CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Agrobiology, Food and Natural Resources

Department of Zoology and Fisheries

Czech University of Life Sciences Prague

Impacts of environmental characteristics and human activities on morphology, behaviour, and physiology of aquatic animals

Doctoral Dissertation

Author : **Chandani Verma, M.Sc.**

Supervisor : **Prof. Ing. Lukáš Kalous, Ph.D.**

Prague 2 0 2 4

Declaration

I, Chandani Verma, hereby declare that I wrote the dissertation: “Impacts of environmental characteristics and human activities on morphology, behaviour, and physiology of aquatic animals” and it was prepared under the guidance of Prof. Lukáš Kalous, based on my own or group projects with my colleagues, using the scientific references that are properly cited and the work was not used to obtain another or the identical academic title. Please note that articles presented in this work that are not ‘Open Access’ are my personal copies, printed here only for dissertation purposes, and can not be further distributed.

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Summary

This thesis investigates the impacts of environmental factors, parasitic infections, pollutants, and biological invasions on the morphology, physiology, and behavior of aquatic organisms, with a focus on fish in the Western Ghats of India and invasive species in Central Europe. The research reveals how environmental pressures drive adaptive changes in species and how human-induced pollutants and invasive species disrupt ecosystem dynamics.

The study first examines micro-adaptations in the lepidophagous catfish *Pachypterus khavalchor*, highlighting how ecological competition shapes specialized feeding adaptations. This species evolved unique morphological features, such as specialized dentition, allowing it to feed on scales and reduce food competition. This adaptation underscores the role of ecological pressures in driving morphological evolution. The thesis also explores the physiological costs of parasitism, focusing on *Contracaecum* nematode infections in hillstream loaches. These parasites induce oxidative stress and muscle damage, impairing host mobility and increasing vulnerability to predators. The research emphasizes the importance of parasitic interactions in influencing host health and behavior, with implications for population dynamics.

Pollution effects on aquatic organisms were another major focus, revealing how microplastics, pharmaceuticals, and phthalates disrupt vital behaviors and physiological functions. Microplastic contamination was prevalent in river and coastal sediments, with eco-morphological features influencing ingestion rates in species like mudskippers. Pollutants like diethyl phthalate affected predator-prey interactions in loaches and impaired regeneration in planarians, demonstrating how chemical contaminants can undermine survival and reproduction. Lastly, the thesis addresses biological invasions, documenting the detection of the invasive Chinese sleeper in Czechia through eDNA methods. This invasive species poses a threat to native ecosystems by altering food web dynamics and competing with indigenous species.

In summary, this research provides a detailed view of the multifaceted pressures on aquatic ecosystems, highlighting the need for integrated environmental management and conservation strategies to mitigate these impacts and protect biodiversity.

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Chapter 1

Literature Review

1.1. General structure and function of aquatic ecosystem

Aquatic ecosystems are dynamic and complex systems that consist of interactions between biological communities and their water environments. These ecosystems are crucial for maintaining ecological balance as they perform various essential functions, such as recycling nutrients, purifying and storing water, maintaining stream flow, and providing habitats for diverse organisms (Schindler & Scheuerell, 2002). Aquatic ecosystems are broadly classified into two primary types: marine ecosystems and freshwater ecosystems (Barange et al., 2010). Marine ecosystems, which cover more than 70% of the Earth's surface, include oceans, coral reefs, estuaries, and coastal areas. On the other hand, freshwater ecosystems, covering less than 1% of the Earth's surface, consist of lentic (still water), lotic (flowing water), and wetland systems. Despite their smaller coverage, both types of ecosystems are vital to global ecological processes, contributing to nearly half of the world's net primary production (Alexander, 1999). Marine ecosystems, due to their vast coverage, play a significant role in regulating the Earth's climate and supporting global biodiversity. They are key players in carbon cycling, with marine organisms such as phytoplankton contributing to oxygen production through photosynthesis, helping to regulate atmospheric carbon dioxide levels. Similarly, freshwater ecosystems are essential in providing resources such as drinking water, sanitation, and agricultural irrigation, making them vital for both ecological balance and human survival. Given their roles in maintaining biodiversity, regulating climate, and supporting life, aquatic ecosystems are recognized as critical components of the Earth's ecological infrastructure. However, human activities such as pollution, overexploitation, and habitat destruction have severely impacted these ecosystems, making their conservation and sustainable management a global priority (Eugene & Oh, 2004; Griffith et al., 2005; Verhougstraete et al., 2015; Beiras, 2018).

Aquatic ecosystems also serve as a source of food, transportation, and recreation. Marine ecosystems provide raw materials for fertilizers, additives, and cosmetics, while freshwater ecosystems are critical for providing potable water for various human activities, including agriculture and industry. The importance of aquatic ecosystems in providing ecosystem services and supporting biodiversity cannot be overstated. The continued degradation of these systems could result in a loss of biodiversity, diminished water quality, and reduced ecosystem services, affecting not only aquatic life but also human populations that depend on these resources (Chivian, E., 2002; Rotter, A. et al., 2021;).

Fig.1. Schematic of an aquatic ecosystem showing few environmental (green) and anthropogenic (orange) factors affecting aquatic life.

