*CHH binding protein* (*CHHBP*), *glycogen phosphorylase* (*GP*), *AMP-activated protein kinase* (*AMPK*), etc.), antioxidant defense (*superoxide dismutase* (*SOD*), *catalase* (*CAT*), *glutathione peroxidase* (*GPX*), etc.), ammonia metabolism (*arginase* (ARG) and *glutamate dehydrogenase* (*GDH*)), DNA repairing (*ataxia-telangiectasia and Rad3-related* (*ATR*) and *DNA-dependent protein kinase catalytic subunit* (*DNA-PKcs*)), and apoptosis (*Caspase 1*, *Caspase 7*, *apoptosis-inducing factor* (*AIF*), etc.) were also found in the DEGs (Fig. 6).

To dissect the functional categories of DEGs, GO enrichment and KEGG pathway analysis were performed. The GO enrichment analysis showed that the DEGs were significantly enriched in 16 GO terms (Fig. 2C). Half of these terms were associated with carbohydrate and protein metabolism, including “carbohydrate metabolic process”, “proteolysis, ”serine-type endopeptidase activity”, “serine-type peptidase activity”, “serine hydrolase activity”, “endopeptidase activity”, “peptidase activity, acting on L-amino acid peptides”, “peptidase activity”. Within these terms, most of the DEGs were downregulated (Table S3), indicating that ammonia exposure resulted in an overall depression of carbohydrate and protein metabolism in the hepatopancreas.

KEGG pathway enrichment analysis showed that the DEGs were

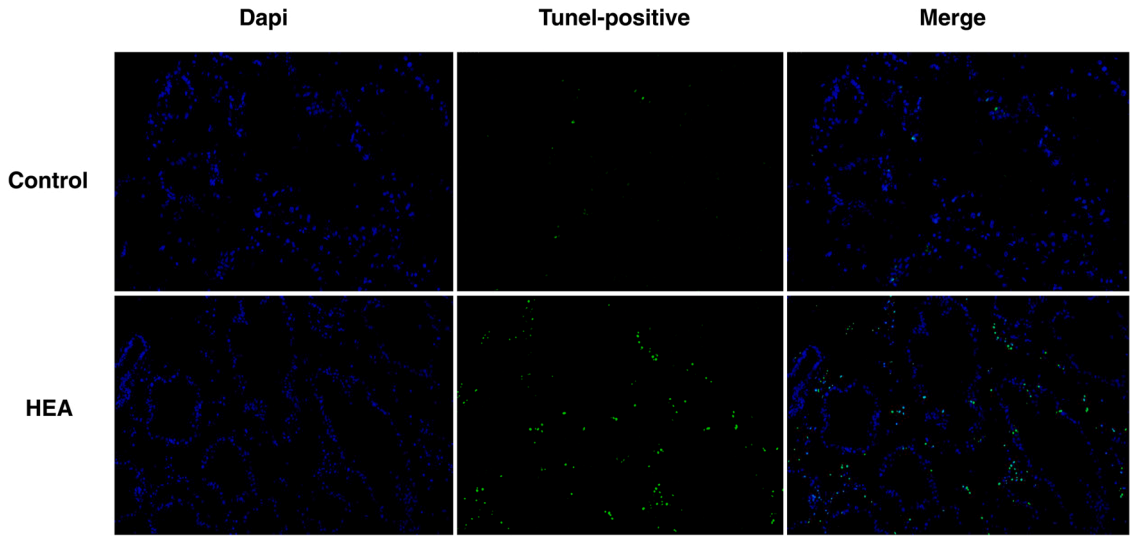

Fig. 4. TUNEL assay of hepatopancreas from the control group (the upper panels) and the HEA group (the lower panels) (n = 3). The apoptotic cells were detected by TUNEL (green) and the nuclei were detected by DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

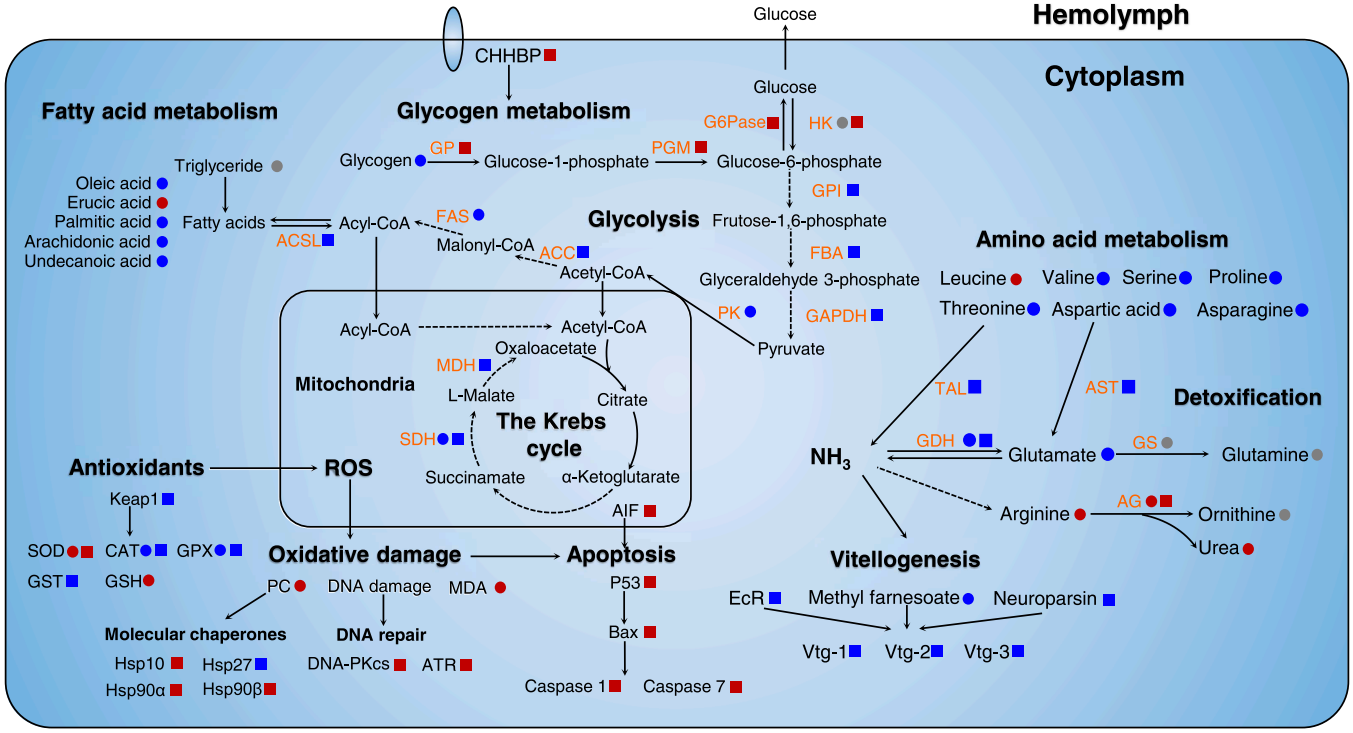

Fig. 6. Schematic overview of the alteration in physiological parameters, transcriptome and metabolome in hepatopancreas of the female swimming crabs after HEA exposure. The dots represent metabolites (n = 6) and physiological parameters (n = 8), and the squares represent genes (n = 3). Red and blue represent upregulation and downregulation, respectively. The solid arrows and dashed arrows represent direct and indirect effects. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

statistically enriched in 24 pathways (Fig. 2D), and 13 of the pathways were related to metabolism of carbohydrate, fatty acid, and amino acid metabolism, including “carbon metabolism”, “starch and sucrose metabolism”, “glycolysis/gluconeogenesis”, “galactose metabolism”, “glycosaminoglycan degradation”, “other glycan degradation”, “pentose and glucuronate interconversions”, “pyruvate metabolism”, “fatty acid biosynthesis”, “arginine and proline metabolism”, “alanine, aspartate and glutamate metabolism”, “beta-alanine metabolism”, “glycine, serine and threonine metabolism”, and “tyrosine metabolism”. Most of the genes in these pathways were downregulated after ammonia exposure, and this result, in consistence with GO analysis, further indicating a metabolic depression after ammonia exposure.

3.3. *Metabolome alteration under ammonia exposure*

Untargeted metabolome analysis was performed to investigate the metabolic changes in hepatopancreas of the swimming crab under ammonia stress. In total, 628 unique metabolites were detected by LC-

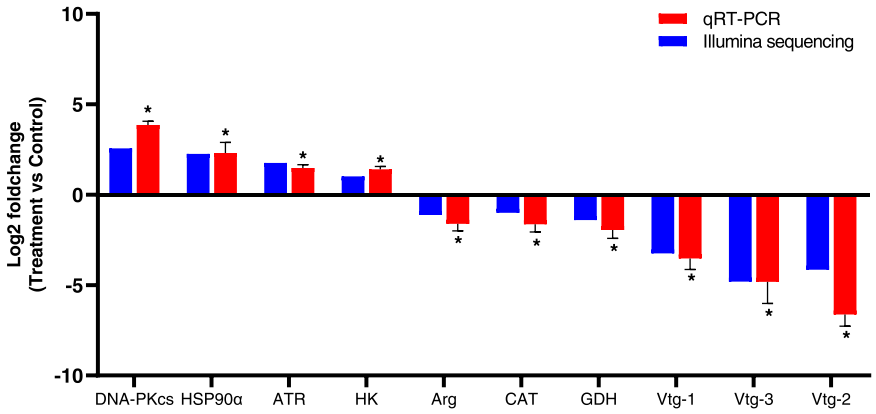

Fig. 5. Comparison of gene expression data between Illumina sequencing and qRT-PCR. Data are presented as mean ± S.D. of three replicates (n = 3). An asterisk indicates statistically significant differences ($P < 0.05$).

MS. To maximize the differentiation between the two groups, the multivariate statistical analysis was carried out using the OPLS-DA model to screen the differential metabolites (Fig. 3A, B). The score plot of OPLS-DA analysis showed a clear separation between the control group and the treatment group (Fig. 3A), indicating a significant change in the metabolite profiles after ammonia exposure. The classification parameters were calculated as R2X = 0.465, R2Y = 0.993, and Q2 = 0.859, which indicated that the model was stable and effective for fitness and prediction.

Based on OPLS-DA analysis, a total of 85 metabolites that significantly changed were identified with the threshold of VIP≥ 1 and $p < 0.05$ (Fig. 3C). Among them, 25 and 60 metabolites were upregulated and downregulated in treatment group, respectively (Table S4). Many of the differential metabolites (DMs) were amino acids (arginine, asparagine, aspartic acid, glutamic acid, leucine, ornithine, proline, serine, threonine, and valine), and fatty acids (arachidonic acid, oleic acid, undecanoic acid, palmitoleic acid, and erucic acid), purines and pyrimidines (adenosine, guanine, uridine, and xanthine) and most of them showed lower levels in the treatment group. After ammonia exposure, the level of methyl farnesoate, a key crustacean reproductive hormone, increased significantly, and glutathione, an important cellular antioxidant, showed a significantly increased level.

To explore the metabolic pathways potentially affected by ammonia stress, KEGG pathway analysis was performed using Metaboanalyst. The DMs were significantly enriched 16 pathways, among which several pathways associated with metabolism of protein, amino acid, and fatty acid, such as “protein digestion and absorption”, “valine, leucine and isoleucine degradation”, “valine, leucine and isoleucine biosynthesis”, and “fatty acid metabolism”, were observed.

3.4. TUNEL results

Apoptosis of hepatopancreatic cells was detected by TUNEL assay (Fig. 4). Few TUNEL-positive cells were detected in the hepatopancreas from the control group, while a markedly increased number of positive cells was observed in the treatment group.

4. Discussion

Our integrated analysis indicated that ammonia exposure leads to significant suppression of cellular metabolism, induce oxidative damage, and results in apoptosis, likely impairing female reproductive output. Transcriptomic and metabolomic data provided further evidence for mechanisms of ammonia toxicity that are discussed in detail below.

4.1. Mobilization of glycogen and depression in energy metabolism

Under ammonia stress, energy reserves are mobilized to meet increased energy expenditure in energy-consuming processes, such as active ammonia secretion through ion transporters, ammonia biotransformation, and tissue damage repair (Marazza et al., 1996). Several studies in decapods at grow-out stage have shown that ammonia exposure results in increased levels of glucose in hemolymph (Racotta and Hernández-Herrera, 2000; Cui et al., 2017). Similar with the results from the previous studies, a significant increase in hemolymph glucose was observed in adult female swimming crab. To date, the regulatory mechanisms for ammonia-induced energy mobilization in crustaceans is still unclear.

In crustaceans, the hepatopancreas functions as a major storage depot for carbohydrate and lipid, mainly in the form of glycogen and triglyceride, respectively (Chang and Thiel, 2015). The mobilization of the energy reserves is critical for survival under stressful conditions (Klepsatel et al., 2016). In this study, no significant differences in triglyceride contents was observed, whereas glycogen levels in hepatopancreas substantially deceased, indicating that hepatopancreas glycogen was utilized as an important energy resource and contributed to the increase in hemolymph glucose under ammonia stress (Hong et al., 2007). Carbohydrate metabolism in crustaceans is under the control of an neurohormone, crustacean hyperglycemic hormone (CHH), secreted from the X-organ-sinus gland complex in eyestalk (Chen et al., 2020). CHH is known to have an adaptive role in response to many types of environmental stresses, and it can mobilize glycogen reserves in the hepatopancreas, leading to hyperglycemia by regulating the expression of the enzymes involved in glycogen metabolism (Chang et al., 1998; Lorenzon et al., 2004; Zhang et al., 2020b). In the present study, *CHHBP*, the receptor for CHH (Li et al., 2017), and *GP*, the enzyme catalyzing the rate-limiting step in glycogenolysis, were significantly upregulated after ammonia exposure. This result indicated that CHH signaling was activated after ammonia exposure and stimulated glycogen mobilization in the hepatopancreas to meet the energy requirements for ammonia tolerance.

AMPK is a conserved master regulator of cellular energy metabolism, which promotes ATP-generating catabolic pathways and inhibits ATP-consuming anabolic processes (Hardie and Sakamoto, 2006; Garcia and Shaw, 2017). In the present study, AMPKβ was significantly downregulated after ammonia exposure. In concordance, the contents of ATP decreased significantly, and several genes and metabolites in glycolysis (*glucose-6-phosphate isomerase* (*GPI*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), and *fructose-bisphosphate aldolase* (*FBA*)), the Krebs cycle (*malate dehydrogenase* (*MDH*) and *succinate dehydrogenase* (*FBA*)), and fatty acid β-oxidation (*long chain fatty acid CoA ligase* (*ACSL*), *lysosomal thioesterase* (*PPT*), and palmitoylcarnitine)

showed significant downregulation in the treatment group. In addition, the activities of the metabolic enzymes including MDH, pyruvate kinase (PK), carnitine palmitoyltransferase I (CPT-I), and fatty acid synthase (FAS) were also decreased following ammonia stress.

Collectively, these results suggested that ammonia exposure resulted in glycogen mobilization and a general decrease in energy metabolism in hepatopancreas of the swimming crab. The glycogen depletion, in accordance with the reduction of ATP level, indicates an increased energy demand for ammonia decomposition. The reduction in energy metabolism is possibly related to internal ionic imbalance. Previous studies have shown that ammonia exposure can enhance the activity of ammonia transporters in gill epithelia such as Na^+-K^+-ATPase, $Na^+/K^+/2Cl^-$-cotransporter, and Na^+/H^+-exchanger, which may lead to ionic imbalance in hemolymph and influence energy metabolism (Romano and Zeng, 2013; Weihrauch and Allen, 2018). In addition, the decreased energy metabolism could also be associated with ammonia-induced cellular damage. It was reported that ammonia can lead to mitochondrial dysfunction and endoplasmic stress in aquatic animals, which in turn could causes metabolic depression (Liang et al., 2016; Zhang et al., 2020a).

4.2. Impairment of reproductive signaling

Despite extensive studies on ammonia toxicity in crustaceans (Liu et al., 2009; Cheng et al., 2019; Si et al., 2019), limited information is available for its toxic effects on reproduction. The only existing report investigated the Cladocera species *Daphnia similis*, which found that elevated ammonia significantly delayed reproductive development and reduced the number of offspring per female (Lyu et al., 2013). Vtgs are the major yolk proteins in oviparous animals, and Vtgs production is critical for oogenesis and embryogenesis (Yeong Kwon et al., 2001). Therefore, vitellogenesis serves as an indicator of female reproductive activity (Tsukimura, 2001). As most of the existing studies were carried out on individuals at grow-out stages, it has not been reported that ammonia stress influences vitellogenesis in decapods. In this study, we identified three isoforms of *vtg* genes from the *P. trituberculatus* hepatopancreas transcriptome, and all of them showed remarkably decreased expression by 89.5%− 96.4% (Fig. 5) after ammonia exposure, clearly demonstrating for the first time that ammonia stress severely impaired vitellogenin synthesis in the hepatopancreas of the female swimming crabs.

Vitellogenesis in crustaceans is coordinated by an intricate network of reproductive regulators and their signaling pathways (Tsukimura, 2001; Jayasankar et al., 2020). In the present study, the levels of several important regulatory factors and components in endocrine signaling pathways were influenced by ammonia exposure. Methyl farnesoate (MF) is a sesquiterpene hormone and plays a critical role in stimulating ovarian maturation in decapods (Laufer et al., 1987; Sagi et al., 1991; Nagaraju, 2011). Many studies have shown that there is a positive correlation between MF level and vitellogenesis, and the MF level is controlled by both anabolism and catabolism (Nagaraju, 2011; Meng et al., 2020). Transcriptome analysis showed that the genes related to both MF anabolism (*FPS*) and catabolism (*JHE-like CXE-1*) pathways exhibited downregulation after ammonia exposure, and metabolome analysis showed a significantly lower level of MF in the treatment group. These results indicated that ammonia stress alters MF metabolism and results in a decreased level of MF in the swimming crab. Furthermore, *EcR*, the nuclear receptor for ecdysone, was also downregulated by ammonia. Previous studies on decapods at grow-out stage found that ammonia influences expression of the genes related to molting, which may contribute to ammonia-induced changes in molting frequency (Lu et al., 2016). However, the downregulation of *EcR* in this study was probably not associated with molting, because the female swimming crab doesn't molt after mating, when it enters reproductive-development stages. It has been demonstrated that EcR also plays an important role in regulating vitellogenesis in decapods (Abdullah-Zawawi et al., 2021). In the mud crab *Scylla paramamosain*, silencing of *EcR* can significantly inhibit *vtg* expression (Gong et al., 2015). The depressed expression of *EcR* in this study suggested that ammonia stress may impair the EcR-mediated signaling pathway and thereby influence vitellogenesis in *P. trituberculatus*. In addition, *neuroparsin*, encoding a conserved neuropeptide in arthropods, also showed decreased expression in the treatment group. In decapods, neuroparsin is involved in regulating ovarian maturation, and knockdown of neuroparsin can cause a significant decrease in *vtg* expression in the hepatopancreas (Yang et al., 2014).

Taken together, our results suggested that ammonia exposure suppressed multiple reproductive signaling pathways, thereby disrupting vitellogenesis in the hepatopancreas of female *P. trituberculatus*. Considering that vitellogenesis is energetically expensive, the nonspecific depression of reproductive endocrine pathways and curtailment of Vtg synthesis may represent a strategy for swimming crabs to cope with ammonia stress (Tsukimura, 2001). Ammonia exposure may lead to increased energy demand for excretion/metabolism and damage repair and an tradeoffs between the allocation of energy to ammonia-tolerance and reproduction. Reallocation of energy may facilitate survival of swimming crabs under ammonia stress. However, prolonged perturbations in Vtg production induced by ammonia stress could have significant adverse ecological consequences, such as reduced female fecundity, egg quality, and larval viability (Subramoniam, 2011).

4.3. Alteration of ammonia metabolism mechanisms

Previous studies in crustaceans have shown that the hepatopancreas can biotransform ammonia into less toxic urea via ornithine-urea cycle (OUC) (Liang et al., 2016; Cheng et al., 2019; Shan et al., 2019). After ammonia exposure, the expression of *ARG* which catalyzes the final step of OUC, converting arginine to ornithine and urea, and its activity were significantly increased. In parallel with this result, arginine and urea showed higher levels in the treatment group. These results indicated that OUC in the *P. trituberculatus* hepatopancreas was enhanced to convert ammonia into urea under ammonia conditions.

The vast majority of the endogenous ammonia in aquatic animals originates from the catabolism of amino acids (Claybrook, 1983). Some studies have reported that fishes can reduce endogenous ammonia production by suppressing protein and amino acid catabolism when subjected to high ambient ammonia (Lim et al., 2001; Bernasconi and Uglow, 2011). However, limited information is available concerning whether this strategy is adopted by crustaceans. The results of KEGG analysis showed that DEGs and DMs were both significantly enriched in the pathways associated with amino acid metabolism (Figs. 2 and 3). In these pathways, most the DEGs and DMs were significantly downregulated after ammonia exposure. Those results suggested that the swimming crab may suppress partial amino acid catabolism to minimize endogenous ammonia production under ammonia stress.

The conversion of ammonia to glutamate by GDH and then to glutamine by glutamine synthase (GS) can allow crustaceans to store toxic ammonia as an oxidative substrate in the hepatopancreas, and is considered an important mechanism to cope with elevated ambient ammonia (Qiu et al., 2018). Interestingly, both GDH mRNA abundance and GDH activity decreased significantly after ammonia exposure. A similar result was observed in a study of the prawn *Macrobrachium rosenbergii* which showed reduced hepatopancreatic GDH activity when exposed to ammonia (Dong et al., 2020). Depression of this mechanism is possibly associated with the activation of CHH signaling. A recent study in the white shrimp *Litopenaeus vannamei* showed that CHH can negatively regulate GDH activity, and *CHH* knockdown can increase GDH activity after ammonia exposure (Zhang et al., 2020b). Considering its importance in ammonia metabolism, the depression of this metabolism mechanism may contribute to the accumulation of ammonia in hemolymph in the ammonia-exposed crabs.

4.4. Disturbance in antioxidant defense and increase in oxidative damage and apoptosis

Oxidative stress is considered one of the apical endpoints of cellular toxicity resulting from environmental stress including ammonia in aquatic animals (Ching et al., 2009; Hegazi et al., 2010; Zhang et al., 2020a). Several studies of juvenile crustaceans have shown that ammonia can cause an overproduction of reactive oxygen species (ROS), and alter the antioxidant defenses (Li et al., 2018; Cheng et al., 2020). Similarly, our data showed that ammonia exposure leads to an increase in SOD activity, while CAT and GPx activities are reduced; SOD catalyzes the dismutation of superoxide (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2), and CAT and GPx decompose H_2O_2 into O_2 and H_2O. Increased SOD activity can lead to elevated levels of H_2O_2, while reduction of CAT and GPx activity can lead to accumulation of H_2O_2. This build-up of H_2O_2 levels can oxidatively damaged lipids and proteins, as indicated by the increased levels of MDA and protein carbonylation (PC) after ammonia exposure.

This mis-match in antioxidant defense is likely a result of altered Nrf2 (erythroid-2 related factor 2)/Keap1 (Kelch-like ECH-associated protein 1) pathway. In vertebrates, the Nrf2 (erythroid-2 related factor 2)/Keap1 (Kelch-like ECH-associated protein 1) pathway plays a central role in cellular defense against oxidative stress through transcriptional induction of numerous cytoprotective genes (Wasik et al., 2017; Suzuki et al., 2019). Recently, we found that this pathway is also critical in protecting against oxidative stress in decapods (Ren et al., 2021). The activity of the Nrf2 transcription factor is primarily controlled by Keap1 which acts as a negative regulator of Nrf2 by targeting it for ubiquitin-dependent degradation (Gañán-Gómez et al., 2013). In the present study, *Keap1* expression was significantly decreased following ammonia exposure, and thus, Nrf2 could exert its transcriptional function. However, the results of the transcriptome analysis showed that the target genes of Nrf2 exhibited the opposite expression pattern; *SOD* was significantly upregulated, whereas *CAT*, *GPX*, and *glutathione-S-transferase* (*GST*) were downregulated. Consistent with that result, SOD activity was enhanced in the treatment group, while the activities of CAT and GPX decreased. These result suggested that the Nrf2/Keap1 signaling pathway in the hepatopancreas may be impaired under ammonia exposure.

In addition to lipids and proteins, oxidative stress may also induce a plethora of DNA lesions in the form of single and clustered DNA damage (Georgakilas, 2008; Hada and Georgakilas, 2008). Compared to the control group, the expression of two important genes in DNA repair, *ATR* and *DNA-PKcs*, were significantly increased in the treatment group. ATR is considered as a sensor and transducer of DNA damage signals in response to DNA double-strand breaks (DSBs), and DNA-PKcs is an key effecter of DNA damage response (Chen et al., 2012). The upregulation of these two genes indicated that ammonia exposure may have caused DNA damage and triggered DNA repair machinery in hepatotactic cell.

The accumulation of oxidatively damaged biological molecules, such as lipid, protein, and DNA, can disturb cellular homeostasis and induce apoptotic cell death (Chandra et al., 2000; Finkel and Holbrook, 2000). In this study, the expression of two mediators of apoptosis, *caspase-1* and *caspase-7*, was significantly upregulated, indicating that ammonia induced apoptosis in the *P. trituberculatus* hepatopancreas, which was further confirmed by the TUNEL assay showing an increased ratio of positive cells in the treatment group. A recent study in juvenile mud crab *Scylla paramamosain* revealed that ammonia triggers apoptosis in the hepatopancreas via p53 signaling (Cheng et al., 2019). P53 can stimulate an array of signals, and consequently triggers caspase-mediated apoptosis (Yang et al., 2006). Consistent with that study, our transcriptome analysis showed that *p53* and its downstream factor *bax* were upregulated after ammonia exposure. These result infer that ammonia can trigger apoptosis in *P. trituberculatus* hepatopancreas through p53-bax mitochondrial apoptotic pathway. In addition, another apoptotic effector *AIF* was found to be upregulated in the treatment group. AIF was identified as a major player in caspase-independent apoptosis, and it can induce chromatin condensation and initial DNA cleavage (Susin et al., 1999; Ye et al., 2002). Therefore, the ammonia-induced apoptosis in hepatopancreas may involve multiple pathways, and further studies are required to understand its mechanisms.

5. Conclusion

In summary, a combined physiological, LC-MS-based metabolomic, and comparative transcriptomic analyses was performed to investigate metabolic and molecular responses in the hepatopancreas of female swimming crab to ammonia exposure. The results revealed that ammonia exposure caused a remarkable decrease in *vtg* expression and a depression of reproductive endocrine signaling, for the first time demonstrating a mechanistic basis for the ammonia induced impaired female reproduction in *P. trituberculatus*. Furthermore, ammonia led to suppression of overall energy metabolism and partial ammonia metabolism and antioxidant machinery, resulting in cellular oxidative damage and increased apoptosis in the hepatopancreas. The cause of metabolic depression following ammonia stress is unclear. This could be a result of total decrease in cells at energetic homeostasis due to increased oxidative damage and consequent apoptosis, which is supported by our findings on significant decreases in overall metabolite levels following ammonia exposure. Further temporal studies with recovery period may help to ascertain if energetic limitation is a mechanism or a consequence of cellular damage, as well as whether the reproductive and energetic depression are reversible. Nonetheless, the findings of this study improve our understanding of the adverse effects of ammonia stress on *P. trituberculatus* and the underlying mechanisms. In addition, it also provides a hint that studies should be carried out on individuals from other population and other benthic brachyura species that may have different thresholds and reactions under ammonia stress, which will provide valuable information for assessing potential ecological risk of environmental ammonia.

CRediT authorship contribution statement

Xianliang Meng: Conceptualization, Software, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Nishad Jayasundara:** Conceptualization, Writing – review & editing. **Jingyan Zhang:** Methodology, Investigation, Software, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Xianyun Ren:** Software, Validation, Formal analysis, Data curation, Writing – original draft. **Baoquan Gao:** Software, Validation, Formal analysis, Data curation, Writing – original draft. **Jian Li:** Resources, Supervision, Project administration. **Ping Liu:** Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113026.

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INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

Plastic ingestion by three species of Scylla (Brachyura) from the coastal areas of Thailand / Soe, K. K., Maae, S., Jaafar, Z., Chuaduangpui, P., Jantarat, S., & Hajisamae, S.

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Plastic ingestion by three species of *Scylla* (Brachyura) from the coastal areas of Thailand

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ABSTRACT

This study marked the first investigation into the presence of plastic particles in the stomachs of three mud crab species (*Scylla olivacea*, *S. paramamosain* and *S. tranquebarica*) collected across the Andaman Sea and the Gulf of Thailand. The highest number of plastic particles in the stomach of crab samples was polyethylene (PE) that contributed 88.5 %; while green was the predominant colour (60.3 %). Ingested particles recovered from the stomachs of crabs differed significantly between species and sites ($p < 0.001$). The average number of plastic particles per individual was 2.3 ± 8.6 in *Scylla olivacea*, 7.2 ± 16.9 in *S. paramamosain*, and 13.5 ± 48.9 in *S. tranquebarica*. Satun, revealed the highest number of plastic particles recovered from mud crabs, while the lowest number of plastic particles were from Pattani. To conclude, species of crab and site of collection plays a crucial factor in the propensity of plastic particles ingested by the genus *Scylla* mud crabs.

1. Introduction

Anthropogenic marine debris comprise solid wastes — glass, metal, paper and plastics (OSPAR, 2007) —from human activities that intentionally or otherwise, enter the marine environment (Coe and Rodgers, 1997; Galgani et al., 2010). Plastic particles, significant components of marine debris, are considered global environmental pollutants (Frias and Nash, 2019 (OSPAR, 2007; Rios et al., 2007). Primary microplastics are plastic fragments <5 mm (NOAA, 2009), and derived from anthropogenic materials such as microbeads and plastic glitter from cosmetic and personal care products (Cole et al., 2011; Ghosh et al., 2023), as well as from manufacturing industries (Lusher et al., 2017). Secondary microplastics, on the other hand, are plastic particles derived from larger plastic pieces, and broken down through processes of ultraviolet radiation, oxidation, or mechanical forces (Ivar do Sul and Costa, 2014; Law and Thompson, 2014; UNEP and NOAA, 2015; Napper et al., 2015; UNEP, 2016; Guzzetti et al., 2018). Terrestrial sources of plastic, transported via freshwater ecosystem networks, is a major contributor of plastic debris in marine environments (Andrady, 2011; Cole et al., 2011; Lebreton et al., 2017; Azevedo-Santos et al., 2021). Every year, an average of 1.2 and 2.4 million tons of plastic waste from rivers enter the oceans; 67 % of these highly-polluting rivers are in Asia (Lebreton et al., 2017). The Mekong River, the longest river in Southeast Asia, discharges approximately 2.28×10^4 tons of plastic waste per year (Lebreton et al., 2017). The plastic particles in aquatic systems can be found either floating within the water column, or embedded within substrate sediments (Law and Thompson, 2014; UNEP, 2015; Capparelli et al., 2022).

Due to this ubiquitous distribution, plastic particles readily enter the marine trophic systems through consumption (Rochman et al., 2015), with certain potential for trophic transfer (Thompson et al., 2004; Farrell and Nelson, 2013; Ward et al., 2022). Larger fragments can be ingested by, or cause entanglements to, marine biodiversity (Walker and Taylor, 1996; Laist, 1997; Gall and Thompson, 2015; Omeyer et al., 2022, 2023). Once ingested, plastic particles cannot be digested, but can transfer to higher trophic levels (Guzzetti et al., 2018) with potential sub-lethal or lethal effects by direct and indirect consequence (Gall and Thompson, 2015; Guzzetti et al., 2018). For instance, the burrowing crab *Neohelice granulata* (Dana, 1851) revealed physical toxicity after ingesting microplastics, especially plastic fibres (Villagran et al., 2020). Several studies have reported instances of plastic particle uptake in

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crabs; through trophic transfers from prey mussels *Mytilus edulis* Linnaeus, 1758 to predator crabs *Carcinus maenas* (Linnaeus, 1758) (Farrell and Nelson, 2013); through respiration and food ingestion of shore crab: *Carcinus maenas* (Akpan, 2014), and wild crabs: *Portunus trituberculatus (Miers, 1876)*, *Charybdis japonica (*A. Milne-Edwards, 1861*)*, *Dorippe japonica (*von Siebold, 1824), and *Matuta planipes* Fabricius, 1798 (Zhang et al., 2021); *through instances of* burrowing by crabs *Neohelice granulata* (Villagran et al., 2020) and *Ucides occidentalis* (Ortmann, 1897) (Aguirre-Sanchez et al., 2022). The quantity of plastic particles in sessile and benthic animals such as bivalves and crabs are correlated to plastic availability of ambient waters (Watts et al., 2014; Van Cauwenberghe and Janssen, 2014; Guzzetti et al., 2018; Waite et al., 2018; Waddell et al., 2020; Ward et al., 2022). Crabs are therefore susceptible to the potential physical toxic effects of microplastics (Villagran et al., 2020).

The genus *Scylla*, distributed throughout the Indo-Pacific region (MacNae, 1968). comprise species with high culture potential due to fast growing rates, large sizes, high reproductive capacities, high disease resistances, and adaptability to farming conditions (Viswanathan and Raffi, 2015). They are opportunistic omnivores that feed on crustaceans, molluscs, fishes, detritus, and miscellaneous items (Laughlin, 1982; Viswanathan and Raffi, 2015) and in turn are prey for higher trophic level invertebrate species and fishes (Hovel and Lipcius, 2001; Waddell et al., 2020). In crabs, plastics particles smaller than 0.5 μm can translocate from the stomach to other tissue (Lusher et al., 2017) and consequently reduce their feeding and growth (Watts et al., 2014). For this reason, plastic particle size and material types are essential information for food security and human health (Guzzetti et al., 2018). They are bio-inert since marine organisms lack suitable enzymatic pathways to break down these synthetic polymers (Andrady, 2011). Like many other crabs, these species are susceptible to plastic ingestion due to foraging and burrowing activities (Yi et al., 2021). The plastic particle loads in species of *Scylla* are unknown despite their economic importance in recreational, traditional and commercial fisheries within southeast Asia (Waddell et al., 2020). This study investigates the extent of plastic particles in the stomachs of three species of *Scylla* from important fishery sites across the Andaman Sea (Ranong, Trang and Satun) and the Gulf of Thailand (Surat Thani, Songkhla and Pattani) to determine the abundance and type of plastic debris; and from the data, discuss these implications of plastic particle accumulation in the economically important *Scylla* species fishery in Thailand.

2. Materials and methods

2.1. Sample collection

Three species of the genus *Scylla*— *S. olivacea* (Herbst, 1796) (n = 1495), *S. paramamosain* Estampador, 1950 (n = 103) and *S. tranquebarica* (Fabricius, 1798) (n = 24)—present in eight study sites were the foci of this investigation (Fig. 1). Study area was illustrated by QGIS program version 3.32.1 (QGIS Development Team, 2023). Mud crab samples were obtained from March to October 2022 within the coastal waters of Thailand; along the coast of the Andaman Sea (Ranong, Trang, Satun) and the Gulf of Thailand (Surat Thani, Songkhla and Pattani). In Pattani, we collected samples from three substations: Bana, Budi, and Bang Pu. All study sites are important *Scylla* fishing grounds for commercial and artisanal fishery economies.

All samples were caught by fisherfolks of the respective study sites, using baited crab nets (Fig. 2). Approximately 50 individuals were caught each month of the study duration, the species identification were followed to the key characters of Keenan et al. (1998). The carapace width (CW), carapace length (CL) of each specimen were measured with digital Vernier calipers to the nearest 0.01 mm and the stomach weight (SW) was obtained using a balance to the nearest 0.01 g. Thereafter, the stomach of each crab specimen was removed with metal forceps and cut open with surgical scissors within a petri dish. The stomach fullness index (FI) was estimated visually based on the distension of the stomach as a proxy of food quantity. Fullness Index was recorded on a scale of 0 to 4 [0 corresponds to empty; 1 corresponds to ¼ full, 2 corresponds to ½ full, 3 corresponds to ¾ full and 4 corresponds to full stomach] (Pillay, 1952; Gomes do Vale et al., 2022). With the aid of a stereomicroscope, plastic particles within the contents of the stomach were removed and placed into a glass vial filled with distilled water. Then, plastic particles were digested with 30 % hydrogen peroxide (H_2O_2) for an hour to

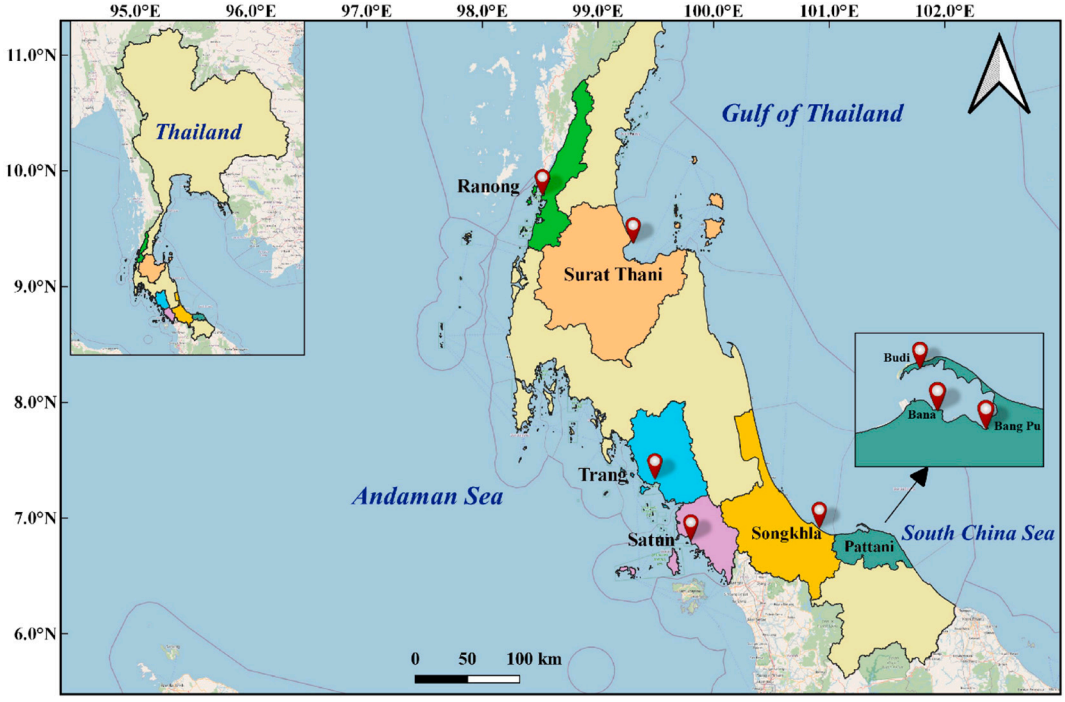

Fig. 1. Sites where mud crabs were sampled for this study in the Andaman Sea (Ranong, Trang and Satun) and in the Gulf of Thailand (Surat Thani, Songkhla and Pattani).

Fig. 2. The fishing method utilized in crab traps with fishes as bait in Satun province.

eliminate organic material (Mathalon and Hill, 2014; Digka et al., 2018). These were then poured over a 5 mm pore size, 47 mm diameter cellulose nitrate membrane filter (Whatman AE98) using 1 stage vacuum pump (RS-1) (Li et al., 2015). The filter paper was set onto a clean petri dish and covered with aluminum foil prior to drying in the oven at 60 °C for two hours. Thereafter, the filter paper, still in the petri dish, was kept in a desiccator until further analyses.

To avoid contamination, no plastic containers were used, and all glassware were rinsed with distilled water prior to use. All windows and doors of the laboratory were closed to minimize ambient sources of plastic, and all work surface areas were wiped with 70 % ethanol (Aguirre-Sanchez et al., 2022). Samples were immediately covered if they were not in use (De Witte et al., 2014). To get accurate results, it is important to minimize contamination in both control and original samples (Noren, 2007; Hidalgo-Ruz et al., 2012). To this end, sample blank test, an 8 cm petri dish filled with a few millimeters of distilled water, was carried out simultaneously with the same laboratory condition to correct the potential airborne contamination (De Witte et al., 2014; Waddell et al., 2020). There were no contaminants observed in blank samples during laboratory work.

2.2. Observation of marine debris

The types, physical characteristics, and length of plastic particles found in the stomachs of the crabs were made with the aid of a stereomicroscope (Olympus SZ-ST). Images of plastic particles were captured by Trinocular Digital HD stereomicroscope (Nikon SMZ 745 T). Additionally, the hot needle test was applied to suspected plastic materials whether they melted or twisted (Hidalgo-Ruz et al., 2012; De Witte et al., 2014).

Recovered plastic particles were categorized into three size classes, microplastics (1–5 mm), mesoplastics (>5–25 mm) and macroplastics (> 25 mm) (Andrady, 2011; Song et al., 2015; Lusher et al., 2017; Bessa et al., 2018). The colour, size and shape of each plastic particle were categorized to determine if the particle is primary or secondary. The colour of plastic particles, and size variations are important for plastic ingestion studies because of the colour preferences in some marine biota (Shaw and Day, 1994; Wright et al., 2013; Ory et al., 2017). This may contribute to the selectivity of marine organisms to plastic particles with colour similar to their prey items (Shaw and Day, 1994). Blue microplastics for instance, resemble copepods, and have been observed to be highly selected by plantktivorous pelagic fishes (Ory et al., 2017).

2.3. Verification of marine debris using ATR-FTIR

About 20 % of filtered samples of common and indeterminate particles were selected and verified with FTIR using an attenuated total reflectance (Platinum ATR) technique connected with OPUS software (version 7.5). To verify the polymer type, fluorescence emission spectra were compared with the well-established polymer spectrum library database (Bruker, Tensor 27 system). The observed spectra of the sample versus the wave number range between 4000 and 400 cm^{-1}.

The identity of the plastic particles was determined by their various components. Conclusively identified: polyethylene (PE), polypropylene (PP), low density polyethylene (LDPE), nylon, acrylic, coconut fibre, sheep wool and natural rubber (Fig. 3). These polymer types are used in a wide range of products including clothing, packaging, and rope material, suggesting that these fragments result from the breakdown of larger items (Thompson et al., 2004).

2.4. Data analyses

Fullness index (FI) was calculated based on non-empty stomachs of 1622 individuals (from a total of 2204 individuals); and correlated with species of crab and sites. Multiple linear regression analysis was tested between ingested plastic particles and different variables (CL, CW, stomach weight, and FI) to determine their relationships. In order to determine the abundance of ingested plastic debris and fullness index (FI) between species and sites, analysis of variance (ANOVA) was used. Prior to ANOVA test, non-parametric Kruskal-Wallis test was applied to test homogeneity of variance without transforming data. If it is statistically varied by ANOVA, Tukey's HSD post hoc test was applied to define the specific groups' means with different parameters of species and sites. All statistical work was carried out using R program version 3.6.2 (R Core Team, 2019).

3. Results

3.1. Characterization of crab samples

A total of 2204 mud crabs were obtained from March to October 2022 but only the stomachs of 1622 individuals were non-empty (1495 *S. olivacea*, 103 *S. paramamosain*, and 24 *S. tranquebarica*). Crabs were obtained from Pattani (789), Trang (217), Ranong (285), Satun (159), Songkhla (126), and Surat Thani (46). The sizes of all crab samples were 60.1 ± 11.5 mm in carapace length, CL and 89.9 ± 17.1 mm in carapace width, CW (Table 1). *Scylla tranquebarica* was the smallest species (54.8 ± 10.0 mm, CL and 89.6 ± 15.2 mm, CW) while *S. paramamosain* was the largest (60.2 ± 11.5 mm, CL and 90.4 ± 17.6 mm, CW).

3.2. Fullness index (FI) and the abundance of ingested plastic debris

The mean FI of all non-empty stomached mud crabs was 2.1 ± 1.1; the FI of *S. tranquebarica* was the highest (2.2 ± 1.3). Crabs (*S. olivacea*

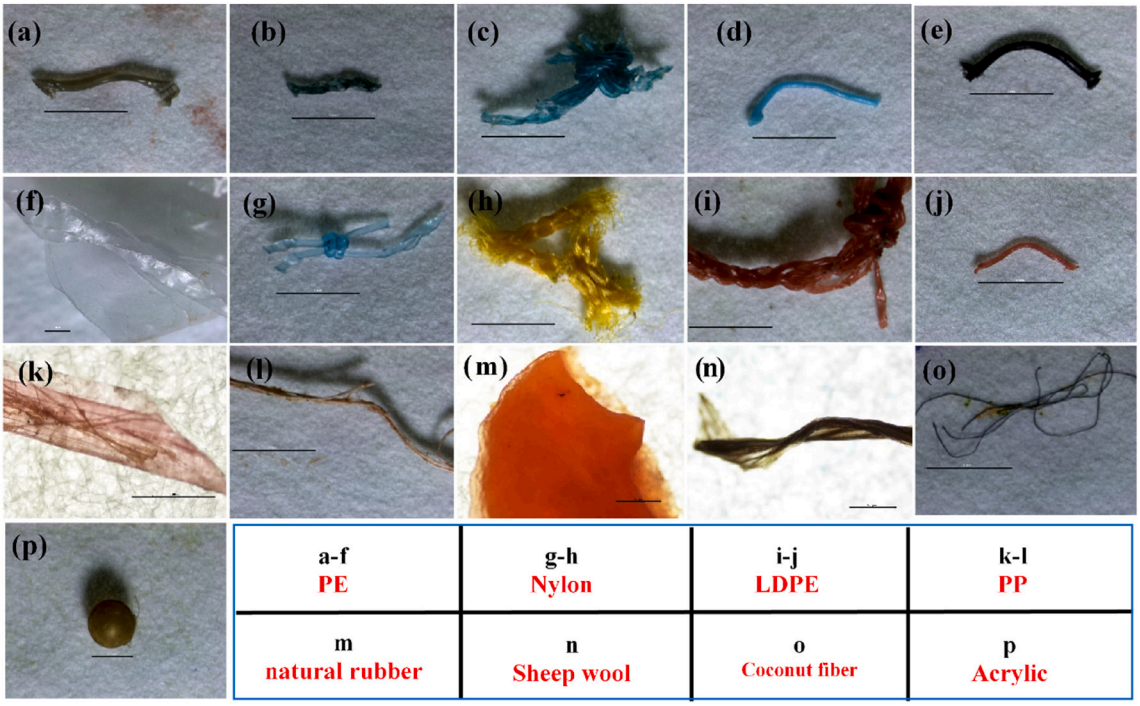

Fig. 3. The shape and colour distribution of polymer types recovered from the stomach contents of mud crabs. Scale bar 1 mm.

Table 1
Size, weight, fullness index and ingestion of plastic particles of mud crabs in coastal waters of Thailand. Number in parentheses represent the crabs from which plastic particles were recovered.

Species	Site	N	% of plastic particles	Number of plastic particles/ individuals	Carapace length	Carapace width	Stomach weight	Fullness index
		1622						
Overall		(358)	22.07	2.76 ± 11.12	60.07 ± 11.47	89.92 ± 17.14	2.03 ± 1.33	2.13 ± 1.14
	Ranong	253 (119)	47.03	6.15 ± 14.25	55.38 ± 8.85	82.35 ± 12.58	1.31 ± 0.79	1.82 ± 1.01
	Trang	206 (40)	19.41	1.21 ± 4.02	56.19 ± 11.22	85.68 ± 17.11	2.47 ± 1.57	2.65 ± 1.18
	Satun	144 (66)	45.83	6.95 ± 15.28	56.39 ± 12.35	83.67 ± 19.04	1.71 ± 1.13	2.37 ± 1.10
	Surat Thani	43 (13)	30.23	3.00 ± 8.87	69.79 ± 11.86	104.93 ± 18.08	2.32 ± 1.26	1.65 ± 1.02
	Songkhla	111 (39)	35.14	2.99 ± 8.65	66.04 ± 8.72	99.30 ± 13.63	2.30 ± 1.08	2.01 ± 1.14
	Bana	276 (26)	9.42	0.26 ± 1.50	64.83 ± 11.69	96.34 ± 17.76	2.07 ± 1.39	1.70 ± 0.93
	Budi	302 (20)	6.62	0.18 ± 0.97	60.21 ± 11.33	90.26 ± 17.02	2.28 ± 1.43	2.33 ± 1.19
S. olivacea	Bang Pu	160 (7)	4.37	0.09 ± 0.53	60.89 ± 9.37	90.46 ± 12.41	2.05 ± 1.24	2.34 ± 1.12
		1495						
Overall		(330)	22.07	2.28 ± 8.62	60.11 ± 11.48	89.90 ± 17.15	2.03 ± 1.33	2.13 ± 1.14
	Ranong	29 (0)	0	13.89 ± 19.54	57.61 ± 9.09	86.20 ± 13.43	1.54 ± 1.05	1.68 ± 1.00
	Trang	8 (5)	62.5	1.75 ± 3.80	56.05 ± 9.26	85.30 ± 14.38	2.22 ± 1.41	2.63 ± 1.18
	Satun	12 (12)	100	24.08 ± 31.61	59.70 ± 9.86	88.97 ± 14.41	1.62 ± 0.70	1.66 ± 0.98
	Surat Thani	2 (0)	0	NA	71.07 ± 18.75	108.56 ± 33.08	3.11 ± 1.13	1.50 ± 0.71
	Songkhla	10 (3)	30	1.60 ± 1.64	69.03 ± 4.92	103.57 ± 8.58	2.49 ± 0.92	2.10 ± 1.19
	Bana	19 (0)	0	1.05 ± 2.83	62.70 ± 16.24	93.95 ± 24.68	1.95 ± 1.38	1.94 ± 1.02
	Budi	11 (0)	0	0.18 ± 0.60	55.88 ± 13.38	84.76 ± 18.13	2.08 ± 1.35	2.36 ± 1.02
S. paramamosain	Bang Pu	12 (0)	0	0.08 ± 0.28	60.28 ± 11.16	90.63 ± 16.73	2.19 ± 1.24	2.41 ± 1.16
Overall		103 (20)	19.41	7.23 ± 16.92	60.17 ± 11.77	90.37 ± 17.61	1.93 ± 1.18	2.00 ± 1.07
	Ranong	3 (0)	0	NA	44.82 ± 0.48	66.38 ± 1.30	0.56 ± 0.13	1.00 ± 0.00
	Trang	3 (1)	33.3	1.00 ± 1.73	65.85 ± 7.08	100.31 ± 11.64	3.25 ± 1.53	2.33 ± 0.57
	Satun	3 (3)	100	86.67 ± 132.85	54.34 ± 1.85	80.07 ± 2.00	1.45 ± 0.37	2.33 ± 1.53
	Surat Thani	1 (0)	0	NA	61.10 ± NA	95.24 ± NA	1.97 ± NA	2.00 ± NA
	Songkhla	5 (2)	40	5.20 ± 7.04	59.81 ± 14.19	102.41 ± 9.65	2.23 ± 0.27	2.40 ± 1.34
	Bana	3 (2)	66.7	12.00 ± 19.92	62.99 ± 7.27	96.13 ± 9.23	3.54 ± 2.11	3.00 ± 1.73
	Budi	4 (0)	0	NA	53.57 ± 9.05	79.06 ± 14.27	1.06 ± 0.69	2.00 ± 1.41
S. tranquebarica	Bang Pu	2 (0)	0	NA	64.22 ± 6.11	98.44 ± 8.12	3.05 ± 1.22	2.50 ± 2.12
Overall		24 (8)	33.33	13.54 ± 48.90	57.79 ± 10.01	89.55 ± 15.21	2.08 ± 1.35	2.21 ± 1.25

and *S. paramamosain*) from Trang province had the highest mean FI (2.7 ± 1.2) compared to crabs from all other sites (range between 1.5 and 2.4; see Table 1). For *S. tranquebarica*, Bana (in Pattani province) had the highest FI (3.0 ± 1.7). The FI of crabs did not differ between species ($p > 0.05$) but significantly differed between sites ($p < 0.001$; Table 2). Tukey HSD test indicated that FI of *S. olivacea* crabs was lower in Ranong, Surat Thani and Bana than other sites. For *S. paramamosain*, there was significant mean FI between Ranong and Trang sites. For *S. tranquebarica*, their FI in Ranong was lower compared to other sites.

Plastic particles were discovered in all three species of *Scylla* sampled. Of the 1622 crabs with contents in their stomachs, 358 crabs (22.1 %) were found to have ingested plastic particles (Table 1). Within each species, plastic particles were present in 22.1 % of *S. olivacea*, 19.4 % from *S. paramamosain* and 33.3 % of *S. tranquebarica*. The average number of plastic particles in individual crabs was 2.3 items in *S. olivacea*, 7.2 items in *S. paramamosain* and 13.5 items in *S. tranquebarica*. The most plastic particles were recovered from crabs collected in Satun— 6.9 items/individual in *S. olivacea*, 24.1 items/individual in *S. paramamosain* and 86.7 items/individuals in *S. tranquebarica* (Table 3 and Fig. 4). Conversely, the least plastic particles were recovered from crabs from Pattani (Bana, Budi, and Bang Pu), and ranged 0.1–12 items/individual.

Plastic particles recovered were not normally distributed between species and sites (Kruskal-Wallis test, $p < 0.001$). Correlation analyses between plastic particles and variables CW, CL, stomach weight and FI for *S. tranquebarica* were negative whilst plastic particles and variables CL and CW for *S. olivacea* and *S. paramamosain* were positive (Fig. 5). Based on the plastic particles counts between samples, there were significant differences between species and sites (ANOVA, $p < 0.01$). An average of 11.7 more plastic particles were recovered from *S. tranquebarica* than *S. olivacea* and S. *paramamosain* ($p = 0.043$). Crabs from Ranong, Satun and Songkhla significantly have ingested a ($p < 0.001$) higher number of plastic debris than crabs from Pattani. Ranong, Satun and Songkhla are areas with high plastic load ($p < 0.05$) as 5.9, 6.7 and 2.7 plastic particles per individual respectively were recovered. Among these three sites, crabs from Satun had ingested more plastic particles than other areas. By these two parameters (species and sites) simulated by multiple linear regression model, *S. paramamosain* from Satun will ingest marine debris by 16.3 items/individual. Meanwhile, *S. tranquebarica* will cause 67.9 items/individuals ($p < 0.05$) in that site. Overall, the multiple R-squared value of 0.208 indicates that 2 % of the variation in the data is explained by this model.

3.3. Plastic material recovered from stomach of crab samples

3.3.1. Sizes of plastic particles

Abundance and particle size composition of plastic recovered from sampled crabs are shown in Fig. 6. Microplastic particles (< 5 mm) accounted for the majority of sizes recovered from all three species — 80.3 % in *S. olivacea*, 76.2 % in *S. paramamosain* and 72.7 % in *S. tranquebarica*. Mesoplastic particles (5–25 mm) were a distant second among plastic particle sizes recovered from all three species — 19.4 % in *S. olivacea*, 22.2 % in *S. paramamosain* and 18.2 % in *S. tranquebarica*. The least number of plastics were the macroplastic (>25 mm), contributing minimally, and only from Songkhla, Ranong and Bana —0.3 % in *S. paramamosain*, 1.6 % in *S. olivacea*, and 9.1 % in *S. tranquebarica* (Fig. 6a-d).

Table 2
Results of ANOAVA on fullness index and number of plastic particles of crabs by species and site. DF = degrees of freedom, SS = sum of squares, MS = mean sum of squares.

	Factor	DF	SS	MS	F value	*P* value
Fullness index of crabs	Species	2	1.95	0.97	0.81	0.44
	Site	7	172.44	24.63	20.67	<0.01
	Species x Site	14	15.21	1.08	0.91	0.55
Plastic particles in crabs	Species	2	8.03	4.01	29.93	<0.001
	Site	7	53.14	7.59	56.72	<0.001
	Species x Site	14	8.26	0.59	4.41	<0.001

3.3.2. Type and colour of plastic particles

A total of 4478 pieces of anthropogenic debris were recovered from the stomachs of 1622 crabs. ATR- FTIR analyses revealed eight polymer types and nine colours (Fig. 7). The component materials of these debris were polyethylene (PE), polypropylene (PP), low-density polyethylene (LDPE), nylon, acrylic, coconut fibre, natural rubber, and sheep wool. Among polymer types, PE was the most abundant in the mud crabs: *Scylla olivacea* (88.6 %), *S. paramamosain* (88.4 %) and *S. tranquebarica* (71.4 %), shown in Table 3 and Fig. 7a. Most of the plastic debris were secondary debris that contributed 88.5 % of crab samples; the majority were PE fibres from the breakdown of fishing nets (Fig. 2).

Nine distinct coloured plastic particles were recovered from the stomach of *S. olivacea,* six from *S. paramamosain*, and three from *S. tranquebarica*. Green plastic debris composed of PE polymer, were the most abundant (61.2 %) in *S. olivacea and S. tranquebarica*, but blue PE plastic debris were dominant (31.9 %) in *S. paramamosain* (Table 4). Green plastic particles made up 60.3 % of total plastic debris recovered from all crab samples, black made up 22.1 %, yellow 7.6 %, blue 5.7 %, white 2.1 %, and pink 0.9 % (Table 4). Green and blue PE particles were highest in total plastic composition (90.9 % and 25.1 %) at Ranong and Songkhla, respectively but black dominated the composition in Satun (50.3 %). Pink polypropylene pieces were only recovered from samples originating from Ranong and Songkhla provinces. Polymer types were characterized by μATR-FTIR technique (Fig. 8).

4. Discussion

Thailand ranks 12th in global fishery production (Department of Fisheries, 2022), and the mud crab is a vital component of this fishery (Nooseng, 2015). Overall, the marine capture production of Thailand, including mud crabs, was 1.5 million tons in 2020, (FAO, 2022). The presence of marine debris was investigated in the stomachs of three mud crab species (*Scylla olivacea*, *S. paramamosain*, and *S. tranquebarica*) from the Andaman Sea and in the Gulf of Thailand. Approximately, 2500 artisanal fishery villages located along coastal areas in Thailand contribute to national fishery outputs (Department of Fisheries, 2022). Plastic debris in aquatic organisms are ingested by humans through the consumption of wild and cultured species of mussels (Farrell and Nelson, 2013; Baroja et al., 2021), oysters (Li et al., 2018), crabs (Watts et al., 2016; Renzi et al., 2020; Villagran et al., 2020; Waddell et al., 2020; Aguirre-Sanchez et al., 2022; McDaid et al., 2023) and fishes (Rochman et al., 2015; Soe et al., 2022; Hajisamae et al., 2022). Our results indicate that some of the anthropogenic debris recovered from crab stomach samples are degraded fishing nets likely to originate from such fishing communities. Their ingested fishing nets particles may also be related to fishing techniques employed at the local or regional levels. For instance, on Satun, fisherfolks wrap bait fish with netting material to increase chances of entanglement before placing them in crab traps as bait (Fig. 2). Similarly, in Brazil, 98 % of marine debris were demonstrated to originate from artisanal fishing activities (Farias et al., 2018). Yet, in India, no correlations were found between microplastic abundance and fishing activities based on sediment samples (Karthik et al., 2018).

Some plastic particles are believed to originate from urban areas (Di and Wang, 2018) and tributaries (Yan et al., 2019) through laundry activities (Napper and Thompson, 2016; Wang et al., 2018; De Falco et al., 2019) as well as commercial and industrial activities from seaports, piers, and domestic zones (Ory et al., 2017; Zaki et al., 2021). Plastic particles from such sources can impact economically important

Table 3
Polymer type of plastic particles in mud crabs. Numbers within parentheses represent the percentage of polymer type.

Species	N	PE	Nylon	PP	Acrylic	Coconut fibre	Natural rubber	LDPE	Sheep wool	Total
Overall	358	3964 (88.50)	346 (7.73)	44 (0.98)	36 (0.80)	28 (0.63)	25 (0.56)	22 (0.49)	13 (0.29)	4478
S. olivacea	330	3860 (88.55)	340 (7.80)	44 (1.01)	35 (0.80)	27 (0.62)	25 (0.57)	15 (0.34)	13 (0.30)	4359
S. paramamosain	20	99 (88.39)	6 (5.36)	0	0	0	0	7 (6.25)	0	112
S. tranquebarica	8	5 (71.43)	0	0	1 (14.29)	1 (14.29)	0	0	0	7
Site										0
Ranong	119	1900 (99.11)	0	4 (0.21)	1 (0.05)	0	0	4 (0.21)	8 (0.42)	1917
Trang	46	199 (81.22)	0	0	18 (7.35)	3 (1.22)	25 (10.2)	0	0	245
Satun	81	1274 (78.35)	340 (20.91)	0	5 (0.31)	2 (0.12)	0	5 (0.31)	0	1626
Surat Thani	13	114 (89.76)	6 (4.72)	0	0	0	0	7 (5.51)	0	127
Songkhla	44	318 (88.58)	0	35 (9.75)	1 (0.28)	2 (0.56)	0	3 (0.84)	0	359
Bana	27	108 (86.40)	0	1 (0.80)	8 (6.4)	7 (5.6)	0	1 (0.8)	0	125
Budi	20	36 (58.06)	0	4 (6.45)	2 (3.23)	14 (22.58)	0	1 (1.61)	5 (8.06)	62
Bang Pu	8	15 (88.24)	0	0	1 (5.88)	0	0	1 (5.88)	0	17

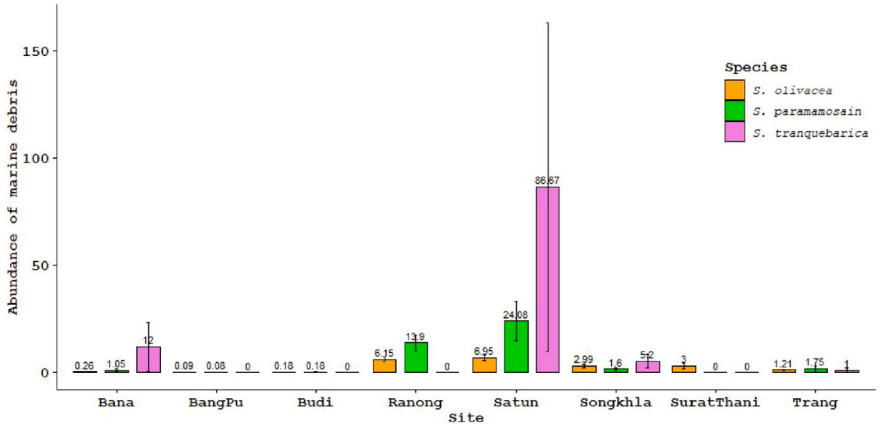

Fig. 4. Abundance of marine debris by species and sites. Number indicating mean value of debris particles.

species (Ivar do Sul and Costa, 2014) such as crustaceans and bivalves (Barboza et al., 2018), and when consumed, raise concerns for human health (Rochman et al., 2015; Li et al., 2015, 2018; Watts et al., 2014; Villagran et al., 2020; Zhang et al., 2021; Ward et al., 2022; Smith et al., 2018). Plastic debris can enter the stomachs of crabs through prey items, such as bivalves and gastropods, and via sediment (Farrell and Nelson, 2013; Watts et al., 2014; Andrady, 2017; Phan et al., 2022). We found marine debris to be present in all three *Scylla* species. Of these three species, *S. tranquebarica* was smallest (89.5 ± 15.2 mm, CW) but had the highest FI (2.2 ± 1.3) and the most abundant debris items/individual (13.5 ± 48.9) (Table 1). However, the average FI did not differ between species ($p > 0.05$), but it was significant between sites.

Altogether, 4478 plastic pieces were recovered in 358 crabs out of 1622 crabs. All three species of crabs recovered from Pattani in the Gulf of Thailand had the highest FI (1.7 ± 0.9 to 3.0 ± 1.7) but less ingested plastic pieces (0.1 to 12 items/individual). Conversely, Satun in the Andaman Sea had lower FI values (1.7 ± 0.9 and 2.4 ± 1.1) but higher abundance of plastic debris (6.9 ± 15.3 and 86.7 ± 132.9 items/individual). It was postulated that the ingested plastic debris by crabs was influenced by the site ($p < 0.001$, Table 2). Also, it has been stated that the ingested plastic particles did not rely on their FI regarding the less correlation value (Fig. 5). There were positive correlations between plastic debris and CW and CL of *S. olivacea,* and *S. tranquebarica,* indicating that larger crabs are capable of ingesting more plastic particles. Conversely, the number of ingested plastic debris is not dependent on size for *S. paramamosain* ($r^2 = -0.17$).

We recovered anthropogenic debris pieces in crabs ranging from 2 mm to 35 mm —the majority (98.5 %) are anthropogenic particles including all types of debris: microplastics (79 %), mesoplastics (20 %) and macroplastics (1 %). We hypothesize that the debris in the stomach of crabs mostly originated from degraded fishing nets and ropes (Fig. 3a-l). We also observed other non-plastic anthropogenic debris such as coconut fibre, natural rubber, and sheep wool that originally were made from organic materials (Fig. 3m-o). Small plastic particles can be introduced into marine animals such as these mud crabs because of their foraging and burrowing behaviors and exacerbated by the high densities of small plastic particles in coastal areas (Jitkaew et al., 2023). The μATR-FTIR analyses indicate that the majority of marine debris recovered from the stomachs of our samples (88.5 %) were PE which are likely to be degraded from fishing nets. The PE and PP particles likely originated from plastic packaging and fishing nets (Claessens et al., 2011; Di and Wang, 2018; Zaki et al., 2021) and cosmetic products (Cole et al., 2011). Our finding corroborates observations of industrial fishing- and domestic-contributed polyethylene plastic materials in the Klang River estuary, Malaysia (Zaki et al., 2021).

Colour is a signal for prey selection in some aquatic animals. Given that plastic pieces are being perceived as prey items, colour is an important parameter in plastic debris studies (Wright et al., 2013). In our study, most of the ingested plastic particles in the stomach of crabs were green and blue PE pieces, likely degraded from monofilament gill nets often used in artisanal and commercial fishing industries along both coasts of Thailand. Green PE fibres made up the majority (60.3 %) of all plastic pieces recovered in the three species of crabs. Blue plastic particles were the most common colour recovered from the gills and stomachs of the burrowing crab *Neohelice granulata*; plastic particles in the stomach were shown to be taken up at the gills during respiration (Villagran et al., 2020). On the other hand, the dominant white, clear, and blue plastic particles recovered might be related to the colour preference of living biota as those colours resemble their prey (Cole et al., 2011; Wright et al., 2013; Syakti et al., 2018). Polymers such as PE

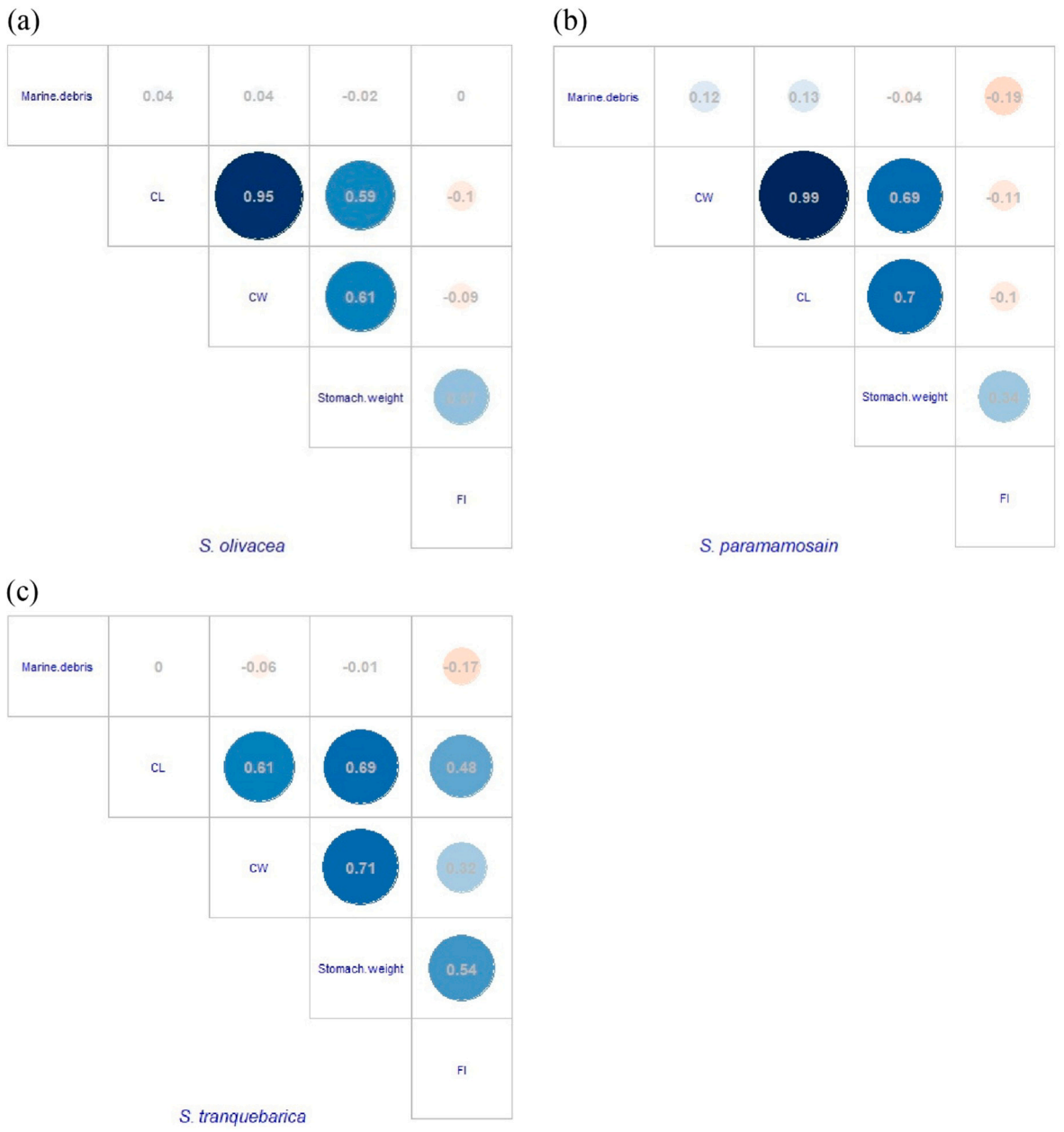

Fig. 5. Correlation plot of marine debris to different variables of carapace length (CL), carapace width (CW), stomach weight and fullness index (FI) of (a) *S. olivacea*, (b) *S. paramamosain* and (c) *S. tranquebarica*.

and PP are transparent fragments that resemble zooplankton, prey items for many marine organisms (Capone et al., 2020). For instance, the FI of European anchovy *Engraulis encrasicolus* (Linnaeus, 1758) in NW Mediterranean Sea, is correlated to the dark plastic fibres ingested (Capone et al., 2020). As congeners, the three mud crab species exhibit remarkably similar foraging strategies, but site-specific plastic availability can explain the differences between sites. The selection of colour between species is difficult to understand, but we hypothesize these to be related to availability at micro-habitat levels.

Knowledge of the effects of plastic particles on marine biota is still evolving; these particles are thought to affect foraging behaviors of crabs due to the tissue damage and false satiation (Watts et al., 2014) as well as malfunction of the digestive system (Brennecke et al., 2015). Burrowing crab (*Neohelice granulata*) and mangrove crab (*Ucides occidentalis*) were reported with higher proportion of microplastic fragments in the gills rather than the digestive tracts (Villagran et al., 2020; Aguirre-Sanchez et al., 2022). In this study, microplastics in the stomachs of wild crabs were significantly higher than those in the gill. The ingestion of plastic particles by crabs is a reflection of their foraging and burrowing habits, but also an indication of high plastic loads in sink areas such as mangrove forests and tidal areas (Villagran et al., 2020; Aguirre-Sanchez et al., 2022) In contrast, crab samples observed by Zhang et al. (2021) gathered from open sea, recovered lower levels of plastic particles through respiration in the gills, but higher in the stomach through ingestion. A study on another species of mangrove crab *U. occidentalis* from Peru discovered that 2–250 μm of plastic fibres and films were commonly found in the gills and digestive tracts of this species (Aguirre-Sanchez et al., 2022).

Our study represents the first investigation on the ingestion of anthropogenic debris in economically and commercially important mud crabs *Scylla* species. Plastic particles were recovered from crab specimens at all study sites. The sites of this study are all fishery areas, and the presence of component plastic particles could be due to breakdown of fishing gear. Our data can be used as evidence for the unintended

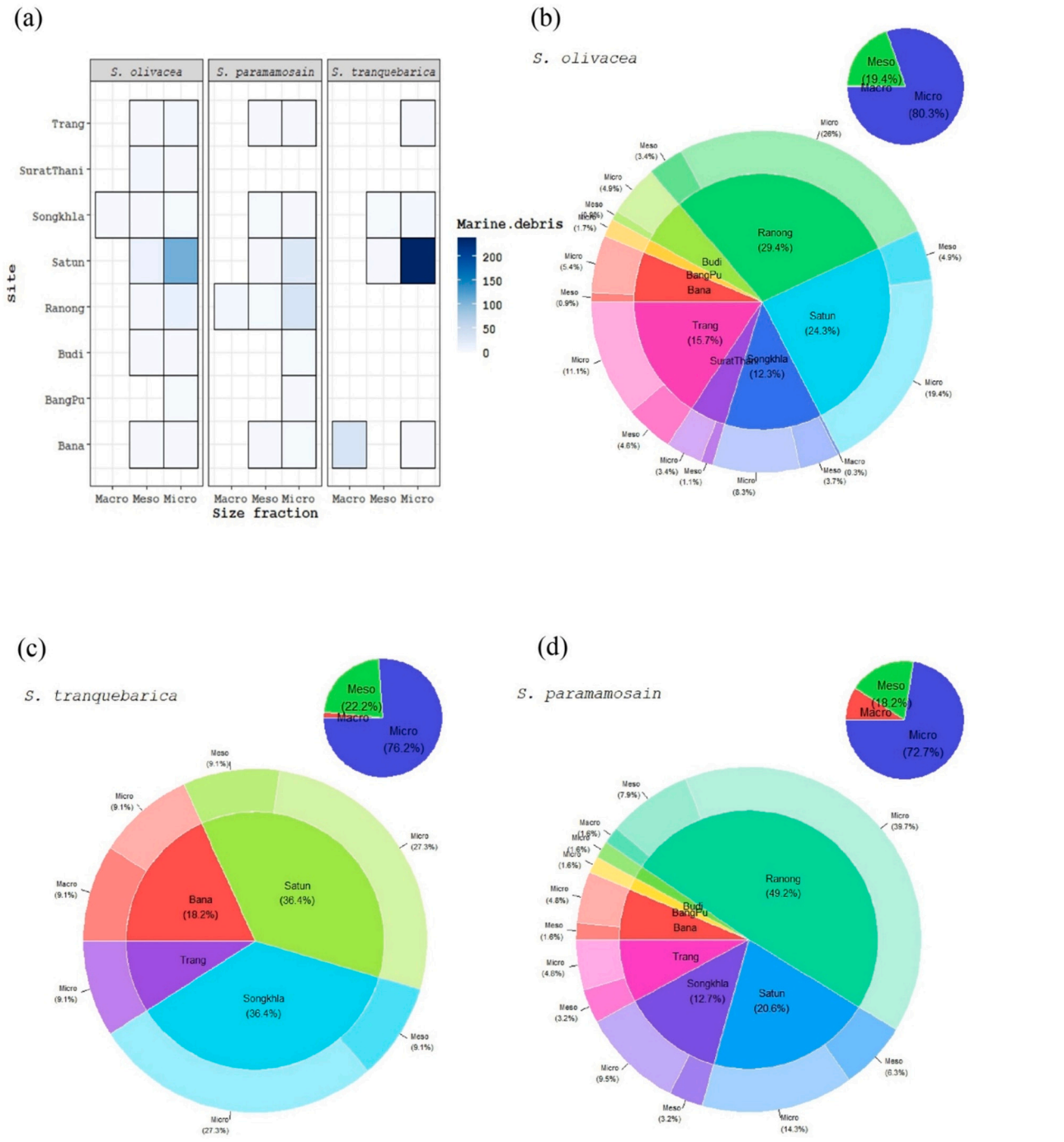

Fig. 6. Distribution of plastic debris and its size fraction of (a) abundance and (b-d) percentage by species and sites.

impacts of fisheries activities to marine biodiversity and habitats. Such data can be co-opted to consider policies and regulations to limit the use of certain plastic material, or implement life spans for fishing gear. Further studies into the ingestion route and translocation of plastic particles from the digestive tract to various organ tissues can further inform food security and safety.

5. Conclusions

Our study is the first to report the presence of plastic debris in the stomach of three mud crab species (*Scylla* spp.), that occur within the territorial waters of Thailand—in the Andaman Sea and Gulf of Thailand. The average number of plastic particles per individual was highest in *S. tranquebarica* followed by *S. paramamosain* and *S. olivacea*. The ingested plastic particles of crabs differed between sites. It was especially highest in Satun province while lowest in Pattani. Our results indicate that Pattani Bay is less anthropogenic debris than other sites; and has a potential as reliable crab fishery production site. Most of the anthropogenic debris recovered from stomach contents of the mud crabs were microplastic particles, most of them were green. These were PE polymers most likely from degraded fishing nets and ropes.

CRediT authorship contribution statement

Kay Khine Soe: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Data curation,

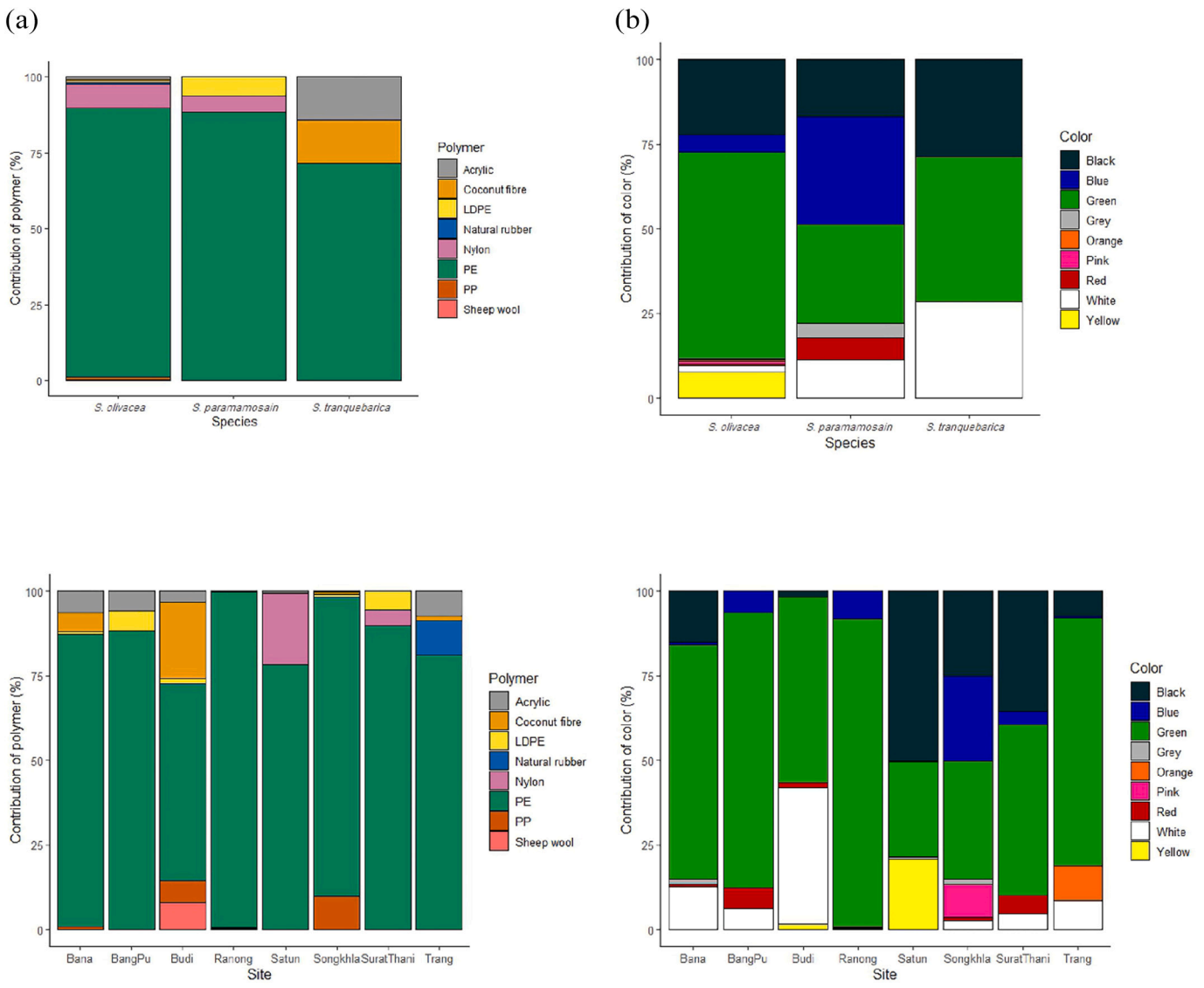

Fig. 7. Contribution of polymer types and colour of plastic particles in the stomachs of mud crabs, (a) species and (b) sites.

Table 4
Plastic particle colour in mud crabs. Numbers within parentheses represent the percentage of colour.

	N	Green (PE, sheep wool)	Black (PE)	Yellow (PE, nylon)	Blue (PE)	White (Acrylic, PE, PP)	Pink (PP)	Orange (Natural rubber)	Red (LDPE, PE)	Grey (PE, PP)	Total
Overall	358	2702 (60.34)	990 (22.11)	341 (7.62)	256 (5.72)	95 (2.12)	39 (0.87)	25 (0.56)	22 (0.49)	8 (0.18)	4478
Species											
S. olivacea	330	2666 (61.17)	969 (22.23)	341 (7.82)	220 (5.05)	80 (1.84)	39 (0.89)	25 (0.57)	15 (0.34)	3 (0.07)	4358
S. paramamosain	20	33 (29.20)	19 (16.81)	0	36 (31.86)	13 (11.50)	0	0	7 (6.19)	5 (4.42)	113
S. tranquebarica	8	3 (42.86)	2 (28.57)	0	0	2 (28.57)	0	0	0	0	7
Site											
Ranong	119	1743(90.92)	0	0	157 (8.19)	9 (0.47)	4 (0.21)	0	4 (0.21)	0	1917
Trang	46	180 (73.47)	18 (7.35)	0	1 (0.41)	21 (8.57)	0	25 (10.20)	0	0	245
Satun	81	456 (28.04)	817 (50.25)	340 (20.91)	1 (0.06)	7 (0.43)	0	0	5 (0.31)	0	1626
Surat Thani	13	64 (50.39)	45 (35.43)	0	5 (3.94)	6 (4.72)	0	0	7 (5.51)	0	127
Songkhla	44	125 (34.82)	90 (25.07)	0	90 (25.07)	10 (2.79)	35 (9.75)	0	3 (0.84)	6 (1.67)	359
Bana	27	87 (69.04)	19 (15.07)	0	1 (0.79)	16 (12.69)	0	0	1 (0.79)	2 (1.60)	126
Budi	20	34 (54.84)	1 (1.61)	1 (1.61)	0	25 (40.32)	0	0	1 (1.61)	0	62
Bang Pu	8	13 (81.25)	0	0	1 (6.25)	1 (6.25)	0	0	1 (6.25)	0	16

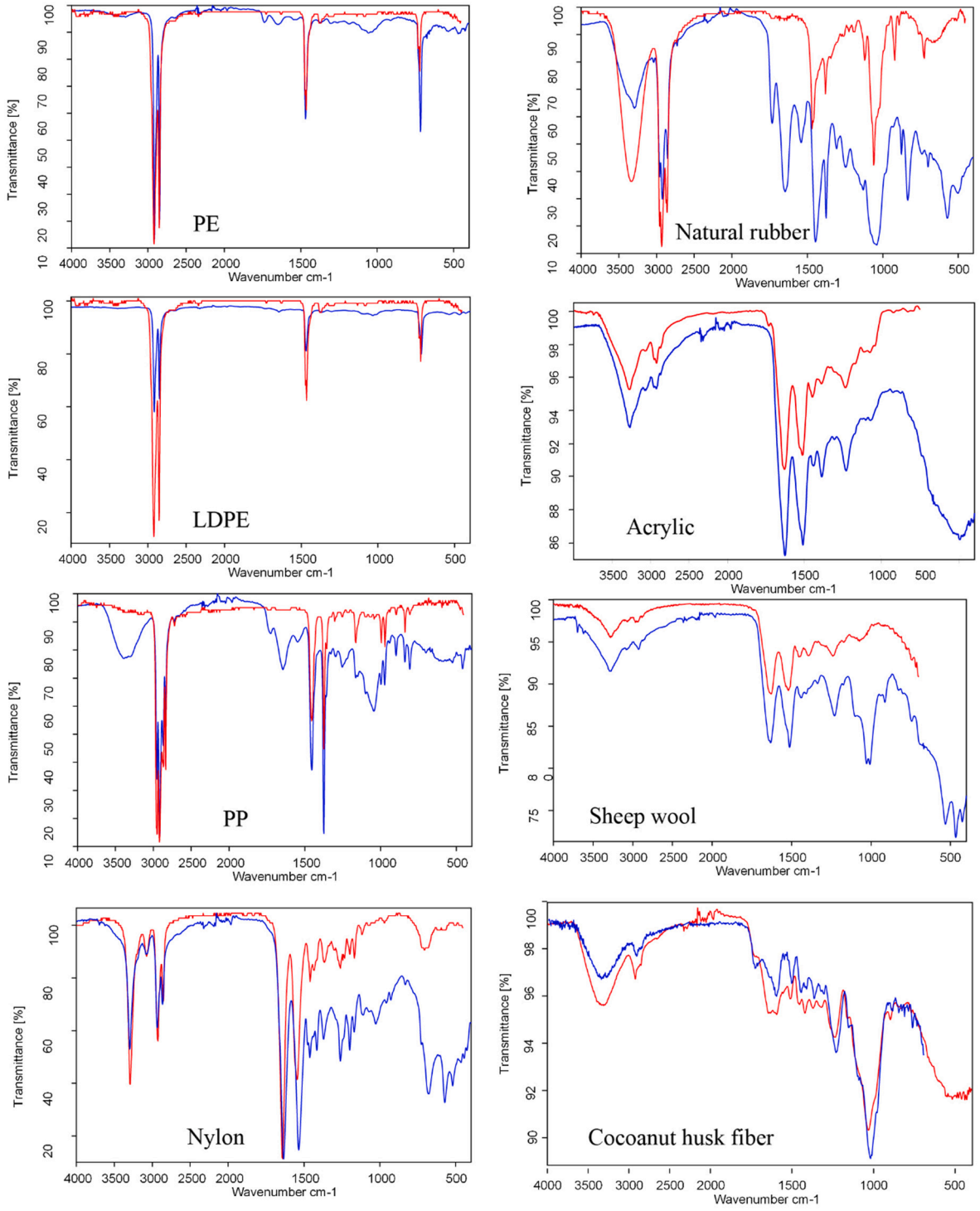

Fig. 8. Characterization of polymer types identified by μATR-FTIR analysis based on the matching percentage greater than 75%. PE = Polyethylene, LDPE = low density PE, PP = Polypropylene, nylon, natural rubber, acrylic, sheep wool and coconut husk fibre. Red line shows a spectrum reference and the blue line represents a marine debris sample of crabs.

Conceptualization. **Sofiyudin Maae:** Writing – original draft, Resources, Methodology, Investigation. **Zeehan Jaafar:** Writing – review & editing, Supervision, Conceptualization. **Pornpimon Chuaduangpui:** Writing – review & editing, Supervision. **Sitthisak Jantarat:** Writing – review & editing, Conceptualization. **Sukree Hajisamae:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

Trace metal accumulations in commercially important fish and crab species from impacted tropical estuary, India: implications on human health risk assessment / Saha, A., Das, B. K., Jana, C., Sarkar, D. J., Sahoo, S., Samanta, S., Kumar, V., Vijaykumar, M. E., Khan, M. F., & Kayal, T.

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ORIGINAL PAPER

Trace metal accumulations in commercially important fish and crab species from impacted tropical estuary, India: implications on human health risk assessment

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Abstract Despite the beneficial role of aquatic food, bioaccumulation of trace metals can increase health risk for consumers. We conducted a comprehensive study to understand the levels of various trace metals (Cd, Co, Cr, Cu, Fe, Ni, Pb, Zn and Mn) in fish (*Nematalosa nasus*, *Gerres filamentosus*, *Arius arius*, *Gerres erythrourus*, *Sardinella fimbriata*, *Caranx ignobilis*, *Etroplus suratensis*, *Mugil cephalus*, *Sillago sihama*, and *Euryglossa orientalis*) and crab (*Portunus pelagicus* and *Scylla serrata)* species collected from Netravathi-Gurupur estuary, India and evaluated the potential health risks to humans by measuring target health hazard (THQ), hazard index (HI), estimated daily (EDI) and weekly (EWI) intake and cancer risk (CR). The hierarchy of toxic metal content in studied species was Fe>Pb>Cr>Mn>Zn>Cu>Ni>Cd>Co. The concentration of heavy metals were distinctly lower than the threshold value as suggested by World Health Organization and Food Safety and Standards Authority of India, except for Cr and Pb in few species. THQ values were below the acceptable limit. However, the estimated mean HI values were >1 for children, indicating they may be vulnerable to health risk due to continuous consumption of contaminated aquatic species from the study area. In contrast, the cancer risk for Cr, Cd, and Pb was below the acceptable range. Principal component analysis (PCA) discerned nearby petrochemical industry, electroplating industry, pesticides and fertilizer from agricultural runoff, as the potential sources of metal bioaccumulation in different tissues. Although the study reveals that metal contamination in aquatic species does not pose any immediate human health effect, continuous monitoring of the study area is recommended, as some metals have demonstrated their ability to accumulate in the tissues.

Keywords Heavy metals · Estuarine fish and crab · Pollution indices · Risk assessment · Netravathi-Gurupur estuary

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Introduction

Estuaries are formed by the mixing of freshwater from rivers and saline water from the sea (Mohammed & Scholz, 2018). Due to this transitional nature,

estuaries are highly productive and provide a habitat for several species, supporting from larva to their entire life cycle (Ahmed et al., 2019). However, with rapid urbanization and industrialization, estuaries are progressively damaged, making them one of Earth's most anthropogenically degraded habitats (Sahu et al., 2023). Trace metals discharged into the estuaries are adsorbed by suspended sediment and eventually settle at the base of the estuary (Yi et al., 2017). Since coastal-estuarine sediment acts as sink for trace metals, different living organisms thriving in this environment can serve as excellent indicators of metal pollution due to their bioaccumulative potential (Patchaiyappan et al., 2023). These trace metals can transfer to humans through the food chain and pose health hazards. While some heavy metals are essential for various metabolic functions in organisms, their excess amount may damage human organs such as kidney, liver, nervous system, reproductive system, and digestive tract due to the consumption of metal contaminated aquatic food (Perumal et al., 2023). Metals like Cd and Pb are classified as non-essential and potential toxic elements.

Trace metals are detected in a wide range of organisms in aquatic ecosystems, including macrophytes, plankton, seafood mussels, and bivalves (Nabi, 2021; Bulut, 2023; Stankovic et al., 2012; Yabanlı et al., 2015). However, fish have historically been the primary models for assessing the effects of potential contaminants in the environment. Fish can be used as bio-indicator for trace metal pollution in coastal-estuarine ecosystems due to their position at a higher trophic level in the food chain, and metal toxicity can cause physiological and behavioural changes in fish (Al-Mahaqeri, 2015; Xie et al., 2020; Yabanli & Tay, 2021). In addition to fish, crustaceans, particularly crabs, are also used as potential metal pollution indicators as they represent typical sediment-dwelling benthic organism and may be considered distinct aquatic species (Yu et al., 2020; Liu et al., 2020).

Therefore, assessing trace metal pollution in aquatic species from contaminated ecosystems are crucial for evaluating the pollution level and the associated human health risks. Since trace metal contamination and human health risks are emerging global concerns (Ahmed et al., 2019), a simple comparison of trace metal levels with legally permitted allowable limits provided by internal/national (WHO, FAO, FSSAI) organization may not be sufficient to estimate their detrimental health effecs. Thus, multivariate statistical techniques and human health risk approaches are employed to evaluate the risks associated with metal pollution (Ustaoğlu et al., 2024). Statistical techniques like principal component analysis, and Pearson's correlation index is commonly used for source identification of trace metal pollution (Ustaoğlu et al., 2024). Moreover, risk model as formulated by USEPA (2019) has been widely used to determine the non-carcinogenic (THQ and HI) and carcinogenic risks (CR) to human health due to consumption of trace metal contaminated fish (Hossain et al., 2023).

India is one of the most rapidly growing economy in the world, with rapid urbanization and industrialization, making pollution an inevitable problem in the coastal-aquatic environment (Patnaik, 2018). Trace metal pollution has severely affected various Indian estuarine systems and their subsequent bioaccumulation in fish (Karthikeyan et al., 2020). The Netravathi-Gurupur estuary, located in the coastal urbanized city of Mangalore, India, is formed by the confluence of the Netravathi and Gurupur rivers (Saha et al., 2023). This estuary provides active fishery year-round and serves as a breeding ground for several fish species (Saha et al., 2024). However, the number of fish breeding sites in this estuary have been destroyed or reduced due to anthropogenic disturbance (Saha et al., 2024; Sahoo et al., 2024). Thus, assessment of trace metal contamination in fishes from this estuary is essential for evaluating the ecosystem's health and conducting human health risk assessment. Unfortunately, there is limited published research on trace metal pollution in fish and crab sepsis from the Netravathi-Gurupur estuary. Heavy metals enter the fish body primarily by two pathways: direct uptake of water and food through digestive tract and through the gills and muscles as non-dietary exposure (Solgi and and Mirmohammadvali, 2021). Since fish muscle is considered as the main edible portion for human diet, it is the preferred tissue for the assessment of human health risks from consumption of metal contaminated fish (Alam et al., 2023). In contrast, fish gills are the primary site for direct uptake of heavy metals from water and are highly sensitive to changes in the surrounding environment, as gill filaments and lamellae are place for direct and continuous contact with pollutants (Olgunoğlu et al., 2015). Hence, studying metal content in muscle and gill is crucial for

evaluating public health risks related to heavy metal contamination. In a bid to bridge this gap, the present work aimed to determine the concentration of trace metals (Cd, Co, Cr, Cu, Fe, Ni, Pb, Zn, and Mn) in the gill and muscle tissues of some commercially important fish and crab species collected from Netravathi-Gurupur estuary. The study also seeks to compare the metal concentrations with internal/national standards, non-carcinogenic and carcinogenic risk for adults and children consumers in India, and identify the sources of trace metal pollution through multivariate statistical technique.

Materials and methods

Study area

Netravathi-Gurupur estuary (latitude 12º51' N and longitude 74º50' E) is formed by the confluence of the Netravathi and Gurupur rivers and is situated in the highly populated, urbanized and industrialized coastal city of Mangalore in the state of Karnataka, India. Due to this industrialization, harbours, urban development, and tourism activities, this estuary's health status is deteriorating daily (Sahoo et al., 2024). Various small and medium industries, like petroleum and oil refineries, metal industry, caustic soda plants, fertilizer industry, food processing, and manufacturing units, and many more industries are situated along the banks of both rivers, as shown in Fig. 1, contributing to the deteriorating health of this estuary. The estuary supports a multitude of organisms, including commercially important fish and crab species. Therefore, detecting trace metals in these fish and crab is essential for human health, as the population consumes aquatic food from this estuary.

Sample collection

The fish and crabs analyzed were acquired in the post-monsoon period (February-March) from the local fishermen fishing in the Netravathi-Gurupur estuary. The selection of fish and crab species was based on their dominance in the estuary, local consumption, and commercial importance. Samples were collected from two sampling sites within the estuary as shown in Fig. 1, and they were pooled together to represent nearly three individuals of each species, all of similar size, for better representation. Ten estuarine fish species (*Nematalosa nasus*, *Gerres filamentosus*, *Arius arius*, *Gerres erythrourus*, *Sardinella fimbriata*, *Caranx ignobilis*, *Etroplus suratensis*, *Mugil cephalus*, *Sillago sihama*, and *Euryglossa orientalis*) and two crab species (*Portunus pelagicus* and *Scylla serrata)* were collected from the estuary (Fig. 2). The collected species were washed with estuary water,

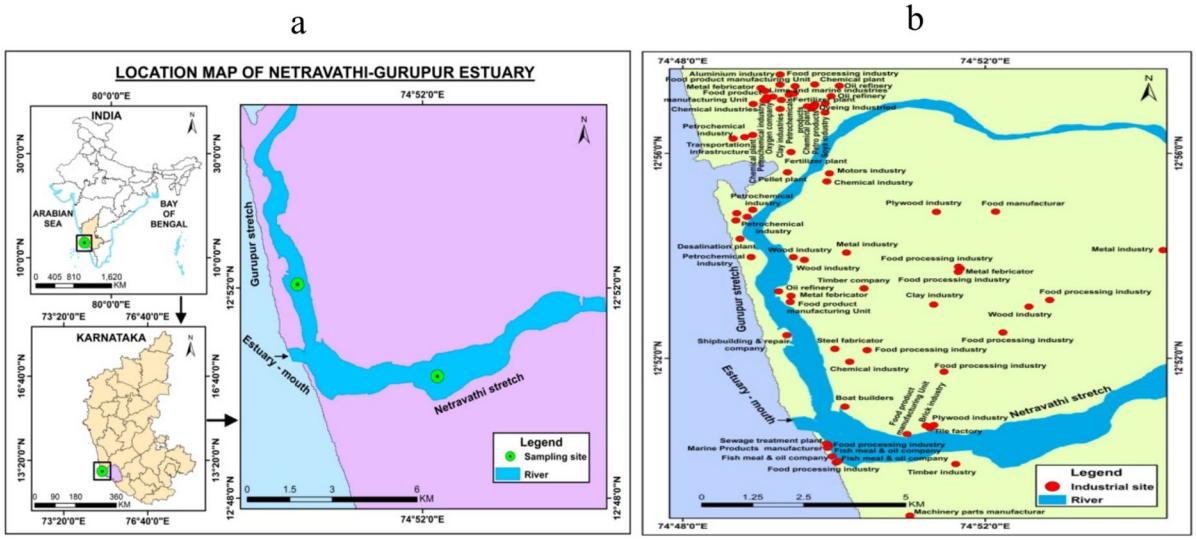

Fig. 1 Study area and location of sampling stations in Netravathi-Gurupur estuary (a) and major industries around the estuary (b)

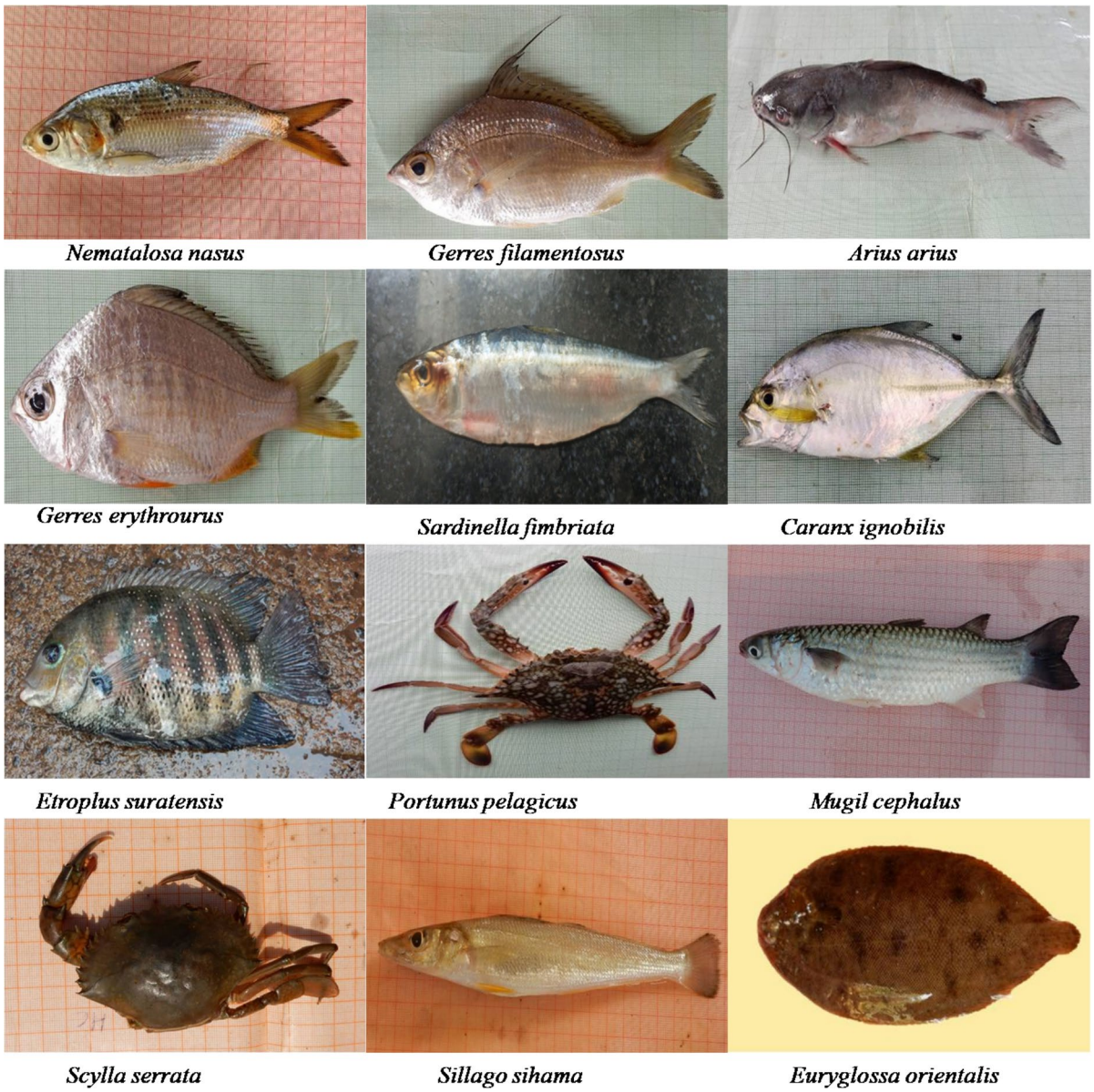

Fig. 2 Photographs showing the fish and crab species

morphologically identified and details about the biometric analysis (weight and length), scientific name, English/common name, habitat, and feeding habits are given in Table 1. Samples were preserved in ice-box, transported to the laboratory, and stored at -20 ºC before analysis.

Sample preparation

Collected samples were de-frosted and cleaned with de-ionized water. Then, the species were dissected with stainless-steel implements to get the tissues [muscle (all species) and gill (for fish species

Table 1 Details of fish and crab sampled, habitat, feeding habits and fishery importance

Sl. No.	Species	Taxonomic group	Trophic guild / feeding niche	Habitat	Ecological importance	Length (cm)	Weight (g)
1.	*Nematalosa nasus*	Gizzard shad	Pelagic	Estuaries and coastal areas	Anadromous, marketed fresh, dried-salted or boiled	18.53 ± 1.14	73.33 ± 15.28
2.	*Gerres filamentosus*	Anchovy	Demersal	Marine, freshwater, brackish	Amphidromous, commercially important	17.93 ± 2.78	90.00 ± 45.83
3.	*Arius arius*	Catfish	Demersal	Marine, brackish	Amphidromous, commercially important	20.77 ± 1.12	83.33 ± 23.09
4.	*Gerres erythrourus*	Anchovy	Demersal	Marine, brackish, reef-associated, oceanodromous, estuaries and coastal lagoons	Minor commercial value	16.73 ± 1.57	86.67 ± 28.87
5.	*Sardinella fimbriata*	Sardine/shad	Pelagic	Coastal water	Marketed fresh, dried-salted, boiled or made into fish balls	14.47 ± 1.67	36.67 ± 11.55
6.	*Caranx ignobilis*	Trevally	Pelagic	Clear lagoon and seaward reefs	Aquaculture, game fish, public aquariums	16.90 ± 0.69	53.33 ± 15.28
7.	*Etroplus suratensis*	Pearl Spot	Benthopelagic	Freshwater and brackishwater, large rivers, reservoirs, lagoons and estuaries	Aquaculture and aquarium	17.00 ± 2.50	108.67 ± 27.23
8.	*Portunus pelagicus*	Crab	Coastal, demersal	Shallow bays with sandy bottoms	Meat consumption	10.47 ± 0.55	53.33 ± 15.28
9.	*Mugil cephalus*	Mullet	Benthopelagic	Marine, freshwater, brackish, catadromous	Marketed fresh, dried, salted, frozen; sold fresh or smoked	24.33 ± 1.53	136.00 ± 18.52
10.	*Scylla serrata*	Crab	Demersal	Estuaries and mangrove swamps, ranging from marine to fresh waters	Meat consumption, highly valued product for the international markets especially chelate legs and meat	12.18 ± 1.74	367.00 ± 119.53
11.	*Sillago sihama*	Sillago	Pelagic	Marine, brackish, reef-associated, amphidromous, beaches, sandbars, mangrove creeks and estuaries	Marketed fresh and frozen, fisheries and aquaculture commercial	25.20 ± 1.73	110.00 ± 26.46
12.	*Euryglossa orientalis*	Sole	Demersal	Marine, freshwater, brackish, anadromous, shallow sand and mud bottoms in coastal waters	Marketed fresh, frozen and dried salted	15.67 ± 2.36	66.67 ± 14.43

only)]. To reduce the surface contamination during dissection, powder-free nitrile gloves were worn. All the implements were washed with 10% HNO_3 followed by Milli Q water after each dissection to avoid cross-contamination. The muscle and gill tissues were blended, homogenized and used for metal contamination analysis.

Sample digestion

For trace metal analysis, 1.0 g of wet weight (muscle and gill) of each species was digested in a hot plate (130–200 °C) with an acid mixture of HNO_3:$HClO_4$:H_2SO_4:10:4:1; v/v. After completion of digestion, samples were cooled to room temperature, diluted with Mili Q water, filtered, and anlysed in inductively coupled plasma mass spectrometry (ICP-MS). Analytical blank was also prepared in the same manner. Metal content in different organs of studied species were expressed as mg/kg wet weight.

Metal analysis through ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) (NexION1000, PerkinElmer, USA) was used to determine the metal residues in tissues. The ICP-MS was used in combination with a Meinhart nebulizer and cyclonic spray chamber. Nebulizer gas flow, plasma argon flow, and auxiliary gas flow rates were 15, 1.5, 2 L min^{-1}, respectively. Radio frequency (RF) was set at 1600 W with a deflector voltage of 20 V.

Data analysis through multiple risk assessment approach

In this study, the degree of metal pollution in the Netravathi-Gurupur estuary was estimated by computing the metal pollution index (MPI) and bioaccumulation factor. Additionally, the estimated daily intake (EDI), estimated weekly intake (EWI), target hazard quotient (THQ), hazard index (HI), and carcinogenic risk (CR) were calculated as recommended by USEPA to evaluate the potential non-carcinogenic and carcinogenic risks associated with the consumption of the studied fish and crab species.

Pollution index

To determine the level of trace metal pollution in the estuary, the metal pollution index (MPI) (Usero et al., 1997) was calculated by multiplication of studied metal concentration (mg/kg) in the studied species and subsequently n^{th} root obtained by using the following equation.

$$MPI = (C_1 \times C_2 \times C_3 \times \ldots \times C_n)^{1/n}$$

Here, C_{i-n} is the concentration of each metal (mg/kg) in fish tissues. Based on MPI values, metal contamination level can be categorized as not-impacted (<2 MPI), very low contamination (2<MPI<5), low contamination (5<MPI<10), medium contamination (10<MPI<20), high contamination (20<MPI<50), very high contamination (50<MPI<100), and extremely high contamination (MPI>100) (Jamil et al., 2014).

Bioaccumulation factor

Bioaccumulation factor (BAF) represents the level of metal contamination and its accumulation in the studied species over time (Ahmed et al., 2019). It is the ratio of metal present in fish tissue (mg/kg) and surrounding estuarine water (mg/l) and is calculated by the following equation

$$BAF = \left[\frac{C_f}{C_w}\right]$$

here, C_f and C_w is the metal in fish and estuarine water, respectively.

According to Arnot and Gobas (2006), metals are categorized as extremely bioaccumulation when BAF >5000, bio accumulation when 1000<BAF<5000, and no probability of accumulation when BAF is <1000

Risk assessment of trace metal contamination

Accumulation of trace metal through consumption of contaminated fish and crab species may pose an irreparable threat to humans and present risks to biota and human health (Khan et al., 2023). In the present investigation, a multiple risk assessment approach, recommended by the USEPA was used to categorize the probable exposure to metal

contamination when humans consume contaminated fish (El-Said et al., 2021).

Estimated daily (EDI) and estimated weekly (EWI) intake

EDI (µg/kg BW/day) was calculated by using the following equation

$$EDI = \frac{C \times WF}{WAB}$$

EWI (µg/kg BW/week) = EDI x 7 days.

Here, C is the trace metal concentration (mg/kg) in fish and crab muscle (Kumar et al., 2023); WF is the daily average consumption of fish, which is generally 0.027 kg/person/day, WAB is the average body weight of an Indian which was taken as 30 kg and 60 kg for children and adult, respectively.

Calculation of target hazard quotient (THQ)

THQ is used to determine the non-carcinogenic health risk through exposure to metals and calculated by using the following equation (USEPA, 2011)

$$THQ = \frac{EDI}{RfD}$$

Here, RfD is the oral reference dose of different trace metals (µg/kg BW/day) [(Cd, 1.0; Co, 30.0; Cr, 30.0; Cu, 40.0; Fe, 700.0; Ni, 11.0; Pb, 4.0; Zn, 300.0; Mn, 140.0 (US EPA, 2000)]. If THQ values are less than 1, it indicates less likely to show any adverse effects due to trace metal exposure to humans, whereas value>1 indicate the possible health risk.

The THQ of each metal is added together to create a new index named hazard index (HI), which is used to determine the health risk due to multi-element exposure of trace metals at a time.

$$\mathrm{HI} = \sum_{i=1}^{n} THQ_i$$

Carcinogenic risk (CR)

Following equation was used to calculate the CR values

$$CR = EDI \times CSF$$

Here, CSF is the oral cancer slope factor (mg/kg/day), which is 0.0085, 6.3, and 0.5 for Pb, Cd, and Cr, respectively (Kaçar, 2024). For other metals the CSF values have yet not been established (USEPA, 2022). Carcinogenic risk is categorized as negligible, acceptable, and unacceptable when the CR value is < 10^{-6} and 10^{-6} to 10^{-4} and > 10^{-4}, respectively.

Statistical analysis

Data were statistically analyzed by using software like SPSS (version 21.0) and R (R Core Team 2019). Metal concentration in each species is expressed as mean ± SD. One-way ANOVA was used to understand the extent of variation in the concentration of heavy metals among different studied species and organs. Multivariate statistical techniques like principal component analysis and Pearson's correlation analysis were carried out to determine the sources identification of trace metal pollution.

Quality assurance/quality control

To get accurate and precise data, quality control and quality assurance protocols were maintained during the analysis of trace metals. Several aspects, viz. preparation of six point calibration curve, preparation of reagent blank, spiked sample analysis, mid-point standard check was done regularly. External quality of analysis were maintained by the specialist analyst, with appropriate management of chemicals and reagents, glassware, sample preparation, instrument etc. Trace metal grade nitric acid (HNO_3, 67-69%), perchloric acid ($HClO_4$, 65-71%) and sulphuric acid (H_2SO_4, 93-98%) as supplied by Fisher Scientific UK Limited were used during analysis. All the glassware and plasticware used in the study were kept in 10% HNO_3 for two days, followed by rinsing with ultrapure water and drying in the oven. ICP-multielement standard solution IV (Certipur®) Merck KGaA, Darmstadt, Germany, was used to prepare metal standards and working standards. Internal quality was checked by regular, constant, and precise monitoring of observed data with accurate statistical and validation method. The limit of detection (LOD; µg/L) and limit of quantification (LOQ; µg/L) were as follows: 0.035 and 0.107 for Cd, 0.028 and 0.085 for Co, 0.060 and 0.182 for Cr, 0.032 and 0.099 for Cu, 1.42 and 4.31 for Fe, 0.017 and 0.052 for Ni, 0.002 and 0.006 for Pb, 0.071 and 0.216 for Zn, and 0.042 and 0.128 for Mn, respectively. The certified reference material of fish muscle (ERM BB422) was obtained from the Joint Research Centre (JRC) Institute for Reference Materials and Measurement (IRMM),

Belgium, to confirm analytical performance (Das et al., 2024). The precision was confirmed through the relative standard deviation (RSD%). The accuracy was obtained by calculating the recovery rate. Metal recovery ranged from 75.2 to 107.0% with RSD<10% (Table S1) which followed the EU requirements (EC/SANTE/11813/2017, 2017).

Results and discussion

Heavy metal accumulations varied significantly among the fish species and between the muscle and gill tissue (Tables 2 and 3). The values obtained in this study were also compared with national and international regulatory limits (Table 4) as well as previous reports of metal contamination in fish from other studied regions (Table 5) and studied fish species from different coastal-estuarine regions of India and other regions of the world (Table 6). The hierarchy of toxic metals (mg/kg) in various species showed as Fe (12.83±6.30)>Pb (3.19±1.86)>Cr (1.18±0.51)> Mn (0.61±0.33)>Zn (0.52±1.13)>Cu (0.19±0.34)>Ni (0.14±0.08)>Cd (0.08±0.07)>Co (0.005±0.01) in muscle and Fe (70.17±24.91)>Pb (3.19±1.21)>Cr (1.61±0.97)>Mn (0.91±0.34)>Zn (0.32±0.37)>Ni (0.17±0.16)>Cu (0.02±0.02)>Cd (0.015±0.01)>Co (0.012±0.01) in gill.

Iron (Fe) is important for human health unless it exceeds the level. Fe is a predominant element both in crustal environment and industrial discharge; therefore, it is frequently detected in coastal environments. In fish, excess Fe may cause gill clogging and respiratory trouble (Dalzell & Macfarlane, 1999). Among the nine trace metals studied, Fe concentration was highest, and sequence of Fe in the muscle of the studied species can be ordered as *E. orientalis*>*G. erythrourus*>*S. fimbriata*>*N. nasus*>*S. serrata*>*G. filamentosus*>*S. sihama*>*E. suratensis*>*A. arius*>*P. pelagicus*>*C. ignobilis*>*M. cephalus*, while in gill the sequence was as *C. ignobilis*>*G. filamentosus*>*A. arius*>*E. suratensis*>*S. fimbriata*>*M. cephalus*>*G. erythrourus*>*N. nasus*>*E. orientalis*>*S. sihama*. In all the fish species, Fe content was higher in the gills than in the muscle, as the gills serve as the main route for metal exchange from water. The larger surface area of the gills facilitates rapid metal diffusion, leading to greater metal accumulation in the gills (Uysal, 2011). Fe concentration in *M. cephalus* was lower than in samples collected from Chilika lagoon, India (17.56 mg/kg) (Parida et al., 2017) but higher than in samples from the coasts of Tanzania (1.79 mg/kg) (Mwakalapa et al., 2019). However, lower Fe levels as compared to the present study were reported for *E. suratensis* from Ennore creek, India (4.35 mg/kg; Jayaprakash et al., 2015), *S. serrata* from Pulicat lake, India (0.21–1.5 mg/kg; Batvari et al., 2016) and *S. sihama* collected from Southeast coast, India (1.58–4.61 mg/kg; Perumal et al., 2023). The mean Fe content was comparatively higher than the value reported for *N. nasus* from Parangipettai Coast, India (39.6,71.5 mg/kg; Raja et al., 2009); *A. arius* from Cochin Backwaters, Kerala, India (266.1–720.0 mg/kg; Jyothirmaye et al., 2022), *E. suratensis* from Cochin Backwaters, India (190.4-712.5 mgkg; Jyothirmaye et al., 2022)and *P. pelagicus* from NW of Arabian Gulf, South Iraq (45.24–68.62 mgkg; Al-Khafaji et al., 2018). The maximum allowable limit of Fe in fish is 100 mg/kg (WHO, 2000) and in our study, the Fe content in all the species did not exceed this limit. Waste from different industries such as fertilizer plants, oil refineries, and municipal and domestic wastewater may be discharged directly or indirectly into this estuary, which could be a significant source ofFe in the Netravathi-Gurupur estuary (Saha et al., 2024). The Mn concentration was highest in the muscle of *S. serrata* (1.22 mg/kg), while the lowest concentration of Mn (0.306 mg/kg) was found in the muscle of *C. ignobilis*. However, the mean concentration of Mn was significantly higher in the gill (0.907 ± 0.18 mg/kg) compared to the muscle (0.606±0.28 mg/kg). Fishes showed significant variation in muscle Mn concentration, while variation in gill Mn concentration was non-significant. Mn concentrations in all the species in this study were lower than the values reported from several other studies (Sivaperumal et al., 2007; Mohapatra et al., 2009; Lakshman et al., 2015; Kumar et al., 2019, 2021). However, the values of Mn in this study are comparable for fishes like *E. suratensis* (0.424 mg/kg) and *S. sihama* (0.4 mg/kg) collected from Ennore creek, India (Jayaprakash et al., 2015); *M. cephalus* from the Gaza strip (0.834 mg/kg; Elnabris et al., 2013) and *E. orientalis* from North Persian Gulf (0.06–1.23 mg/kg; Soltani et al., 2021). The detected values (100 mg/kg) of Mn in this study were lower than the suggestive guidelines of FAO (FAO, 1989).

Table 2 Concentration of trace metals in different organs of fish and crab species collected from Netravathi-Gurupur estuary

Species	Body part/organ	Concentration (mg/kg)								
		Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn
Nematalosa nasus	Muscle	0.072 ± 0.003	BDL	1.088 ± 0.724	0.014 ± 0.009	15.450 ± 5.056	0.100 ± 0.032	2.651 ± 0.564	0.014 ± 0.004	0.485 ± 0.122
	Gill	0.017 ± 0.007	BDL	1.524 ± 0.360	0.010 ± 0.009	58.162 ± 35.006	00.093 ± 0.026	1.979 ± 0.167	0.095 ± 0.015	0.991 ± 0.163
Gerres filamentosus	Muscle	0.158 ± 0.021	0.010 ± 0.004	0.890 ± 0.070	0.044 ± 0.012	13.287 ± 5.724	0.101 ± 0.055	5.030 ± 1.499	0.398 ± 0.040	0.625 ± 0.234
	Gill	0.014 ± 0.007	0.021 ± 0.009	1.405 ± 0.254	0.022 ± 0.007	92.367 ± 8.993	0.074 ± 0.076	1.680 ± 0.565	0.016 ± 0.005	0.693 ± 0.111
Arius arius	Muscle	0.090 ± 0.027	0.007 ± 0.006	1.642 ± 0.215	0.010 ± 0.001	9.596 ± 8.999	0.235 ± 0.069	4.349 ± 2.109	0.077 ± 0.043	0.401 ± 0.018
	Gill	0.010 ± 0.003	0.013 ± 0.013	0.537 ± 0.034	0.017 ± 0.005	89.306 ± 20.614	BDL	5.077 ± 1.742	1.108 ± 0.302	0.873 ± 0.042
Gerres erythrourus	Muscle	0.110 ± 0.040	0.007 ± 0.012	0.345 ± 0.053	0.029 ± 0.003	19.670 ± 1.684	BDL	6.263 ± 2.552	0.385 ± 0.149	0.855 ± 0.344
	Gill	BDL	0.009 ± 0.016	0.810 ± 0.076	0.026 ± 0.008	60.557 ± 20.485	0.050 ± 0.056	2.205 ± 1.003	0.008 ± 0.007	1.032 ± 0.176
Sardinella fimbriata	Muscle	0.139 ± 0.065	0.009 ± 0.008	0.759 ± 0.124	0.076 ± 0.020	17.170 ± 3.939	0.098 ± 0.083	5.533 ± 1.204	0.400 ± 0.089	0.869 ± 0.052
	Gill	0.011 ± 0.003	0.016 ± 0.006	0.824 ± 0.046	0.017 ± 0.008	79.457 ± 17.088	0.019 ± 0.018	4.300 ± 0.361	0.672 ± 0.224	1.205 ± 0.499
Caranx ignobilis	Muscle	0.040 ± 0.020	BDL	1.467 ± 0.331	0.043 ± 0.022	5.783 ± 2.177	0.154 ± 0.081	2.064 ± 0.752	0.030 ± 0.020	0.306 ± 0.125
	Gill	0.018 ± 0.011	BDL	2.270 ± 0.14	0.011 ± 0.007	92.766 ± 20.719	0.229 ± 0.062	3.177 ± 0.172	0.032 ± 0.000	0.769 ± 0.300
Etroplus suratensis	Muscle	0.024 ± 0.007	BDL	1.241 ± 0.071	0.389 ± 0.180	11.267 ± 6.214	0.171 ± 0.061	2.645 ± 0.885	0.133 ± 0.057	0.501 ± 0.445
	Gill	BDL	0.018 ± 0.003	2.335 ± 1.058	0.032 ± 0.007	81.320 ± 20.385	0.249 ± 0.034	3.513 ± 1.241	0.538 ± 0.314	0.894 ± 0.300
Portunus pelagicus	Muscle	0.023 ± 0.000	0.006 ± 0.005	1.078 ± 0.907	0.147 ± 0.076	9.495 ± 4.653	0.119 ± 0.058	1.876 ± 0.221	0.118 ± 0.010	0.310 ± 0.129
Mugil cephalus	Muscle	0.024 ± 0.009	BDL	0.980 ± 0.118	0.024 ± 0.014	5.262 ± 0.366	0.132 ± 0.032	1.740 ± 0.269	0.117 ± 0.100	0.333 ± 0.041
	Gill	0.017 ± 0.008	0.015 ± 0.005	3.391 ± 0.687	0.063 ± 0.023	67.611 ± 5.216	0.370 ± 0.142	3.731 ± 0.366	0.316 ± 0.101	1.088 ± 0.880
Scylla serrata	Muscle	0.230 ± 0.084	0.012 ± 0.010	1.632 ± 0.180	1.198 ± 0.180	14.012 ± 6.740	0.230 ± 0.076	2.126 ± 0.226	3.839 ± 1.812	1.220 ± 0.412
Sillago sihama	Muscle	0.060 ± 0.024	0.005 ± 0.009	1.249 ± 0.205	0.257 ± 0.089	12.500 ± 4.397	0.168 ± 0.059	1.961 ± 0.149	0.736 ± 0.339	0.651 ± 0.149
	Gill	0.034 ± 0.021	0.013 ± 0.011	1.176 ± 1.198	0.036 ± 0.021	38.863 ± 14.264	0.427 ± 0.126	3.133 ± 0.153	0.293 ± 0.072	0.887 ± 0.016
Euryglossa orientalis	Muscle	0.024 ± 0.003	BDL	1.775 ± 0.327	0.023 ± 0.011	20.462 ± 3.487	0.211 ± 0.014	2.023 ± 0.069	0.015 ± 0.013	0.721 ± 0.231
	Gill	0.028 ± 0.016	0.012 ± 0.000	1.849 ± 0.355	0.007 ± 0.012	41.259 ± 4.832	0.229 ± 0.026	3.117 ± 0.115	0.079 ± 0.038	0.644 ± 0.046

BDL: below detection limit

Table 3 One-way ANOVA among metal accumulations observed in different species and organs in the fishes and crabs collected from Netravathi–Gurupur estuary

Trace metals	Between gill and muscle (*P* value)	Between fish (gill) (*P* value)	Between fish (muscle) (*P* value)
Cd	1.66e-07 ***	0.0109 *	1.25e-06 ***
Co	0.000432 ***	0.0739	0.192
Cr	0.0211 *	0.000194 ***	0.00444 **
Cu	0.0091 **	0.000558 ***	5.59e-14 ***
Fe	< 2e-16 ***	0.0105 *	0.0147 *
Ni	0.251	1.49e-06 ***	0.00218 **
Pb	0.578	0.000952 ***	0.000159 ***
Zn	0.306	3.99e-07 ***	3.28e-07 ***
Mn	9.36e-05 ***	0.677	0.00178 **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Higher concentrations of Zn in fish tissue can damage gill tissue, induce chronic toxicity, and ultimately lead to fish mortality. Zn contamination in estuarine environments may be due to the galvanization of trawlers and fishing vessels, the use of zinc-based fertilizer which eventually finds their way to estuarine biota (Patchaiyappan et al., 2023). In muscle, the highest and lowest concentrations of Zn were found in *S. serrata* (3.839 mg/kg) *and N. nasus*, respectively (0.014 mg/kg) while in gill the corresponding maximum and minimum values were found in *A. arius* (1.108 mg/kg) and *G. erythrourus* (0.008 mg/kg). A significant difference was observed in Zn concentration among the species and between gill and muscle tissue. However, the values are far lower than those reported for *S. serrata* collected from Mahandi estuary (287.6 mg/kg Mohapatra et al. 2009), lower Gangetic Delta (10.4–239 mg/kg Rudra et al., 2016), and Ashtamudi lake of India (14.38–92.85 mg/kg; Williams et al., 2022). Zn accumulation in *S. sihama* in this study was far lower than values reported for the same species collected from Southeast coast, (1.78–7.26 mg/kg; Perumal et al., 2023) and Ennore creek of India (3.81 mg/kg; Jayaprakash et al., 2015). Zn accumulation in *M. cephalus* (0.117 mg/kg) in this study was comparatively lower than the value observed in the same species collected from the Gaza strip (12.783 mg/kg; Kamal et al., 2013) and Cochin backwaters (41.07-629.75 mg/kg), India (Mohan et al., 2012). Zn accumulation in *M. cephalus* was almost similar to the observed values for the fishes from Vellar and Uppanar estuaries (0.176±0.09 mg/kg), India (Sulieman and Suliman 2019). However, far higher concentration of Zn were reported from the fishes of coastal Borneo, Malaysia (160.5–435 mg/kg; Anandkumar et al., 2019); Gulf of Mannar, India (6.75–65.08 mg/kg; Karunanidhi et al., 2017) and Gulf of California (22.3–67.3 mg/kg; Serviere-Zaragoza et al., 2021). Maximum guidelines for Zn in fish are 30 and 100 mg/kg by WHO (WHO, 1989) and FAO (FAO, 1989), respectively, and therefore, human health risk exposure to Zn is comparatively lower than other elements like Pb, Cr, and Cd. In the current study, all the Zn concentrations were below the permissible limit; hence, this metal concentration does not pose any threat through consumption of studied fish and crab species. Similar to Zn, copper (Cu) concentrations in the studied species were generally quite low; nevertheless, in this study, *S. serrata* had the highest mean concentration in muscle (1.198 mg/kg), while *A. arius* had the lowest (0.010 mg/kg). In gill tissue, the maximum Cu content was found in *M. cephalus* (0.063 mg/kg) and the lowest was in *E. orientalis* (0.007 mg/kg). The mean concentrations of Cu in the studied species were below the guideline value proposed by FAO

Table 4 Standard regulatory limits (µg/g wet weight) of toxic heavy metals in fish muscle according to national and international guideline values

Reference	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn
FAO (1989)	0.5	–		30	–	8.97	0.5	100	100
FSSAI (2011)	0.3	–	12	–	–	–	0.3	–	–
WHO (1989)	0.5	–	1	30	–	–	0.5	30	–
EC (2014)	–	–	–	0.3	–	–	0.3	–	–

FAO, Food and Agriculture Organization; FSSAI, Food Safety and Standards Authority of India; WHO, World Health Organization; EC, European Commission

Table 5 Comparison of trace metal level (mg/kg) in fish and crab species from Netravathi-Gurupur estuary and otherecosystem

Locations	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn	References
Parangipettai coast, India	0.18–0.54	0.05–0.28	0.65–0.92	0.12–0.31	24.1–50.3	0.38–1.54	–	14.1–33.5	0.31–1.20	Raja et al. (2009)
Pulicat lake, India	0.18	–	–	0.76	–	–	3.28	0.32	–	Prabhu Dass Batvari et al. (2013)
Southeast Aegean Sea, Turkey	0.03	–	–	0.17–0.22	–	–	0.10–0.12	4.95–5.04	–	Yabanli and Alparslan (2015)
Ennore creek, India	0.11	0.36	1.67	7.33	6.53	0.44	1.33	4.91	0.43	Jayaprakash et al. (2015)
Chilika lake, India	–	–	0.16–2.65	3.95–11.02	22.29–79.92	–	–	10.01–19.85	–	Parida et al. (2017)
Coastal Borneo, Malaysia	0.25–0.45	2.05–4.15	14.25–16.6	7.5–15.2	–	10.25–12.2	2.3–4.25	160.5–435	41.7-51.95	Anandkumar et al. (2019)
Pulicate Lake, India	0.0824–0.1541	–	0.0753–0.1227	3.19–8.51	–	–	0.0722–0.1504	8.01–12.65	–	Pandion et al. (2022)
Puducherry coast, India	0.02–10.5	–	0.04–1.33	0.32–26.1	–	–	0.02–1.48	1.77–25.03	–	Patchaiyappan et al. (2023)
Tuscany coast	0.001–0.35	–	0.05–5.40	0.17–43.7	–	0.03–6.63	0.001–3.56	2.30–58.5	–	Bonsignore et al. (2018)
Southeast coast, India	0.01–0.29	–	–	0.01–2.44	3.16–44.4	–	0.06–0.6	1.072–7.26	–	Perumal et al. (20230
Gulf of California, Mexico	0.13–0.21	–	–	0.56–1.6	21.69–27.06	–	0.75–1.67	22.3–67.3	–	Serviere-Zaragoza et al. (2021)
Adyar estuary, India	–	0.44	2.07	8.6	7.54	0.58	1.66	4.47	0.52	Rubalingeswari et al. (2021)

Table 6 Comparison of trace metal concentration (mg/kg) in the studied species with earlier studies from different ecosystems for same species

Sl no.	Species	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn	Location	Reference
1.	*Nematalosa nasus*	0.15–0.22	0.25–0.41	0.61–1.52	0.10–0.21	39.6–71.5	0.72–1.83	–	17.6–43.0	0.80–1.50	Parangipettai Coast, India	Raja et al. (2009)
		0.02–0.22	0.2–1.05	9.26–15.98	2.69–7.61	–	–	0.001-3.9	4.08–18.04	3.12–7.9	Fishing harbours, Mumbai, India	Kumar et al. (2021)
2.	*Gerres filamentosus*	0.03–0.04	0.15–0.52	3.24–6.23	1.66–1.83	–	–	0.16–0.35	18.32–21.08	0.68–15.77	Fishing harbours, Mumbai, India	Kumar et al. (2021)
		0.01–0.02	–	–	0.25–0.51	8.39–15.39	–	0.07–0.36	1.7-2.746	–	Southeast coast, India	Perumal et al. (2023)
3.	*Arius arius*	3.5–6.86		19-47.5	9.4–45.2	266.1–720	21.2–31	8.8–17.9	63-166.03	–	Cochin Backwaters, India	Jyothirmaye et al. (2022)
	Arius parkii	0.114	0.324	2.037	8.051	7.818	0.385	1.516	11.353	0.45	Ennore creek, India	Jayaprakash et al. (2015)
	Arius sp.	0.03–0.62	0.21–1.93	2.24–14.33	0.7-19.23	–	–	0.11–2.29	9.04–37.78	0.86–12.78	Fishing harbours, Mumbai, India	Kumar et al. (2021)
	Arius sp.	-	–	0.2–16.5	66.3-148.3	–	–	0.01	2.5–44.1	–	Pulicat Lake, India	Akila et al. (2022)
4.	*Sardinella fimbriata*	2.62 ± 2.26	–	0.05 ± 0.02	21.70 ± 1.26	–	–	1.48 ± 1.05	15.36 ± 0.59	–	Puducherry coast, India	Patchaiyappan et al. (2023)
		0.01–1.76	0.49–7.72	1.87–10.36	4.03–9.47	–	–	0.002–1.47	8.33–29.36	2.65–7.2	Fishing harbours, Mumbai, India	Kumar et al. (2021)
5.	*Caranxsex fasciatus*	BDL-0.04	–	–	–	–	BDL-6	BDL-0.01	-	-	Kendari Region, Indonesia	Saputri et al. (2023)

Table 6 (continued)

Sl no.	Species	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn	Location	Reference
6.	*Etroplus suratensis*	BDl-1.32	BDL-0.67	0.31–0.53	1.62–6.25	–	BDL-0.69	0.27–0.76	6.06–12.3	0.57–1.94	Cochin estuary, India	Sivaperumal et al. (2007)
		–	–	0.51	4.51	20.05	–	–	9.48	–	Chilika Lagoon, India	Parida et al. (2017)
		0.117–0.126	–	0.088–0.099	5.87–6.83	–	–	0.088-0.115	10.78–11.39	–	Pulicate Lake, India	Pandion et al. (2022)
		BDL-14.93	–	–	14.68–42.23	–	–	ND-8.42	21.93–484	–	Cochin backwaters, India	Mohan et al. (2012)
		0.104	0.316	1.499	6.915	4.351	0.391	1.45	3.289	0.424	Ennore creek, India	Jayaprakash et al. (2015)
		4-7.86		34.76–46.1	11.24–51.15	190.4-712.5	23.5-45.57	8.33–28.3	84.9-252.9		Cochin Backwaters, India	Jyothirmaye et al. (2022)
7.	*Portunus pelagicus*	0.6	3.8–6.8	5.1–7.2	33.5–85.7	–	2.4–3.1	4-4.1	319–394	7.2–15.9	Coastal Borneo, Malaysia	Anandkumar and Suliman (2019)
		0.43–0.74	–	–	23.16–45.24	45.24–68.62	–	0.78–1.74	9.67–18.98	–	NW of Arabian Gulf, South Iraq	Al-Khafaji et al. (2018)
		0.41–5.86	–	0.2–2.39	3.86–25.29	–	–	0.28–4.72	–	–	Northern Bay of Bengal	Karar et al. (2019)
		BDL-30.23	BDL-3.84	BDL-81.7	BDL-267.26	–	BDL-32.77	BDL-8.68	–	BDL-225.63	South East coast of India	Kumar et al. (2019)
		0.004 ± 0.43	–	–	0.061 ± 0.25	–	–	0.013 ± 0.08	0.259 ± 0.08	–	Vellar and Uppanar estuaries, India	Sulieman and Suliman (2019)
		-	–	–	4.8–7.4	15.18.1	–	–	141–198	3.7–5.4	Chilika Lagoon, India.	Lakshman et al.(2015)

Table 6 (continued)

Sl no.	Species	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn	Location	Reference
8.	*Mugil cephalus*	–	–	0.045	3.63	17.56	–	–	5.047	–	Chilika Lagoon, India	Parida et al. (2017)
		BDL	–	–	0.91	–	0.978	0.172	12.87	0.83	Gaza strip	Elnabris et al. (2013)
		24.62 ± 12.11	–	0.43 ± 0.66	33.48 ±15.54	–	BDL	10.59 ± 9.12	–	–	Creek in Woji, Nigeria	Ihunwo et al. (2020)
		0.02	0.03	0.01	BDL	1.79	0.05	1.39	0.88	NA	Coasts of Tanzania	Mwakalapa et al. (2019)
		0.127–0.146	–	0.097–0.112	5.5–6.54	–	–	0.094–0.13	9.25–10.65	–	Pulicate Lake, India	Pandion et al. (2022)
		BDL-20.42	–	–	11.51–88.31	–	–	BDL-22.52	41.07-629.75	–	Cochin backwaters, India	Mohan et al. (2012)
		BDL	–	0.34 ± 0.01	0.38 ± 0.01		0.26 ± 0.02	0.03 ± 0.014	3.1 ± 0.24	–	Tuscany coast	Bonsignore et al. (2018)
		0.005 ± 0.001	–		0.085 ± 0.11	–	–	0.026 ± 0.25	0.176 ± 0.09	–	Vellar and Uppanar estuaries, India	Sulieman and Suliman (2019)
		2.21	–	4.6	2.4	–	2.6	4.9	–	–	Ennore estuary, India	Karthikeyan et al. (2020)
9.	*Scylla serrata*	3.3		11.4	22.1	–	6	8.9	–	–	Ennore estuary, India	Karthikeyan et al. (2020)
		0.25–0.65	1.05–3.85	0.027–12.9	27.1–34		0.65–5.65	1.9–16.7	247.5-428.7	1.25–53.5	Coastal Borneo, Malaysia	Anandkumar et al. (2019)
		-	–	–	117.2	167.3	–	0.22	287.6	12.7	Mahandi estuary, India	Mohapatra et al. (2009)
		BDL-8.89	–	–	30.09-189.44	–	–	BDL-28.09	10.4–239	–	Lower Gangetic Delta, India	Rudra et al. (2016)

Table 6 (continued)

Sl no.	Species	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn	Location	Reference
		0.033–0.308	–	BDL-0.204	BDL-0.3	0.213-1.5	–	0.64–2.33	–	–	Pulicat lake, India	Batvari et al. (2016)
		0.024–0.976		0.82–47.4	2.795–16.53	–	–	0.699–22.58	14.38–92.85	–	Ashtamudi lake, India	Williams et al. (2022)
		0.13–1.1	–	–	0.21–10.6	–	–	–	14.26–44.94	–	Tuticorin, India	Yogeshwaran et al. (2020)
		–	–	–	5.4–9.5	14.4–18.4	–	–	143–211	3.8–5.5	Chilika Lagoon, India.	Lakshman et al. (2015)
10.	*Sillago sihama*	0.01–0.03	–	–	0.25–0.53	1.58–4.61	–	0.07–0.41	1.78–7.26	–	Southeast coast, India	Perumal et al. (2023)
		0.06	0.39	1.09	6.99	6.69	0.41	0.97	3.81	0.4	Ennore creek, India	Jayaprakash et al. (2015)
		2.28 ± 1.61	–	0.15 ± 0.02	0.73 ± 0.09	–	–	0.07 ± 0.02	7.49 ± 0.91	–	Puducherry coast, India	Patchaiyappan et al. (2023)
11.	*Euryglossa orientalis*	0.001–1.14	0.002–0.21	0.03–0.14	0.18–130.3	2.72–474.2	0.028–0.07	0.014–0.029	3.61-50	0.06–1.23	North Persian Gulf	Soltani et al. (2021)

BDL: below detection limit

and WHO (Table 4) indicating that Cu concentration in fish and crab species from the Netravathi-Gurupur estuary does not pose any serious health risk for human through their consumption. Although the Cu concentration in *S. serrata* was maximum, the values are much lower than those reported for the same species collected from Mahandi estuary (117.2 mg/kg; Mohapatra et al., 2009) and Ashtamudi lake (2.8–16.53 mg/kg; Williams et al., 2022) of India but comparable to the samples collected from Pulicat lake, India (Batvari et al., 2016). Cu content in the muscle of *E. suratensis* was after *S. serrata*, and the values are lower than the values reported from Pulicate lake (5.87–6.83 mg/kg; Pandion et al., 2022), and Cochin backwaters (14.68–42.23 mg/kg; Mohan et al., 2012) of India. Gill of *M. cephalus* contains maximum Cu concentration. However, the values are comparable to *M. cephalus* samples collected from Vellar and Uppanar estuaries (0.085 ± 0.11 mg/kg), India (Sulieman and Suliman 2019). The Cu content in the muscles of *M. cephalus* here was lower than the value reported for the same species collected from the Gaza strip (0.907 mg/kg; Kamal et al., 2013), and the Creek in Woji southern Nigeria (33.48 ± 15.54 mg/kg; Ihunwo et al., 2020). Similarly, the average Cu content in the muscle of *P. pelagicus* was lower than that of the same species collected from Kuwait coast (123.8 mg/kg; Al-Mohanna & Subrahmanyam, 2001).

Here, the highest mean cobalt (Co) bioaccumulation in muscle was 0.012 mg/kg in *S. serrata*, while in gills, it was maximum up to 0.21 mg/kg in *G. filamentosus*. However, there is no regulatory limit proposed for Co in fish by various organisations. Co concentrations in the studied species were either very low or undetected. The maximum Ni concentration was found in the muscle of *A. arius* (0.235 mg/kg), which was comparatively lower than that reported for the same species collected from Cochin Backwaters, India (21.2–31 mg/kg; Jyothirmaye et al., 2022). The mean Ni concentration in *S. serrata* (0.23 mg/kg) was comparatively higher than the value reported from Thane creek, India (0.04–0.09 mg/kg, Krishnamurti & Nair, 1999), but lower than the reported values from coastal Borneo, Malaysia (0.65–5.65 mg/kg; Anandkumar et al., 2019) and from Ennore estuary, India (6 mg/kg; Karthikeyan et al., 2020). The species-wise variations in Ni concentration was significantly different; however, the values were not significantly different between gills and muscles though the highest accumulation was observed in the gills. Moreover, Ni levels in the gills and musclesof all the studied species were below the permissible level set by FAO (FAO, 1989).

In this study, the highest cadmium (Cd) concentration (0.23 mg/kg) was recorded in the muscle of *S. serrata*, while the lowest concentration was found in the gills of *A. arius* (0.010 ± 0.0 mg/kg). Cd was not detected in the gills of *G. erythrourus* and *E. suratensis.* The Cd concentration in *S. serrata* was close to that in specimens collected from Pulicat lake, India (0.033–0.308 mg/kg; Batvari et al., 2016). After *S. serrata*, the second-highest Cd concentration was found in the muscle of *G. filamentosus (*0.158 mg/kg). These values were higher than in the same species collected from the fishing harbours, Mumbai (0.03–0.04 mg/kg) (Kumar et al., 2021) and Southeast coast India (0.01–0.02 mg/kg) (Perumal et al., 2023). Cd concentration in *M. cephalus* in this study was higher than values recorded for the same species from the coasts of Tanzania (0.02 mg/kg) (Mwakalapa et al., 2019) and Vellar and Uppanar estuaries (0.005 ± 0.001 mg/kg) (Sulieman and Suliman 2019). Among the species studied, *S. fimbriata* contains a relatively higher amount of Cd (0.139 mg/kg), although the values are much lower than those previously reported (Kumar et al., 2021; Patchaiyappan et al., 2023). Cd accumulation was significantly different between tissues and species. The Cd concentration was higher in the muscle than in the gill. However, all the values were below the limits recommended by FAO/WHO for fish. Industrial processes like smelting or electroplating, and fertilizer use may increase the Cd concentration in aquatic environments. Although this area is surrounded by industrial activities, the low concentration of Cd found in this study is a positive sign. However, continuous monitoring is recommended to protect the coastal-estuarine taxa. In the muscle, lead (Pb) had the highest mean concentration of 6.26 mg/kg in *G. erythrourus*, and the lowest concentration of 1.74 mg/kg in *M. cephalus* (Table 2). Pb bioaccumulation in the gills of the studied fish species varied from 5.1 to 1.68 mg/kg with *A. arius* having the highest, and *G. filamentosu* having the lowest bioaccumulation. There was a significant species-wise difference in mean Pb concentration, but the differences between gill and muscle were non-significant. Notably, the mean Pb

concentration in most of the studied species exceeded the recommended limit set by India (FSSAI, 2011) and other international organizations (FAO, 1983; WHO, 1989). The permissible limit for Pb in fish is 0.3 mg/kg according to FSSAI (FSSAI, 2011) and 0.5 mg/kg according to WHO (WHO, 1989). This low level of the permissible limit for Pb makes it hazardous for fish consumption (Vu et al., 2017). Compared to these thresholds, all the studied species are above the recommended limit (WHO, 1989). Lead is a non-essential element with no known biological role in living organisms (Bibi et al., 2023). It is a potential neurotoxic, nephrotoxic and can slow down the growth, metabolism and survival of the mammals (Satarug et al., 2020). Lead has the ability to induce behavioral deficits and learning disabilities in vertebrates which makes it as a potential biomarker for metal contamination in environment. Lead contamination in estuarine environment is often due to antifouling painting, dye industries, and vehicular emissions (Kamaruzzaman et al., 2011). Lead is extremely toxic to humans and consuming Pb contaminated fish could cause chronic toxicity in the local population (Tolkou et al., 2023). Lead concentration in *M. cephalus* was lower than those found in the coasts of Tanzania (1.39 mg/kg; Mwakalapa et al., 2019) and Vellar and Uppanar estuaries (0.026 ± 0.25 mg/kg; Sulieman and Suliman 2019). However, the Pb levels observed in *G. filamentosus* and *S. sihama* in the present study was far higher than those reported from fishing harbours in Mumbai, India (Kumar et al., 2021) and Southeast coast, Tamil Nadu, India (Perumal et al., 2023). Lead accumulation in *E. suratensis* was much lower than the value reported for same species from Cochin backwaters, India (upto 8.42 mg/kg; Mohan et al., 2012; 8.33–28.3 mg/kg; Jyothirmaye et al., 2022) but higher than those from Pulicate lake (0.0876-0.115267 mg/kg; Pandion et al., 2022) and Ennore creek of India (1.45 mg/kg; Jayaprakash et al., 2015). Lead content in *S. serrata* was higher than in the Tuticorin coast, India (0.09–0.72 mg/kg; Yogeshwaran et al., 2020) but lower than the values reported for *Scylla serrata* from the lower Gangetic Delta (BDL-28.09 mg/kg; Rudra et al., 2016) and Pulicat lake of India (0.64–2.332 mg/kg; Batvari et al., 2016). Lead contamination in the fishes of Netravathi-Gurupur estuary may be due to its proximity to industrial district Mangalore. High Pb concentrations have also been reported in fishes from the Cachorros river Brazil (Santos et al., 2019).

In this study, chromium (Cr) bioaccumulation ranged from 0.34 to 1.78 mg/kg in the muscle of the examined species, and from 0.537 to 3.391 mg/kg in the gills. The maximum concentration of Cr was found in *E. orientalis*, which was ten times higher than the value recorded for the same species from the North Persian Gulf (0.03–0.14 mg/kg; Soltani et al., 2021). A very high concentration of Cr (19-47.5 mg/kg) was also reported in *A. arius* collected from Cochin Backwaters, Kerala, India (Jyothirmaye et al., 2022). The Cr concentration in *E. suratensis* (1.24–2.34 mg/kg) in this study was higher than the value reported for the same species from Cochin estuary (0.31–0.53 mg/kg; Sivaperumal et al., 2007) and Chilika lagoon (0.514 mg/kg; Parida et al., 2017), India. Chromium accumulation in crab species from this estuary was comparatively higher than that of Pulicat lake, India (*S. serrata;* BDL-0.204 mg/kg; Batvari et al., 2016). Moreover, Cr concentration in most of the studied species was higher than the reported values from Parangipettai coast, India (0.65–0.92 mg/kg; Raja et al., 2009), Pulicate lake, India (0.0753–0.1227 mg/kg; Pandion et al., 2022) and Meghna estuary, Bangladesh (0.76 mg/kg Ahmed et al., 2019). The daily endurable Cr concentration suggested by the FSSAI is 12 mg/kg (FSSAI, 2011), whereas the WHO suggested 1 mg/kg (FEPA, 2003; WHO, 1995). The study revealed that for Cr content in muscle, eight of the tweleve species (fish and crab) exceeded the recommended limit for Cr while for gills, it was seven out of ten fish species. Long term exposure to Cr may cause damage to skin, lungs, stomach and even death (Guertin et al., 2004). Sometimes, excessive intake of Cr through food can cause acute pulmonary disorders (Kawser Ahmed et al., 2016; Forti et al., 2011). High Cr concentrations in fish and crab species collected from the Netravathi-Gurupur estuary may pose a risk to human health. The high concentration of Cr in this estuary may be due to agriculture runoff, boat painting, and leaching from Cr-bearing minerals. Saha et al. (2024) also reported a comparatively high value of Cr from the sediment of this estuary. Nonetheless, the values are consistent with reported values in literature (Parida et al., 2017; Mohiuddin et al., 2023; Patchaiyappan et al., 2023).

As observed, the levels of trace metals found in this study were relatively similar to or lower than those reported in most studies. Variations in heavy metal concentrations in different tissues from various regions of the world may result from differences in metal levels and physical, chemical characteristics of the water from which the fishes were sampled (Bahnasawy et al., 2009; Solgi & Mirmohammadvali, 2021). In general, gill tissue contains a higher amount of trace metal than muscle tissue, but it was not always the case for all species. Higher metal accumulation in gill tissue may be due to the mucus layer on the gills, which facilitates the rapid accumulation (Jayaprakash et al., 2015). Moreover, the highly branched structural organization of the gills allow them to pass large volumes of water, and have more contact with surrounding water than other organs (Evans et al., 2005). All these factors make this organ as prime site of metal accumulation.

Although all the species live in similar ecological conditions, trace metal content varied across the species. This variation may be due to their difference in feeding habits, trophic levels and trace metal pollution gradient (Ahmed et al., 2019). Based on total metal content (including gill and muscle), the studied fish species can be arranged in the following descending order *G. filamentosus* (116.83 mg/kg; demersal) > *A. arius* (113.35 mg/kg; demersal) >*S. fimbriata* (111.57 mg/kg; Pelagic)>*C. ignobilis* (109.16 mg/kg; Pelagic)>*E. suratensis* (105.27 mg/kg; Benthopelagic)>*G. Erythrourus* (92.4 mg/kg; demersal)>*M. cephalus* (85.21 mg/kg; benthopelagic)>*N. nasus* (82.74 mg/kg; Pelagic)>*E. orientalis* (72.48 mg/kg; demersal)>*S. sihama* (62.45 mg/kg; Pelagic). This indicates that demersal fish species and fish-feeding benthos are more likely to accumulate metals due to their close connection with sediment than the pelagic species (Yi et al., 2017). In contrast, for pelagic species, diet mainly consists of water and phyto-plankton, leading to lower accumulation of metals. Recent studies also support the pattern obtained in this study (Sahu et al., 2023; Traina et al., 2019). However, metal accumulation depends not only on the feeding behavior but also on factors such as age, size, metabolic activity, reproductive behaviour and life cycle (Teunen et al., 2021).

Assessment of trace metal level in different species using MPI.

In an aquatic ecosystem, fishes are exposed to multiple metals simultaneously, so it is important to assess the overall metal toxicity (Pinkey et al., 2024). The metal pollution index (MPI) helps to determine the combined potential harm from exposure to multiple metals. A higher value of MPI indicates a higher accumulation of metal in fish tissues. MPI values 10–20, 5–10, 2–5, and < 2 indicates medium, low, very low, and no toxicity, respectively (Jamil et al., 2024). In terms of MPI (Fig. 3) values in muscle tissue, the studied species were ranked as follows: *S. serrata* (1.35) > *G. erythrourus* (0.66) > *G. filamentosus* (0.58) > *S. sihama* (0.55) > *S. fimbriata* (0.49) > *E. suratensis* (0.48) > *E. orientalis* (0.38) > *P. pelagicus* (0.33) > *A. arius* (0.30) > *C. ignobilis* (0.26) ≈ *N. nasus* (0.26) > *M. cephalus* (0.25), while in gill the order was *E. suratensis* (0.66) > *M.cephalus* (0.57) > *G. erythrourus* (0.53) > *S. sihama* (0.52) > *A. arius* (0.48) > *G. filamentosus* (0.44) > *N. nasus* (0.43) > *C. ignobilis* (0.40) > *E. orientalis* (0.37) > *S. fimbriata* (0.30). The highest MPI value was found

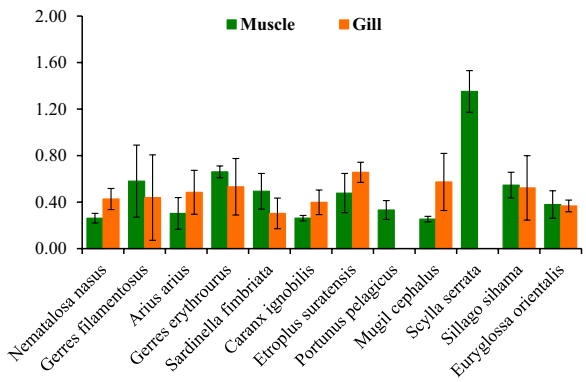

Fig. 3 Metals pollution indices in various fish and crab species of the Netravathi-Gurupur estuary, India

to be below 2.0, indicating the absence of toxicity to the studied species from this estuary. The average MPI values in the studied species were comparable to those for the fishes from Tanguar Haor, Bangladesh (Pinkey et al., 2024) and Kalapakkam coast, Bay of Bengal (Pandion et al., 2023), while they were higher than those for the fishes from Ennore coast, India (Kumar et al., 2021) and Karnaphuli River Estuary, Bangladesh (Rahman et al., 2022). Although the MPI values are low, constant and regular monitoring of metal accumulation in costal-estuarine fish species is important to prevent the metal-associated human health risks.

Bioaccumulation factor

Bioaccumulation factor (BAF) is the ratio of metal concentration in a particular organ of aquatic species and the concentration of that particular metal in the surrounding environment. The impact of metal concentration at any trophic level of an aquatic species depends on the bioaccumulation factor specific to the respective metals and species (Ahmed et al., 2017). BAFs of trace metal for studied fish species are given in Fig. 4. Overall, the accumulation of metal in muscle of selected species varied in the following descending order: Cr (1968.3) > Pb (1296.8) > Co (321.8) > Ni (119.2) > Fe (71.0) > Mn (51.9) > Cu (37.0) > Cd (27.9) > Zn (10.8) while in gill the order was Cr (2691.9) > Pb (1297.9) > Co (806.9) > Fe (388.1) > Ni (144.8) > Mn (77.7) > Zn (6.5) > Cd (5.0) > Cu (4.8). Among the metals, Cr was found to be the most bioaccumulative in the studied species. BAFs of both Cr and Pb were > 1000 indicating their potential chronic effects. Previous studies also suggest that the different fish species can easily absorb Pb and Cr (Lipy et al., 2021). The results showed that BAFs were higher in gill tissues than muscle. Species-wise, the BAF varied across different species. Bioaccumulation of metals depend on various factors such as habitat, life cycle, feeding nature, ecology, exposure duration, process absorption, and elimination (Anandkumar et al., 2017). Due to these factors, the metal accumulation in the studied fish and crab species collected from Netravathi-Gurupur estuary was different.

Human health risk evaluation

The accumulation of contaminants in fish species may affect human health of the consumers due to regular consumptions of contaminated studied species. Hence, health risk assessment is necessary for the fish species coming from the studied estuary. Though

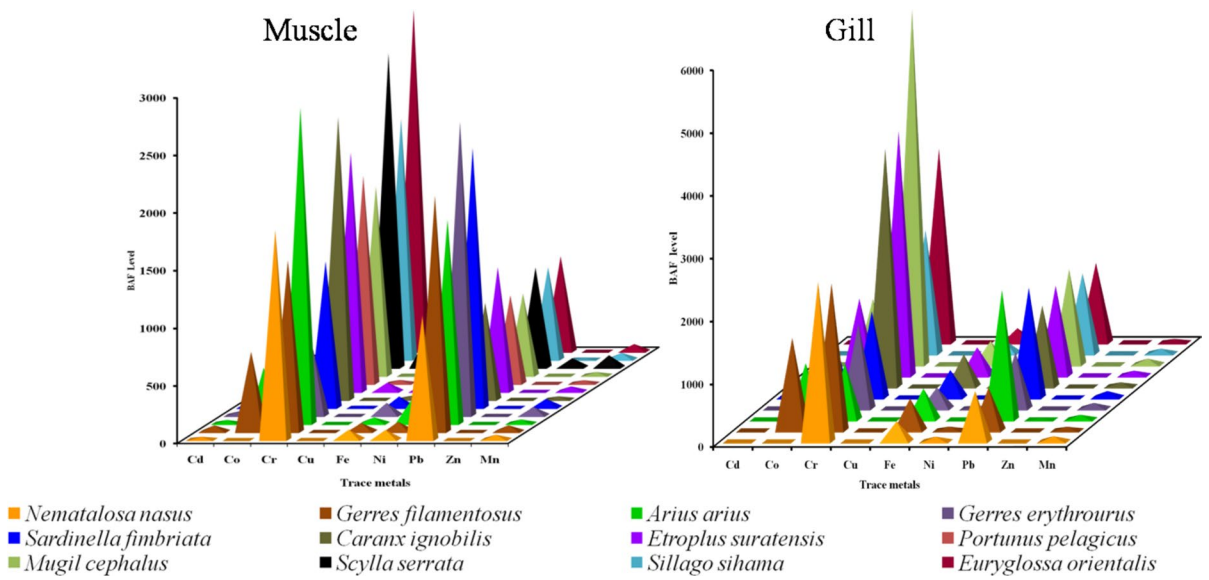

Fig. 4 Bioaccumulation factors of different trace metals in muscle and gill tissues in the targeted species of the Netravathi-Gurupur estuary, India

extensive studies have been carried out on metal contamination in aquatic organisms, human health risk assessment approach has only been recently explored. Thus to evaluate the risk associated with consumption of contaminated fish species, we have estimated the target health hazard (THQ), hazard index (HI), estimated daily (EDI) and weekly intake (EWI), and cancer risk (CR). These metrics act as a valuable approach for determining potential health hazard (Rahman et al., 2019; Tore et al., 2021).

Estimated daily (EDI) and estimated weekly (EWI) intake of heavy metals through estuarine fish and crab consumption

Calculation of EDI helps to determine the daily dietary exposure to trace metals through the consumption of contaminated fish species in both adults and children. This is the first step for assessing the non-carcinogenic and carcinogenic risks involved in consuming contaminated fish. Irrespective of age group, the average EDI values for Cd, Co, Cr, Cu, Fe, Ni, Pb, Zn and Mn were 0.056, 0.005, 0.796, 0.127, 8.66, 0.105, 2.15, 20.352, 0.409 µg/kg/BW/day respectively. Table 7 shows that the EDI values are lower than the RfD values, which estimate the daily exposure level to which the human population may be continually exposed over a lifetime without a considerable risks of harmful effects. According to the New York Health Department (2007), if the EDI value is equal to or less than the RfD, the risk to the human population is minimal. However, the fact that doses are lower than the RfD does not necessarily mean that metal concentration fall within an "acceptable limit" or "unacceptable limit". Nevertheless, the EDI values calculated in this study are also lower than the provisional maximum tolerable daily intake (PMTDI) limit proposed jointly by the FAO and WHO (JECFA 1999). This indicates that metal exposure is less, and fish consumption is safe for human health at present. However, there may be possibilities of an increased accumulation of trace metals in fish over time and long term consumption of contaminated fish species from this estuary may pose serious health risks (Mohiuddin et al., 2022). Therefore, as a precaution highly contaminated fish species should be avoided for consumption.

The sequence of mean EDI (µg/kg BW/day) was Fe (5.8–11.55) > Pb (1.44–2.87) > Cr (0.53–1.061) > Mn (0.27–0.55) > Zn (0.24–0.47) > Cu (0.085–0.169) > Ni (0.07–0.14) > Cd (0.037–0.075) > Co (0.004–0.007). The measured mean EDIs (µg/kg/day) for muscles of fishes were arranged in the descending order: *G. erythrourus* (2.33) > *E. orientalis* (2.13) > *S. fimbriata* (1.88) > *S. serrata* (1.84) > *N. nasus* (1.68) > *G. filamentosus* (1.54) > *E. suratensis* (1.38) > *S. sihama* (1.32) > *A. arius* (1.23) > *P. pelagicus* (0.988) > *C. gnobilis* (0.834) > *M. cephalus* (0.727). Estimated weekly intake values were also similar to EDI (Table 7), which were well below the tolerable metal levels compared to PTWI (provisional tolerable weekly intake) values. In most cases, the mean EDI and EWI values were higher in demersal species than pelagic species, suggesting that feeding behaviour influences metal accumulation. This also indicates that the consumption of demersal fish species may increase metal exposure to children and adults. The EDI value is higher in children than adults, supported by other literature (Khan et al., 2023) indicating that younger age groups are more vulnerable to metal exposure than adults.

Target hazard quotient (HQ) and hazard index (HI) for non-carcinogenic risk

The hazard quotient (HQ) is used to estimate non-carcinogenic effect, which is the ratio of EDI to RfD of individual metal and is widely used to evaluate xenobiotics risk factor. THQ value < 1 indicates safe condition, while THQ value > 1 indicates a susceptible health condition.

Table 8 summarizes the THQ values of different metals due to fish and crab species consumption from the Netravathi-Gurupur estuary. Descending level of average THQ value was as follows: Pb, Cr, Cd, Fe, Ni, Cu, Mn, Zn, and Co ranged from 0.359 to 0.717, 0.17 to 0.354, 0.0373 to 0.0745, 0.00825 to 0.0164, 0.0064 to 0.0127, 0.0021 to 0.0042, 0.00195 to 0.0039, 0.00078 to 0.0016, and 0.00012 to 0.00024 in both children and adult. All THQ values were < 1, signifying no potential risk. However, THQ values were higher in children than in adults. Since long-term exposure may pose a risk to health in any age group, regular monitoring is required. The mean THQ, irrespective of age group, indicates that benthic feeder species accumulate higher levels of metals compared to species living in the middle or upper zone of the water column. The results confirm that

Table 7 Estimated daily [EDI; g/kg BW/day] and weekly intake [EWI; µg/kg BW/week] of heavy metals through consumption of studied species by the human population

Species	Age group	Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn
Nematalosa nasus	Children	0.455 (0.065)	BDL	6.853 (0.979)	0.091 (0.013)	97.335 (13.905)	0.63 (0.090)	16.702 (2.386)	0.091 (0.013)	3.059 (0.437)
	Adult	0.224 (0.032)	BDL	3.423 (0.489)	0.042 (0.006)	48.671 (6.953)	0.315 (0.045)	8.351 (1.193)	0.042 (0.006)	1.526 (0.218)
Gerres filamentosus	Children	0.994 (0.142)	0.063 (0.009)	5.607 (0.801)	0.273 (0.039)	83.706 (11.958)	0.637 (0.091)	31.689 (4.527)	2.506 (0.358)	3.941 (0.563)
	Adult	0.497 (0.071)	0.035 (0.005)	2.8 (0.400)	0.14 (0.020)	41.853 (5.979)	0.322 (0.046)	15.848 (2.264)	1.253 (0.179)	1.967 (0.281)
Arius arius	Children	0.567 (0.081)	0.042 (0.006)	10.346 (1.478)	0.063 (0.009)	60.452 (8.636)	1.477 (0.211)	27.398 (3.914)	0.483 (0.069)	2.527 (0.361)
	Adult	0.287 (0.041)	0.021 (0.003)	5.173 (0.739)	0.028 (0.004)	30.226 (4.318)	0.742 (0.106)	13.699 (1.957)	0.245 (0.035)	1.26 (0.180)
Gerres erythrourus	Children	0.693 (0.099)	0.042 (0.006)	2.17 (0.310)	0.182 (0.026)	123.921 (17.703)	BDL	39.459 (5.637)	2.422 (0.346)	5.383 (0.769)
	Adult	0.343 (0.049)	0.021 (0.003)	1.085 (0.155)	0.091 (0.013)	61.964 (8.852)	BDL	19.733 (2.819)	1.211 (0.173)	2.695 (0.385)
Sardinella fimbriata	Children	0.875 (0.125)	0.056 (0.008)	4.781 (0.683)	0.483 (0.069)	108.171 (15.453)	0.616 (0.088)	34.86 (4.980)	2.52 (0.360)	5.474 (0.782)
	Adult	0.441 (0.063)	0.028 (0.004)	2.387 (0.341)	0.238 (0.034)	54.089 (7.727)	0.308 (0.044)	17.43 (2.490)	1.26 (0.180)	2.737 (0.391)
Caranx ignobilis	Children	0.252 (0.036)	BDL	9.24 (1.320)	0.273 (0.039)	36.435 (5.205)	0.973 (0.139)	12.999 (1.857)	0.189 (0.027)	1.925 (0.275)
	Adult	0.126 (0.018)	BDL	4.62 (0.660)	0.133 (0.019)	18.221 (2.603)	0.483 (0.069)	6.503 (0.929)	0.091 (0.013)	0.966 (0.138)
Etroplus suratensis	Children	0.154 (0.022)	BDL	7.819 (1.117)	2.45 (0.350)	70.98 (10.140)	1.078 (0.154)	16.667 (2.381)	0.84 (0.120)	3.157 (0.451)
	Adult	0.077 (0.011)	BDL	3.906 (0.558)	1.225 (0.175)	35.49 (5.070)	0.539 (0.077)	8.33 (1.190)	0.42 (0.060)	1.575 (0.225)
Portunus pelagicus	Children	0.147 (0.021)	0.035 (0.005)	6.79 (0.970)	0.924 (0.132)	59.822 (8.546)	0.749 (0.107)	11.816 (1.688)	0.742 (0.106)	1.953 (0.279)
	Adult	0.07 (0.010)	0.021 (0.003)	3.395 (0.485)	0.462 (0.066)	29.911 (4.273)	0.378 (0.054)	5.908 (0.844)	0.371 (0.053)	0.973 (0.139)
Mugil cephalus	Children	0.154 (0.022)	BDL	6.174 (0.882)	0.154 (0.022)	33.152 (4.736)	0.833 (0.119)	10.962 (1.566)	0.735 (0.105)	2.093 (0.299)
	Adult	0.077 (0.011)	BDL	3.087 (0.441)	0.077 (0.011)	16.576 (2.368)	0.413 (0.059)	5.481 (0.783)	0.371 (0.053)	1.05 (0.150)
Scylla serrata	Children	1.449 (0.207)	0.077 (0.011)	10.283 (1.469)	7.546 (1.078)	88.277 (12.611)	1.449 (0.207)	13.391 (1.913)	24.185 (3.455)	7.686 (1.098)
	Adult	0.721 (0.103)	0.035 (0.005)	5.138 (0.734)	3.773 (0.539)	44.142 (6.306)	0.728 (0.104)	6.699 (0.957)	12.089 (1.727)	3.843 (0.549)
Sillago sihama	Children	0.378 (0.054)	0.035 (0.005)	7.868 (1.124)	1.624 (0.232)	78.75 (11.250)	1.057 (0.151)	12.355 (1.765)	4.641 (0.663)	4.102 (0.586)
	Adult	0.189 (0.027)	0.014 (0.002)	3.934 (0.562)	0.812 (0.116)	39.375 (5.625)	0.525 (0.075)	6.174 (0.882)	2.317 (0.331)	2.051 (0.293)
Euryglossa orientalis	Children	0.154 (0.022)	BDL	11.186 (1.598)	0.147 (0.021)	128.912 (18.416)	1.33 (0.190)	12.747 (1.821)	0.098 (0.014)	4.543 (0.649)
	Adult	0.077 (0.011)	BDL	5.593 (0.799)	0.07 (0.010)	64.456 (9.208)	0.665 (0.095)	6.37 (0.910)	0.049 (0.007)	2.275 (0.325)
RfD(µg/kg BW/day) (US EPA, 2014)		1.0	30	3.0	40.0	700.0	11.0	4.0	300	140.0
PMTDI (µg/kg BW/day) (WHO, 2016)		1			500	800		4.0	300–1000	5000
PTWI (FAO/WHO, 2011)		7	700	233	500	5600[a]	35[b]	25	420	5000[c]

The figures in the parenthesis are the EDI values; BDL: below detection limit

[a]Source: Younis et al. (2024)

[b]Source: Miri et al. (2017)

[c]Source: Egbe et al. (2023)

Table 8 Target hazard qoutient (THQ) and hazard index (HI) of heavy metals content through consumption of fish and crab species from Netravathi-Gurupur estuary

Fish species	Age group	THQ									
		Cd	Co	Cr	Cu	Fe	Ni	Pb	Zn	Mn	HI
Nematalosa nasus	Children	0.0648	BDL	0.326	0.0003	0.0199	0.0082	0.596	0.00004	0.0031	1.019
	Adult	0.032	BDL	0.163	0.0002	0.0099	0.0041	0.298	0.00002	0.0016	0.51
Gerres filamentosus	Children	0.142	0.0003	0.267	0.001	0.0171	0.0083	1.132	0.00119	0.004	1.572
	Adult	0.071	0.0002	0.133	0.0005	0.0085	0.0041	0.566	0.0006	0.002	0.786
Arius arius	Children	0.081	0.0002	0.493	0.0002	0.0123	0.0192	0.978	0.00023	0.0026	1.587
	Adult	0.041	0.0001	0.246	0.0001	0.0062	0.0096	0.489	0.00012	0.0013	0.793
Gerres erythrourus	Children	0.099	0.0002	0.103	0.0007	0.0253	BDL	1.409	0.00115	0.0055	1.644
	Adult	0.049	0.0001	0.052	0.0003	0.0126	BDL	0.705	0.00058	0.0027	0.822
Sardinella fimbriata	Children	0.125	0.0003	0.228	0.0017	0.0221	0.008	1.245	0.0012	0.0056	1.637
	Adult	0.063	0.0001	0.114	0.0009	0.011	0.004	0.623	0.0006	0.0028	0.818
Caranx ignobilis	Children	0.036	BDL	0.44	0.001	0.0074	0.0126	0.464	0.00009	0.002	0.963
	Adult	0.018	BDL	0.22	0.0005	0.0037	0.0063	0.232	0.00004	0.001	0.482
Etroplus suratensis	Children	0.022	BDL	0.372	0.0087	0.0145	0.014	0.595	0.0004	0.0032	1.03
	Adult	0.011	BDL	0.186	0.0044	0.0072	0.007	0.298	0.0002	0.0016	0.515
Portunus pelagicus	Children	0.021	0.0002	0.323	0.0033	0.0122	0.0097	0.422	0.00035	0.002	0.794
	Adult	0.01	0.0001	0.162	0.0017	0.0061	0.0049	0.211	0.00018	0.001	0.397
Mugil cephalus	Children	0.022	BDL	0.294	0.0005	0.0068	0.0108	0.392	0.00035	0.0021	0.728
	Adult	0.011	BDL	0.147	0.0003	0.0034	0.0054	0.196	0.00018	0.0011	0.364
Scylla serrata	Children	0.207	0.0004	0.49	0.027	0.018	0.0188	0.478	0.01152	0.0078	1.258
	Adult	0.103	0.0002	0.245	0.0135	0.009	0.0094	0.239	0.00576	0.0039	0.629
Sillago sihama	Children	0.054	0.0002	0.375	0.0058	0.0161	0.0137	0.441	0.00221	0.0042	0.912
	Adult	0.027	0.0001	0.187	0.0029	0.008	0.0069	0.221	0.0011	0.0021	0.456
Euryglossa orientalis	Children	0.022	BDL	0.533	0.0005	0.0263	0.0173	0.455	0.00005	0.0046	1.058
	Adult	0.011	BDL	0.266	0.0003	0.0132	0.0086	0.228	0.00002	0.0023	0.529

BDL: below detection limit

feeding habit influences metal accumulation. However, for individual metals, the THQ of Pb was > 1 in some fish species (*G. filamentosus*, *G. erythrourus*, and *S. fimbriata*) exceeding the permissible limit of 1. Hence, excessive and continuous consumption of these species may pose non-carcinogenic risks.

Hazard index

Since humans may be exposed to multiple elements simultaneously, estimating the cumulative impact of trace metals through hazard index (HI) is important (Pandion et al., 2023). HI is the summation of the THQ value for each element in individual species and indicates the potential non-carcinogenic risk to humans. According to the USEPA (US EPA 1999), based on HI, impacts can be categorized as negligible, low significant health impacts, medium significant health impacts and very high risk when HI values are < 0.1, 0.1 < HI < 1, 1 < HI < 4 and HI > 4, respectively.

The HI value ranged from 0.36 to 0.82 in adults and 0.73 to 1.64 in children (Table 8). This implies that children are facing two times more health risks than adults. Moreover, HI values were more than 1 for children in most of the observations, indicate medium significant health effects, while for adults the values were < 1, indicating low significant health effects. This suggests that more attention should be given to the health of children around the Netravathi-Gurupur estuary by avoiding overconsumption of contaminated aquatic food species. Increased industrialization around the Netravathi-Gurupur estuary might have contributed to

increased metal accumulation in some fish species (Saha et al., 2024).

Determination of carcinogenic risk

Carcinogenic risk (CR) was calculated from the Cd, Cr, and Pb concentration in the muscle tissue of fish and crab species. CR value for Cd, Pb and Cr was 6.5×10^{-5}-6.51×10^{-4}, 6.7×10^{-6}-2.4×10^{-5}, 7.8×10^{-5}-3.99×10^{-4} respectively in adults, while the values were 1.3×10^{-4}-1.3×10^{-3}, 1.33×10^{-5}–4.79×10^{-5}, 1.55×10^{-4}–7.99×10^{-4}, respectively in children (Table 9). Results indicate that CR exposure for all metals was negligible except for Cd in *S. serrata.* Moreover, CR exposure to Pb was negligible for both children and adults. Nonetheless, the cumulative cancer risk was measured and found to be 9.18×10^{-4}, 1.33×10^{-3}, 1.28×10^{-3}, 8.25×10^{-4}, 1.17×10^{-3}, 9.01×10^{-4}, 7.15×10^{-4}, 6.30×10^{-4}, 5.92×10^{-4}, 2.05×10^{-3}, 9.17×10^{-4}, and 9.50×10^{-4} in children, and 4.59×10^{-4}, 6.66×10^{-4}, 6.41×10^{-4}, 4.12×10^{-4}, 5.87×10^{-4}, 4.50×10^{-4}, 3.57×10^{-4}, 3.15×10^{-4}, 2.96×10^{-4}, 1.03×10^{-3}, 4.59×10^{-4} and 4.75×10^{-4} in adult for *N. nasus*, *G. filamentosus*, *A arius*, *G. erythrourus*, *S. fimbriata*, *C. ignobilis*, *E. suratensis*, *P. pelagicus*, *M. cephalus*, *S. serrata*, *S. sihama* and *E. orientalis*. This indcates that the consumption of some fish species may pose carcinogenic risk and may not be safe for human consumption. The results further indicatethat children are more vulnerable to CR exposures than adult, corroborating previous study (Khan et al., 2023). Comparing the cumulative CR value, *G. filamentosus*, *A. arius*, *S. fimbriata*, and *S. serrata* may present moderate risk of cancer due to Cd, Pb, and Cr exposure. Therefore, the carcinogenic health risks linked to these trace metals should not be ignored.

Source identification of trace metal pollution through multivariate statistical analysis

This study used multivariate statistical analysis like principal component analysis (PCA), and Pearson's correlation analysis to identify the relationship between individual trace metal pollution and discern their likely sources along the Netravathi-Gurupur estuary, India (Khan et al., 2023).

Pearson's correlation analysis

To assess the association among the trace metals in different species, Pearson's correlation analysis was carried out as shown in Fig. 5. Inter-metal interactions indicate the source of trace metal in the selected species. Figure 5 depicts a strong correlation between Fe and Mn, indicating Fe/Mn flocculation takes place and their similar elemental behavior as they are present in all species. Their strong association also points to both natural occurrence and external sources (Saha et al., 2024). There was a strong relation of Cd

Table 9 Lifetime carcinogenic risk of consumption of different fish and crab species from Netravathi-Gurupur estuary due to presence of Cd, Cr and Pb as contaminants

Species/age groups	Cd		Pb		Cr		Cumulative CR	
	Child	Adult	Child	Adult	Child	Adult	Child	Adult
Nematalosa nasus	4.08 E-04	2.04 E-04	2.03E-05	1.01E-05	4.89 E-04	2.45 E-04	9.18 E-04	4.59 E-04
Gerres filamentosus	8.94 E-04	4.47 E-04	3.85E-05	1.92E-05	4.00 E-04	2.00 E-04	1.33 E-03	6.66 E-04
Arius arius	5.10 E-04	2.55 E-04	3.33E-05	1.66E-05	7.39 E-04	3.70 E-04	1.28 E-03	6.41 E-04
Gerres erythrourus	6.22 E-04	3.11 E-04	4.79E-05	2.40E-05	1.55 E-04	7.8 E-05	8.25 E-04	4.12 E-04
Sardinella fimbriata	7.90 E-04	3.95 E-04	4.23E-05	2.12E-05	3.41 E-04	1.71 E-04	1.17 E-03	5.87 E-04
Caranx ignobilis	2.25 E-04	1.12 E-04	1.58E-05	7.89E-06	6.60 E-04	3.30 E-04	9.01 E-04	4.50 E-04
Etroplus suratensis	1.36 E-04	6.88 E-05	2.02E-05	1.01E-05	5.58 E-04	2.79 E-04	7.15 E-04	3.57 E-04
Portunus pelagicus	1.30 E-04	6.5 E-05	1.44E-05	7.18E-06	4.85 E-04	2.43 E-04	6.30 E-04	3.15 E-04
Mugil cephalus	1.38 E-04	6.9 E-05	1.33E-05	6.66E-06	4.41 E-04	2.21 E-04	5.92 E-04	2.96 E-04
Scylla serrata	1.30E-03	6.51 E-04	1.63E-05	8.13E-06	7.34 E-04	3.67 E-04	2.05 E-03	1.03 E-03
Sillago sihama	3.40 E-04	1.70 E-04	1.50E-05	7.50E-06	5.62 E-04	2.81 E-04	9.17 E-04	4.59 E-04
Euryglossa orientalis	1.36 E-04	6.8 E-05	1.55E-05	7.74E-06	7.99 E-04	3.99 E-04	9.50 E-04	4.75 E-04

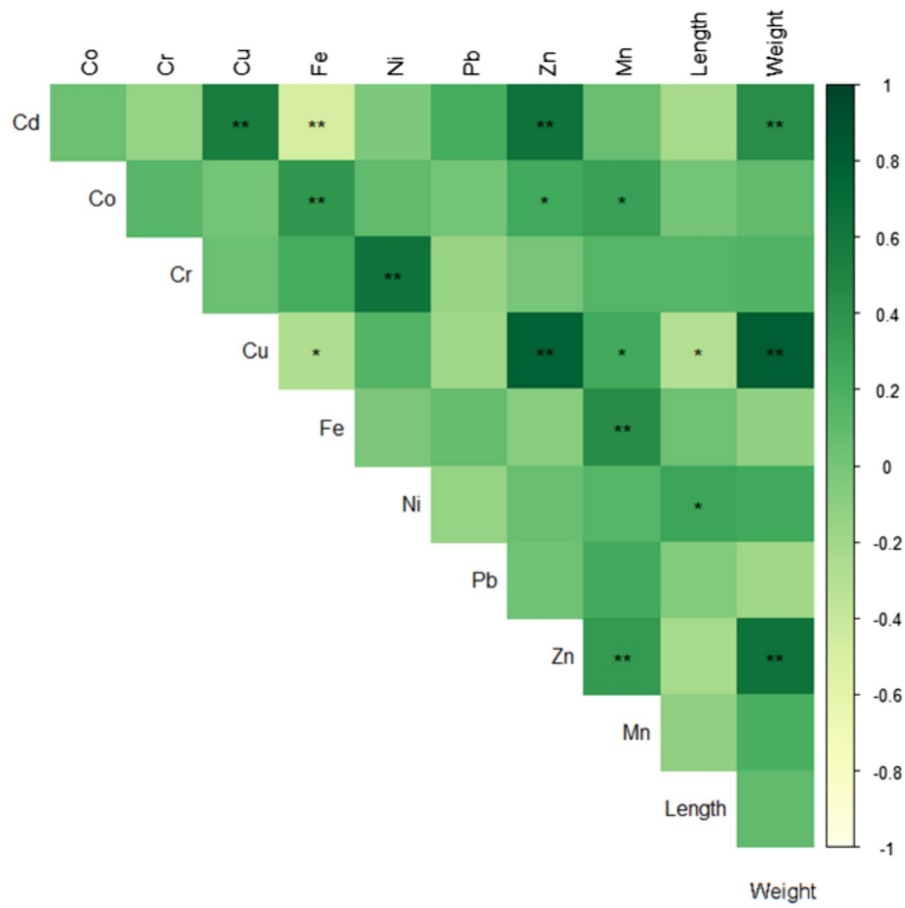

Fig. 5 Pearson's correlation matrix showing the significant correlation for trace metals concentration in fish and crab species collected in the Netravathi-Gurupur estuary, India

with Cu and Zn indicating their cumulative presence is of concern and suggesting their similar source as they are coming from electroplating, petrochemical, and chemical-intensive industries. However, Cd and Cu concentrations have a significant negative correlation with Fe in fish, which is attributed to their dissimilar accumulation pathways. The positive correlation between Cr and Ni indicate that they may have common sources and their subsequent accumulation in the estuarine species (Patchaiyappan et al., 2023). The significant positive correlation between Fe and Co, indicates these metals may originate from similar parent materials. Overall, interdependence among various trace metals point to their common source of origin. The body size (weight and length) of aquatic organisms play an important role in the bioaccumulation of heavy metals in their tissue; thus, understanding their relationship is important. It was observed that there were no significant correlations between most of the trace metals in the tissue and length. Only Ni showed a positive correlation, and Cu showed a negative correlation with length. A significant negative correlation between metal and both fish weight and length was reported by Arulkumar et al. (2017) and Velusamy et al. (2014). However, a significant positive correlation was observed between fish weight and Cd, Cu, and Zn. Consequently, there was no clear and consistent relationships between the heavy metals in the tissues of the fish species and their size. Overall, the results suggest that the heavy consumption of large fish may pose health risks to consumers. However, the accumulation of trace metal in livingorganisms also depends on their uptake, detoxification and elimination mechanisms, which also controls the interdependency among the trace metals.

Principal component analysis

Principal component analysis (PCA) interprets the association of metal in the studied estuarine species

Table 10 Principal component analysis of trace metals

	PC1	PC2	PC3
Cd	0.46	-0.19	0.05
Co	0.18	-0.37	-0.23
Cr	0.08	0.45	-0.56
Cu	0.50	0.03	0.04
Fe	- 0.28	- 0.28	- 0.58
Ni	0.18	0.43	- 0.43
Pb	- 0.16	- 0.51	- 0.26
Zn	0.50	- 0.06	- 0.02
Mn	0.34	- 0.30	- 0.21
Eigenvalues	1.968	1.678	1.0264
Proportion of variance (%)	43.03	31.29	11.71
Cumulative proportion (%)	43.03	74.32	86.02

and distinguishes their sources of bioaccumulation, whether geo-genic or anthropogenic, aiding in remediation measures and future metal pollution management (Khan et al., 2023). Based on eigenvalues, three principal components (PCs) explain almost 86.02% of the total variance (Table 10). PC1 represents 43.03% of the total variance while PC2 contributes 31.29% of the total variance and PC3 represents only 11.71%. PC1 is loaded with Cd, Cu and Zn, which are attributed to sources such as petrochemical industry, electroplating industry, pesticides and fertilizers from agricultural runoff. PC2 showed the considerable accumulation of Cr and Ni in estuarine fish and crab species, attributing to weathering minerals, petroleum waste, and agricultural runoff. PC3 showed no significant loading of any metals, indicating their minimal influence on the dataset. This indicates that industrial waste and agricultural chemicals are the significant contributors to metal pollution, with minor influence of geo-genic sources. The PCA biplot shows the relative positions of the fish species (Fig. 6). It is apparent that species like *S. fimbriata*, *G. filamentosus*, *A. arius*, *G. erythrourus* and *S. serrata* are distinctly different from other species. The feeding behavior of these fish species is primarily demersal, except for *S. fimbriata*, indicating that most trace metal accumulation is strongly associated with feeding behaviour.

Conclusions

Regarding aquatic food safety, human health issues, and sustainable coastal-estuarine management, it is crucial to regularly monitor the presence of toxic metal in commercially important species captured from this estuary. This study is the first scientific report on metal contamination in aquatic food species from the Netravathi–Gurupur estuary and the assessment of human health risks associated with their consumption, focusing on both non-carcinogenic and carcinogenic impacts. The WHO, FAO and FSSAI

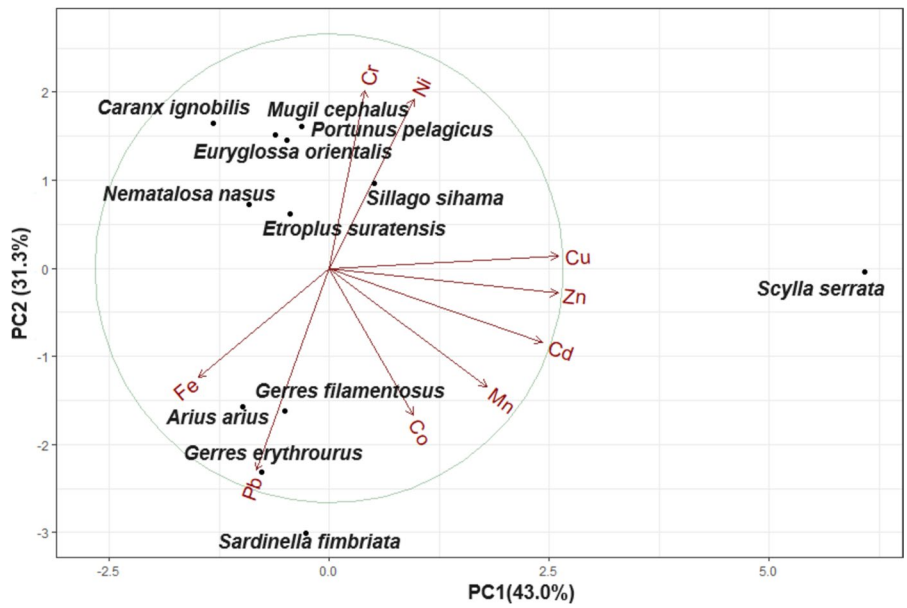

Fig. 6 Principal component analysis of PC1 and PC2 of different species from Netravathi–Gurupur estuary with trace metals

threshold limits were used in this investigation and it has been observed that most aquatic species exhibited significantly higher amounts of Pb and Cr than the threshold levels, while other metals were found at elevated levels only to a few species. Therefore, regular monitoring of trace metal in both abiotic and biotic components, including aquatic species, is essential to evaluate the future trends of trace metal accumulation in aquatic food and their impact on human health and to support sustainable management of the coastal-estuarine ecosystem.

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Data availability No datasets were generated or analysed during the currentstudy.

Declarations

Competing interests The authors declare no competing interests.

Author contribution Ajoy Saha: Conceptualization, sample collection and analysis, data analysis, manuscript preparation; B. K. Das: Contributed to the study conception and revising the article critically for important, intellectual content; Chayna Jana: Statistical analysis; D. J. Sarkar: Heavy metal analysis; Sonalika Sahoo: Manuscript editing and reviwieng; S. Samanta: Provided contribution through manuscript review and editing; Vikas Kumar: Calcualte the different metal index; M. E. Vijaykumar: Sample collection, and formal analysis; M. Feroz Khan: Guidance in manuscript preparation; Tania Kayal: Preparation of study area map.

Ethics approval The authors declare that they have strictly followed all the rules and principals of ethical and professional conducts while compeleting the research work.

Consent to participate Not applicable.

Consent to Publish All the author's provided consent to publish the study work.

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ARTICLES FOR FACULTY MEMBERS

INFLUENCE OF ANTHROPOGENIC POLLUTANTS ON MUD CRAB ECOSYSTEMS TO ENSURE SUSTAINABLE AQUACULTURE AND SAFE CONSUMPTION TO HUMAN HEALTH

The influence of ocean acidification and warming on responses of Scylla serrata to oil pollution: An integrated biomarker approach / Baag, S., & Mandal, S.

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The influence of ocean acidification and warming on responses of *Scylla serrata* to oil pollution: An integrated biomarker approach

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ABSTRACT

Anthropogenic activities primarily combustion of fossil fuel is the prime cause behind the increased concentration of CO_2 into the atmosphere. As a consequence, marine environments are anticipated to experience shift towards lower pH and elevated temperatures. Moreover, since the industrial revolution the growing demand for petroleum-based products has been mounting up worldwide leading to severe oil pollution. Sundarbans estuarine system (SES) is experiencing ocean warming, acidification as well as oil pollution from the last couple of decades. *Scylla serrata* is one of the most commercially significant species for aquaculture in coastal areas of Sundarbans. Thus, the prime objective of this study is to delineate whether exposure under ocean warming and acidification exacerbates effect of oil spill on oxidative stress of an estuarine crab *S. serrata*. Animals were separately exposed under current and projected climate change scenario for 30 days. After this half animals of each treatment were exposed to oil spill conditions for 24 h. Oxidative stress status superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), lipid peroxidation (LPO level) and DNA damage (Comet assay) were measured. Augmented antioxidant and detoxification enzyme activity was noted except for SOD but failed to counteract LPO and DNA damage. The present results clearly highlighted the detrimental combined effect of OWA and pollution on oxidative stress status of crabs that might potentially reduce its population and affect the coastal aquaculture in impending years.

1. Introduction

Environmental perturbations as a result of global climate change pose serious threats in marine ecosystems. Industrialization and human population growth worldwide are identified to be the major drivers of global climate change (Hashmi and Alam, 2019; Ghazali and Ali, 2019). Anthropogenic activities, primarily fossil fuel combustion, have increased the concentration of CO_2 in the atmosphere, altering the global carbon cycle and affecting climate patterns globally (Friedlingstein et al., 2019). The pH of marine waters is reducing (known colloquially as 'ocean acidification') and ocean temperatures are rising, with marine heat waves becoming more frequent (Caldeira and Wickett, 2003; Sokolov et al., 2009). Ocean acidification and warming are occurring concurrently, and the response of animals to projected conditions are likely the result of the cumulative effects of these and other key drivers such as habitat destruction, overfishing, pollution and contamination (Boyd et al., 2018; Britton et al., 2020). Moreover, since the industrial revolution the growing demand for petroleum-based products has been mounting up worldwide, leading to severe oil pollution in coastal ecosystems from various sources, majorly including oil spills and leakage from transportation.

Sundarbans, the largest mangrove forest in the world, is a UNESCO world heritage site and recently declared Ramsar site (Bhowmik and Mandal, 2021). However, the area is far from pristine, with enormous local and industrial waste discharges having been observed in the Sundarbans estuarine system (SES) (Zanardi-Lamardo et al., 2019). Additionally, the coastal Bay of Bengal including the SES has been experiencing steady surface water warming for the past couple of decades (Mitra et al., 2009; Samanta et al., 2018), and ocean pH has also altered due to the excessive absorption of CO_2 by the coastal waters in this area (Sarma et al., 2021). Fuel and oil spills from nearby Diamond Harbour and Haldia port are common (Panigrahy et al., 2014). Such spills introduce polycyclic aromatic hydrocarbons (PAHs) into coastal environments (Martins et al., 2011; Abreu-Mota et al., 2014). PAHs are presumed to affect aquatic biota in numerous ways, e.g., oxidative stress (Lushchak, 2011). Escalation in temperature also leads to overproduction and accumulation of reactive oxygen species (ROS) that cause oxidative damage to various cellular components (Lesser, 2006;

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González et al., 2010; Zhou et al., 2010).

Basal levels of ROS are generated as a result of routine metabolic/physiological processes like cell signalling and homeostasis. Living organisms are equipped with a well-developed antioxidant defence mechanism to eliminate and balance excessive ROS (Lushchak and Bagnyukova, 2006; Sookruksawong et al., 2013). The dynamic balance between the producing and eradicating ROS is pivotal to maintain an animal's health. The antioxidant defence system is comprised of several antioxidant and detoxifying enzymes, predominantly superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) requisite for ROS detoxification. If ROS scavenging is not efficient enough, lipid peroxidation (LPO) level increases in cell, which further reduces defence mechanisms (Regoli and Giuliani, 2014). Unessential ROS production incapacitates the antioxidant defence machinery and likely damages cellular DNA (Bhagat et al., 2017). Biomarkers can be used to detect ROS and determine the production-eradication balance, though results from individual enzymatic and non-enzymatic biomarkers are often idiosyncratic. Multiple-biomarker approaches can be more useful (Beliaeff and Burgeot, 2002), with scores of biomarkers from several levels of biological grades generating an enhanced understanding of stress response mechanisms. Moreover, integrating multiple biomarkers enables a holistic estimation of animal health status, which can be indicative of ecosystem stress levels.

Crab fisheries are valued worldwide as contributors to economies and livelihoods. Decapod crustaceans are also ecologically important as regulators of trophic dynamics and nutrient cycling in coastal ecosystems (Grilo et al., 2011; Pachelle et al., 2016; Madeira et al., 2018). They are vital components of a food web as detritivores, predators of small invertebrates, and prey for fish and birds (Chiodi Boudet et al., 2013; Nandy et al., 2021). They are regarded as excellent sentinel species as well as reliable environmental bio-monitors (Zheng et al., 2019). The mud crab (*Scylla serrata*) is a species of economic interest because it is one of the most commercially significant species for aquaculture in coastal areas of India and specifically in Sundarbans. The reported landing of crabs from the West Bengal coast was about 4500 tons in 2018, which decreased by about 56% from the preceding year (CMFRI, 2019).

The biological repercussions of several global and local environmental drivers have received attention independently howbeit; studies on their interactions are still very limited. Coastal species are among the most vulnerable to global environmental stressors as they are already jeopardized due to frequent exposure to local pollution like oil spill (Ghosh and Mandal, 2021). Environmental factors tend to modulate the resilience capacity of animals exposed to contaminants (Maulvault et al., 2019; Maynou et al., 2021; Baag and Mandal, 2022a). Multiple environmental perturbations might cause oxidative stress in an organism owing to incapacitated antioxidant defence mechanisms. Diverse categories of stressors in combination have a greater negative impact on oxidative stress responses and DNA damage of marine ectotherms (Braga et al., 2020). Besides affecting organisms' sensitivity to oil pollution, global environmental factors might also alter their toxicity potential by altering the bioavailability. Pollutants can hinder animal's homeostasis under climatic acclimation, which in turn might diminish the population persistence and cause additional ramifications on ecosystem functioning (Baag and Mandal, 2022b).

It is imperative to study the antioxidant defence mechanism of *S. serrata* under global change scenarios and pollution, as it holds a crucial position in India's aquaculture and rural economy. Considering meagre information is available regarding interactions involved in global environmental drivers (ocean warming and acidification) and local driver (oil pollution), the present study aims to elucidate the sensitivity and resilience of *S. serrata*, an economically important crustacean from SES, to multiple stressors using antioxidant based indicators. We theorise that multiple environmental perturbations will incapacitate antioxidant defence mechanisms and cause oxidative stress, as combinations of stressors tend to have greater negative impacts on oxidative stress responses and DNA damage in marine ectotherms (Braga et al., 2020). Besides affecting organismal sensitivity to oil pollution, global environmental factors might also alter their toxicity potential by altering the bioavailability. Pollutants can impact the ability of animals to maintain homeostasis in a changing climate, which may in turn diminish population persistence and alter ecosystem functioning (Baag and Mandal, 2022b). Studies of multiple stressors are crucial for fisheries and aquaculture and ecosystem based management, as climate change-related stressors are unlikely to occur in isolation (Baag and Mandal, 2022b).

The collective effect of multiple drivers can be additive or multiplicative and can work synergistically or antagonistically. The intention of our study was to compare and decipher the manifestation of multiple stressor effects and specifically whether global environmental drivers (ocean warming and acidification) aggravate the effects of a local driver (oil pollution) on antioxidant responses of *S. serrata*. We evaluated multiple biomarkers (antioxidant and detoxification defence mechanisms, lipid peroxidation levels, DNA damage) of the species to understand responses. Stress levels were quantified using an "Integrated Biomarker Response (IBR)" approach. To our knowledge, no prior studies have investigated acute exposures of oil pollution on the oxidative stress status of crabs acclimated under climate change conditions (combined ocean warming and acidification). The present work aims to understand the oxidative stress status of *S. serrata* when exposed to oil in a tropical area where the animals are near their thermal tolerance limits. We believe this study can provide an outline for more integrated management of this commercially exploited crab populations, and offer insights into coastal fisheries and aquaculture prospects as the climate changes.

2. Materials and methods

2.1. Species collection and experimental setup

Adult *Scylla serrata* (carapace length: 63.41 ± 4.08 mm, weight: 48.35 ± 4.79 g) were collected from the tidal creek of river Matla in Sundarbans and acclimated for two weeks (Temperature: 27.95 ± 0.36 °C, DO: 7.88 ± 0.07 mg L^{-1}, pH: 8.092 ± 0.025 and salinity: 22 psu) prior to experimentation. The crabs were fed with dried fish pellets till satiation and unconsumed food particles were removed from the aquaria (40 × 28 × 20 cm) daily. The artificial seawater (salinity = 22 psu) was replaced every day.

After acclimation, 120 animals were treated for 30 days under two scenarios separately (60 crabs in each scenario, 10 crabs in each aquaria): a) current environmental scenario (pH 8.1, 28 °C) and b) projected future climate change scenario for 2100 (pH 7.7, 34 °C). The intended temperatures were achieved by submerging digital thermostats in water baths holding experimental aquaria for the complete experimental period. Seawater acidification scenario was controlled using a pH stat system (Cole-Parmer, USA). The pH values were set and controlled by means of a computerized system connected to individual pH probes with automatic cut off mechanism controlling the CO_2 gas flux. After this experimental duration, 50% animals (30 crabs) from each treatment were exposed to 5 mg L^{-1} of marine diesel oil (MDO) for 24 h (maintaining the water quality parameters intact for each treatment group) and the other 50% were left uncontaminated. MDO is a blending of gasoil and heavy fuel oil used in marine diesel engines. MDO was purchased from a standard local fuel station near to fishing port for our experiment. The oil type and concentration were chosen to match a realistic oil spill scenario based on previous studies from different geographic locations (Bechmann et al., 2010; Sagerup et al., 2016; Mansour et al., 2017; Arnberg et al., 2019) and also from the present geographic location (Ghosh and Mandal, 2021). This resulted in 4 different treatment groups (triplicate aquaria per treatment with 10 crabs per aquaria, i.e., 30 crabs per treatment): 1) pH 8.1 and 28 °C in the absence of MDO; 2) pH 8.1 and 28 °C with MDO; 3) lower pH 7.7 and

elevated temperature 34 °C in the absence of MDO and 4) pH 7.7 and 34 °C with MDO. Multiple biomarkers were measured after exposure to treatment conditions.

Temperature, pH and salinity of artificial seawater were monitored continuously throughout the experimental duration. Total alkalinity was measured twice weekly using an alkalinity checker (HANNA HI755). CO_2SYS software (Lewis and Wallace, 1998) was used to calculate the carbonate chemistry parameters with constants from Mehrbach et al. (1973) and further refitted by Dickson and Millero (1987) (Table 1 for a summary of water quality parameters).

2.2. Biochemical assays

To analyse the biochemical biomarkers, the hepatopancreatic tissues were dissected and homogenised in a cold tissue homogenizer containing phosphate buffer (pH 7.2, 0.1 M, 4 °C). It was centrifuged at 10,000 rpm at 4 °C for 20 min and collected supernatant was used for the assays. The results were normalized as per the total protein content estimated by Bradford method (Bradford, 1976) using BSA as the standard.

Superoxide dismutase (SOD) activity was evaluated by measuring the rate of haematoxylin autooxidation inhibition following Martin et al. (1987). One unit of enzyme activity is equivalent to the enzyme concentration required to inhibit haematoxylin autooxidation by 50%. Catalase (CAT) activity was assessed by quantifying the rate of hydrogen peroxide degradation, the substrate of the enzyme (Aebi, 1984). Glutathione-S-transferase (GST) activity was determined in accordance to Habig et al. (1974) using CDNB as a substrate. Lipid peroxidation (LPO) level was measured following the method described in Ohkawa et al. (1979), by estimating formation of thiobarbituric acid reactive substances (TBARs).

2.3. DNA damage assay

An alkaline version of the comet assay was performed to assess DNA damage following methods described in Singh et al. (1988) with minor alterations as mentioned in Bhagat et al. (2017). The comet scoring was performed following Baag et al. (2021). Comet scoring was carried out using Image J with Open Comet plugin. Results are expressed in terms of the percentage of DNA migrated from the comet's head to tail region as Tail DNA (TDNA) and Olive tail moment (OTM), which is the product of the tail length and the fraction of total DNA in the tail.

2.4. Integrated biomarker response

Integrated biomarker response (IBR) was calculated following Beliaeff and Burgeot (2002) with modifications suggested by Devin et al. (2014) following Baag et al. (2021). Every individual biomarker response data was standardized using the formulae $Y = (X - m)/s$, where Y is the standardized biomarker response, X is response value of each biomarker at a given time; m and s are mean value and standard deviation, respectively. Z was then calculated using $Z = -Y$ or $Z = Y$, responding to a biological effect respectively for an inhibition or an induction. The scores (S) for the biomarker were computed as $S = Z + |\mathrm{Min}|$, where $|\mathrm{Min}|$ is the minimum value for each biomarker calculated from the standardized biomarker response. Star plots were then used to display score results (S) and to calculate the integrated biomarker response (IBR) as:

$$\mathrm{IBR} = \sum_{1}^{n} Ai.$$

$$A_i = S_i^{*} S_{i+1}^{*} sin(2\pi/k)/2$$

where, A_i is the triangular area represented by two consecutive biomarker scores on the star plot, S_i and S_{i+1} represent the individual biomarker scores (calculated from standardized data) and their successive star plot radius coordinates, and k represents the number of radii corresponding to the biomarkers used in the experiment.

2.5. Statistical analysis

PRIMER 6 software (Clarke and Gorley, 2006; Clarke et al., 2008) with the PERMANOVA + add on (Anderson et al., 2008) was used for statistical analyses. Permutational analysis of variance (PERMANOVA) was applied to investigate the effects of environmental factors and oil exposure individually and together on multiple biomarkers. The main test was conducted using environmental scenario and oil exposure as fixed factors. Only whenever the main test revealed statistical significance ($p \leq 0.05$), pairwise evaluations were performed.

3. Results

3.1. Biochemical assays

There were significant individual and interactive effects of climate change factors and oil on SOD activity (Table 2). A significant increase in the enzyme activity was observed only at oil treatment of control condition but in both the climate change (CC) scenario the activity significantly decreased compared to the control (Fig. 1a). The SOD activity was also statistically insignificant between the two CC treatments (OWA and OWA + Oil).

Significant effects of climate change factors and oil was recorded in CAT activity individually, albeit not among their interactions (Table 2). A significant increase was observed in the enzyme activity of all treatments compared to control (Fig. 1b). Among the treatment groups, the highest activity was found in combined OWA scenario and oil treatment (pH 7.7 and 34 °C with MDO) while the lowest being in the treatment with MDO at present environmental scenario (pH 8.1 and 28 °C with MDO).

GST activity remarkably varied with climate change factors and oil, as well their interaction (Table 2). A significant difference was noted among all the treatment groups. Significant increases in the enzyme activity were observed in all treatment groups with respect to control (Fig. 1c). The highest activity was found in the OWA scenario (pH 7.7 and 34 °C in absence of MDO) while the lowest being in the treatment with MDO at present environmental scenario (pH 8.1 and 28 °C with MDO).

LPO levels differed significantly across all treatments and were affected by climate change factors and oil both independently and interactively (Table 2). Significant higher LPO levels were observed in all treatment groups with respect to control (Fig. 1d). Treatment with oil in both the environmental scenario induced significant LPO levels with the highest in combined exposure (pH 7.7 and 34 °C with MDO).

Table 1
Water quality parameters (Temperature, Salinity, pH, TA (total alkalinity), DIC (dissolved inorganic carbon), pCO2 (partial pressure of carbon dioxide), Ωcal (saturation state for calcite), Ωara (saturation state for aragonite) maintained in different treatments throughout the experimental period in the aquaria containing the animals. Values represent mean ± SD.

	Control (28 °C, pH 8.1)	OWA (34 °C, pH 7.7)
Temperature (°C)	27.95 ± 0.36	33.97 ± 0.31
Salinity	22	22
pH	8.092 ± 0.025	7.706 ± 0.012
TA (μmol kg^{-1})	2374.5 ± 50.44	2170.8 ± 60.49
DIC (μmol kg^{-1})	2116.41 ± 51.84	2055.76 ± 57.24
pCO_2 (μatm)	416.34 ± 33.70	1060.17 ± 34.89
Ωcal	5.501 ± 0.25	2.91 ± 0.12
Ωara	3.485 ± 0.16	1.878 ± 0.07

Table 2
Summary of the main test of PERMANOVA exposed to different treatments ($p \leq 0.05$).

	SOD			CAT			GST		
Source of variation	MS	F	P	MS	F	P	MS	F	P
OWA	126.1	387.56	0.002	46.395	77.649	0.002	765.04	255.15	0.002
Oil	2.5697	7.8977	0.023	143.21	239.69	0.003	162.83	54.304	0.005
OWAXOil	6.2676	19.263	0.004	2.6747	4.4766	0.061	453.99	151.41	0.002

	LPO			Tail DNA			OTM		
Source of variation	MS	F	P	MS	F	P	MS	F	P
OWA	807.89	74.399	0.005	240.52	120.38	0.005	91.905	4.4621	0.075
Oil	1638.7	150.91	0.003	3.1159	1.5595	0.255	36.721	1.7829	0.217
OWAXOil	293.52	27.03	0.003	13.684	6.8488	0.033	1.3946	0.06771	0.81

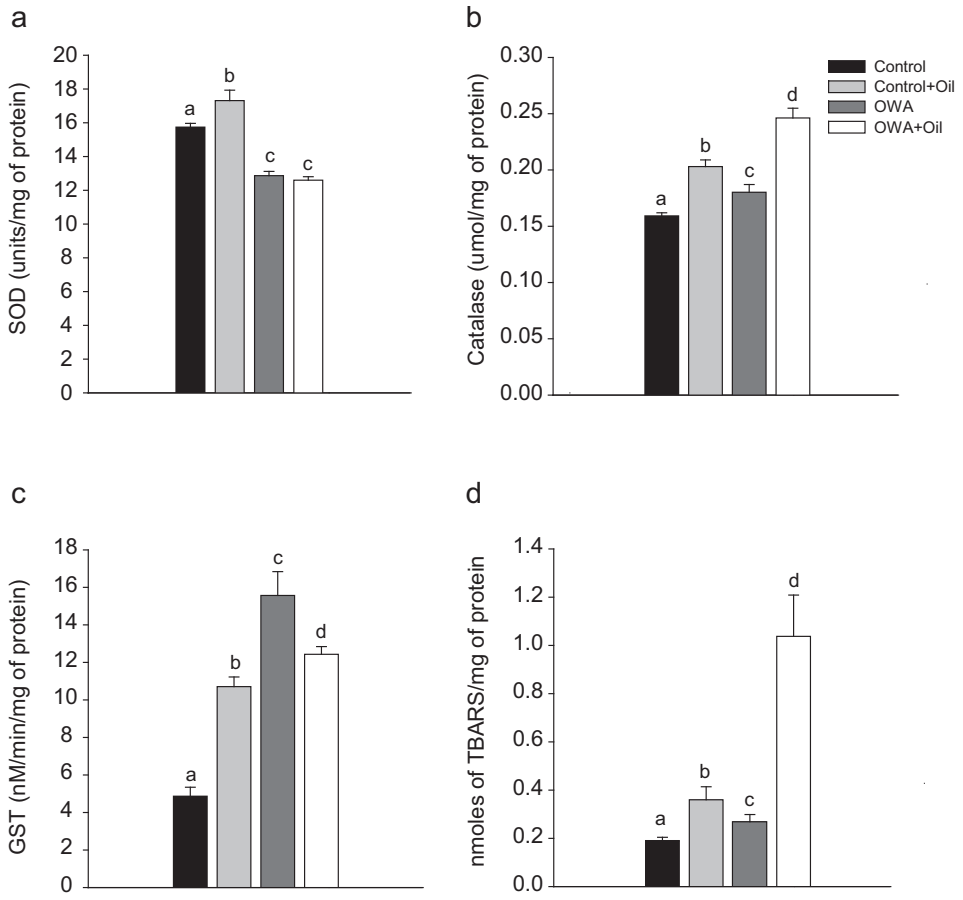

Fig. 1. Changes in SOD activity (a), CAT activity (b), GST activity (c), LPO levels (d) of *Scylla serrata* under different treatments of the experiment. Values represent mean ± SD ($n = 9$, $p < 0.05$). Different letters (a, b, c, d) indicate significant differences between treatment groups.

3.2. DNA damage assay

A statistically significant difference was noticed in Tail DNA percentage between climate change factors and its interaction with oil but not under oil exposure independently (Table 2). Tail DNA percentage was significantly high in both CC scenario irrespective of oil exposure (pH 7.7 and 34 °C with MDO and in absence of MDO) (Fig. 2a). Olive tail moment (OTM) was statistically indistinguishable between climate change factors, oil and their interaction (Fig. 2b).

3.3. Integrative biomarker response

The IBR was assessed with five biomarkers (SOD, CAT, GST, LPO and Tail DNA%) for all the treatments. The standardized biomarker responses are projected in a star plot (Fig. 3a) used in the IBR calculation. The IBR results are presented in the Fig. 3b. IBR results confirmed that SOD and CAT show the most responsiveness whereas GST and LPO were the least responsive among biomarkers in this crab species (Table 3).

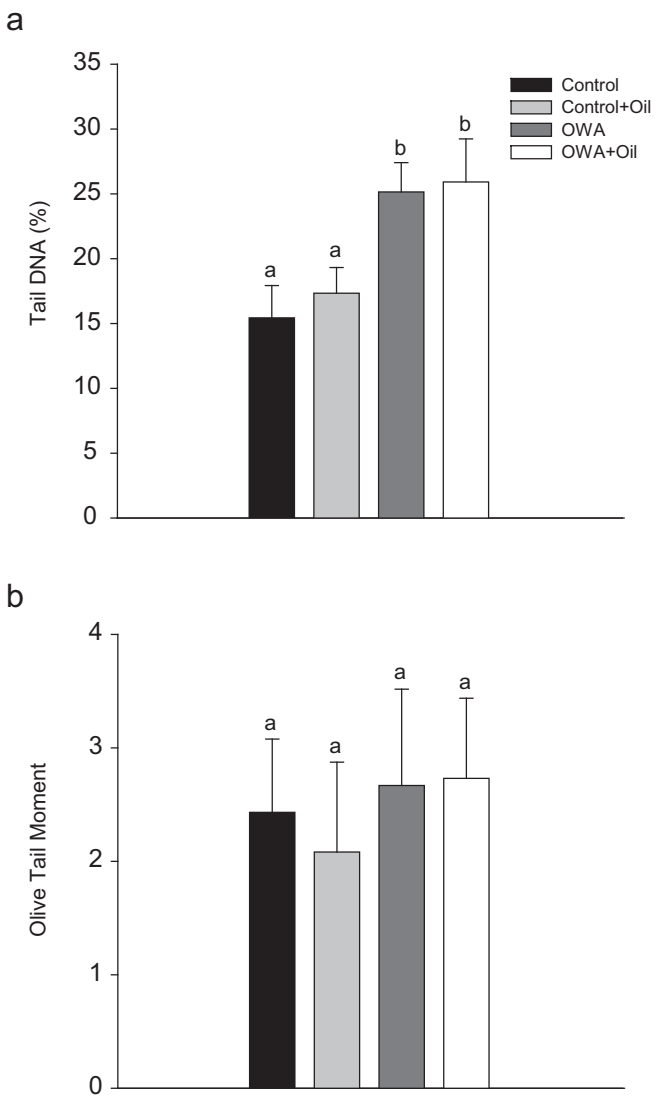

Fig. 2. Changes in Tail DNA percentage (a) and Olive Tail Moment (b) of *Scylla serrata* under different treatments of the experiment. Values represent mean ± SD ($n = 6$, $p < 0.05$). Different letters (a, b, c, d) indicate significant differences between treatment groups.

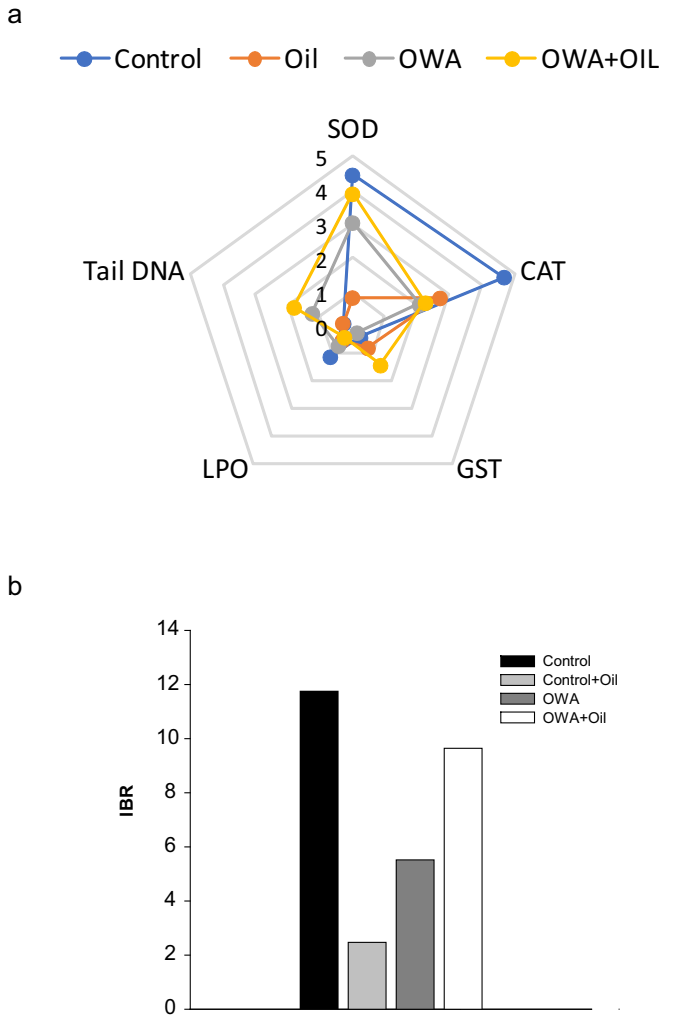

Fig. 3. Star plots representing standardized biomarker response (SOD, CAT, GST, LPO and Tail DNA) (a), Integrated biomarker response (IBR) score (b) for different stress treatments in *Scylla serrata* exposed to different treatments.

Table 3
Standardized biomarker response values for different stress biomarkers- SOD, CAT, GST, LPO and Tail DNA in *Scylla serrata.*

	Control	Oil	OWA	OWA + OIL
SOD	4.45	0.84	3.05	3.89
CAT	4.68	2.7	2.07	2.25
GST	0.4	0.77	0.22	1.41
LPO	1.1	0.5	0.69	0.39
Tail DNA	0.26	0.29	1.23	1.8

4. Discussion

4.1. Biochemical assays

SOD and CAT are first line antioxidant defence mechanisms against ROS in crustaceans and are sensitive to environmental perturbations (Pinheiro and Oliveira, 2016). In the present study, SOD activity increased in only oil treatment at control condition but in both the OWA scenario the activity significantly decreased irrespective of oil exposure. A similar pattern in SOD activity have been described in the intertidal clam *Ruditapes decussatus* exposed to forecasted OWA scenario in presence of the drug diclofenac (Costa et al., 2020a) and the antimicrobial agent triclosan cross treatment (Costa et al., 2020b). Oil exposure activated defence mechanisms in crabs as mirrored by high SOD activity. However, OWA scenario negatively affected the SOD activity by decreasing their antioxidant capacity and revealed a greater sensitivity of crabs to contaminants after exposure to warming and acidified conditions for one month. The inhibition of enzyme activity in cross stress reveals compromised defences and higher adaptive capability under individual stressors. SOD activity showed a significant increase in *Macrobrachium borellii* exposed to petroleum (Lavarías et al., 2011) and crab *Scylla serrata* exposed to PAH and PCB (Ragunathan, 2017) to counter increased ROS production. In contrast, SOD activity decreased in response to ocean acidification scenario in the crab *Homarus gammarus* suggesting disrupted extracellular buffering capacity in acidic conditions (Rato et al., 2017) which corroborates our finding.

CAT activity significantly increased in crabs exposed to oil at both current and projected environmental scenarios in our experiments here. Analogous results were observed for the clam *R. decussatus* exposed to a forecasted CC scenario in the presence of diclofenac (Costa et al., 2020a) and triclosan cross treatments (Costa et al., 2020b). CAT activity was significantly high in mussels *Mytilus coruscus* (Hu et al., 2015; Khan et al., 2021) and gastropods *Trochus niloticus* co-exposed to ocean acidification and warming (Zhang et al., 2021). Shrimp *Litopenaeus*

vannamei (Muralisankar et al., 2021) and scallops *Flexopecten glaber* (Nardi et al., 2018) exposed to acidified seawater also showed elevated CAT activity. These findings are in agreement with our results and suggest that acidification promotes oxidative stress by generating peroxides. CAT activity was also found to be elevated in *M. borellii* exposed to petroleum compared to their control counterparts (Lavarías et al., 2011). These results validate our findings that CAT activity increased for regulation of ROS. Exposure to oil caused an overall activation of CAT activity regardless the climate scenario.

The activity of the biotransformation enzyme GST was significantly elevated in OWA conditions irrespective of contamination. Similar findings were reported in clams *Ruditapes* sp. where GST activity was significantly elevated in contaminated organisms exposed to OWA scenario (Costa et al., 2020a, 2020b). GST activity was higher in *M. borellii* exposed to petroleum in comparison to controls (Lavarías et al., 2011). In crabs *Carcinus maenas* high GST activity was recorded because of hydrocarbon exposure. The response of GST to chemical pollutants might be triggered as part of the phase II biotransformational pathway. GST activity was also elevated in crab *Minuca rapax* exposed to copper at high temperature (Capparelli et al., 2019). Acidified conditions also promoted GST activity in the scallop *F. glaber* (Nardi et al., 2018). The copepod *Calanus pacificus* had higher GST activity exposed to combined OWA (Engström-Öst et al., 2019). In Antarctic scallop *Adamussium colbecki*, similar elevation in enzyme activity was noticed when exposed to OWA in presence of contaminant (Benedetti et al., 2016). The upsurge activity of GST under combined multiple drivers confirms activation of the antioxidant and detoxification mechanisms. This also confirms the induction of oxidative stress owing to enhanced ROS production. We accentuate that CAT and GSTs play the central role as effective barriers against oxidative stress in this study.

An increase in intra and extracellular ROS can amplify the consumption of reducing equivalents, accelerating protein oxidation and lipids peroxidation (de Oliveira et al., 2015; Grilo et al., 2018). Lipid peroxidation is regarded as one of the chief molecular mechanisms associated with oxidative damage. In present study, LPO levels were higher in organisms exposed to multiple stressors, highest in the cross-stress (OWA + Oil) treatment. This observation indicates inefficient activation of defence mechanisms by crabs. Similar findings were also reported in studies of other species around the globe. Higher LPO values were observed in contaminated clams *R. decussatus* under both current and projected environmental scenarios (Costa et al., 2020b). Significant increase in LPO was noted in L. *vannamei* exposed to acidification which confirmed that the acidic environment could initiate membrane damage (Nardi et al., 2018). Increase in the LPO was observed in crabs *S. serrata* and *C. maenas* due to higher concentration of petroleum hydrocarbon in water (Ragunathan, 2017). Similar observation was noted in prawn *M. borellii* exposed to petroleum (Lavarías et al., 2011). Under combined elevated temperature and acidification condition, the antioxidant defences of *Littorina obtusata* (Cardoso et al., 2017) and gastropod *T. niloticus* (Zhang et al., 2021) were suppressed leading to peroxidative damage which was also observed in our study. Similar damage was observed when scallop *A. colbecki* was exposed to OWA in presence of a contaminant (Benedetti et al., 2016). Higher LPO levels in cross stress treatments suggests that antioxidant defence system could not counterbalance excessive ROS produced resulting in oxidative damage.

4.2. DNA damage assay

A functioning DNA repair system requires an equilibrium between ROS formation and elimination. Amplified ROS formation overwhelms the antioxidant defence mechanism in cells which may lead to DNA damage. A handful of prior studies have considered genotoxicity in marine crustaceans. Ocean acidification significantly increased the toxicity responses to metal contaminants in shellfishes regarding DNA damage (Roberts et al., 2013; Lewis et al., 2016). These findings corroborate our results and clearly demonstrate the potential of projected OA conditions to increase the susceptibility of shellfishes to various contaminants. Under ambient conditions, oil contamination did not cause significant DNA damage to affect the ability of the crabs to use their antioxidant defences to prevent damage or to activate various efficacious DNA repair pathways. Contrastingly, in the amphipods *Gammarus locusta,* the combined OWA and contamination treatment did not damage the DNA of haemocytes, suggesting adaptation potential (Cruzeiro et al., 2019).

4.3. Integrative biomarker response

Analysing the effects of environmental stressors is a challenging task, especially when multiple stressors interplay. In our results the IBR analysis reflected the highest value in control treatment, which is a rare observation in such experiments. In presence of stress and toxic chemicals, antioxidant defence might decline as a consequence of enhanced catabolic rate and/or a direct inhibition wielded by the pollutant (Baag et al., 2021). A decrease in the IBR value of SOD clearly explains the decrease in enzyme activity in treatments compared to control. IBR value of CAT also reflected a similar trend and justified the insignificant effect of stressors' interaction. Higher IBR value of Tail DNA percentage in the CC scenario, irrespective of oil exposure, also explains the comet scoring patterns. The present results accentuate the vulnerability of crab population to climate change and acute pollution exposure.

5. Conclusion

The present study provides insights into the antioxidant defence mechanism of crabs exposed to acute oil pollution after acclimation under combined interactive effects of ocean acidification and warming. This study provides a clear understanding about biochemical responses of crabs exposed to acute pollution and global environmental drivers. The results suggest that future climate is expected to exacerbate the effects of acute pollution and cause oxidative stress leading to DNA damage in *S. serrata*. Integrated biomarker approaches increased our understanding of the antioxidant responses in multiple stressor scenarios relative to individual biomarkers in isolation. Our work emphasizes the importance of including environmentally relevant drivers, such as climate stressors, in toxicological studies. The present work also highlights the role of natural stresses in enhancing a pollutant's toxicity to aquatic animals. With the projected risk of OWA and oil spills in the study region, the population of *S. serrata* may face local extinction in the absence of resilience capability which might have significant repercussion to the local aquaculture industry in Sundarbans. This will significantly jeopardize the rural economy as well the ecological balance of the largest mangrove ecosystem in the world. Future research on impacts of global environmental drivers combined with contamination on crab ecotoxicological responses would facilitate understanding of future stock production and envisioning ramifications in the future oceans.

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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