Aquatic organisms play a fundamental role in the functioning of aquatic ecosystems, contributing to both ecological processes and food webs. The diversity of organisms found in aquatic environments is vast, ranging from microorganisms such as bacteria and phytoplankton to larger species such as fish, birds, and marine mammals (Postel et al., 2012). Phytoplankton, for example, are tiny plants that perform photosynthesis, producing approximately 50% of the world's oxygen and forming the base of aquatic food chains. Zooplankton, small animals that feed on phytoplankton, are critical intermediaries in the transfer of energy and nutrients through aquatic food webs. Other aquatic organisms, including invertebrates, fish, and mammals, rely on these lower trophic levels for sustenance, highlighting the importance of maintaining biodiversity in these ecosystems (Karlusich et al., 2020; Dahlin et al., 2021). Microbes are another essential component of aquatic biodiversity, as they play significant roles in biogeochemical cycles, including the cycling of carbon, nitrogen, phosphorus, and sulfur. Microbial communities facilitate the transport of nutrients and the degradation of organic matter, contributing to the overall health of aquatic ecosystems (Simon et al., 2002; Sang et al., 2018). The structure and function of these microbial communities are closely linked to the environmental conditions within an ecosystem, influencing nutrient cycling and pollutant mitigation. The diversity of these microbial communities ensures that ecosystems remain resilient in the face of environmental stressors, making them a critical focus for ecological research (Huang et al., 2022).

In addition to their ecological importance, aquatic organisms are economically valuable. Fisheries, which rely on healthy populations of aquatic species, provide food and livelihoods for millions of people worldwide (Finegold, C., 2009). However, overfishing and unsustainable fishing practices threaten the balance of these ecosystems by depleting key species and disrupting food webs (Sumaila, U., 2020). For example, the depletion of predatory fish can lead to an overabundance of smaller species, which in turn affects the entire food chain. Sustainable practices, including the establishment of marine protected areas and the implementation of fishing quotas, are essential to preserving aquatic biodiversity and ensuring the continued provision of ecosystem services (Sumaila, U., 2020). Aquatic ecosystems, through their biological diversity and ecological functions, maintain the health of global ecosystems. Organisms at different trophic levels, from phytoplankton to top predators, interact in complex ways that sustain life and contribute to the overall functioning of these ecosystems. Understanding these interactions and the role of various species in the food web is essential for managing aquatic environments sustainably (Dahlin et al., 2021). As the pressures on aquatic ecosystems continue to increase due to human activities, the need for conservation efforts that address both ecological and economic concerns becomes even more critical.

1.2. Role of physical factors in aquatic ecosystem

Water temperature plays a critical role in the metabolic processes, growth, and reproductive cycles of aquatic organisms. According to Shelford's law of tolerance, each organism has an optimal environmental range in which it can thrive, including a specific temperature range (Dong & Tian, 2023). When temperatures fall outside this range, organisms may experience stress, leading to decreased growth rates and lower survival. Changes in water temperature can affect not only individual organisms but also broader ecosystems, including the dynamics of food chains and energy flow. For instance, increased water temperatures can result in oxidative stress for aquatic organisms, influencing their physiological responses and immune systems, especially in the interconnected food web of plankton, invertebrates, and fish (Lydy et al., 1999). Climate change has significantly influenced temperature patterns in aquatic environments, leading to warming surface waters and shifts in species distributions (Benedetti et al., 2021). As temperatures rise, the metabolic rates of aquatic organisms increase, which can alter their life cycles and reproduction timings. For species that rely on specific temperature conditions, such as certain fish during spawning seasons, these shifts can disrupt their reproductive success (Reid et al., 2019). Moreover, higher temperatures have been linked to increased prevalence of marine diseases and a higher risk of hypoxia in aquatic ecosystems. This underscores the urgency of understanding temperature-induced changes and developing strategies to mitigate their impact on aquatic ecosystems.

Dissolved oxygen is another crucial factor that directly affects the survival and behavior of aquatic organisms. Oxygen is required for respiration, which sustains the metabolism of most aquatic species. However, dissolved oxygen levels can fluctuate due to various factors such as temperature, salinity, and water movement. Lower oxygen levels, often caused by increased water temperature, can lead to hypoxic conditions, which have profound effects on the health of aquatic ecosystems (Domenici et al., 2007). Hypoxia forces fish and other species to exhibit stress behaviors, such as moving to the water's surface to obtain oxygen, which increases their vulnerability to predators. In environments with low oxygen levels, fish and other aquatic species face physiological challenges that limit their ability to escape from predators or efficiently hunt prey. Hypoxic waters create scenarios where predator-prey dynamics are altered, often to the detriment of prey species, which are less capable of escaping predation (Meager & Batty, 2007). Hence, understanding the dynamics of dissolved oxygen levels and their effect on aquatic species is essential in addressing the increasing stress on aquatic ecosystems due to environmental changes. The pH of water is a significant environmental factor in aquatic ecosystems, influencing the survival of organisms and the overall health of ecosystems. Acidic waters, caused by acid rain or industrial pollutants, lower the pH and can lead to harmful ecological changes, including the degradation of water quality and the loss of biodiversity. When the pH drops too low, it can increase the solubility of toxic metals, such as aluminum, which can be lethal to fish and invertebrates (Driscoll, & Wang, 2019). Additionally, organisms sensitive to pH fluctuations, like certain fish species and plankton, may experience physiological stress, impacting their reproduction and survival. Acidification is closely linked to the broader issue of climate change, as increased carbon dioxide levels lead to the formation of carbonic acid in water, lowering pH levels (Reid et al., 2019). Acidified waters particularly affect calcifying organisms, such as mollusks and corals, which rely on calcium carbonate to build their shells and skeletons. These organisms are especially vulnerable to reduced pH levels, as it impedes their ability to grow and survive (Doney et al., 2009; Van de Waal et al., 2013). Consequently, acidification is a growing concern for aquatic ecosystems, as it poses long-term threats to biodiversity and ecosystem stability.

Light availability plays a crucial role in photosynthesis, which is vital for aquatic plants and phytoplankton, the primary producers in aquatic ecosystems. Photosynthesis supports oxygen production and forms the basis of aquatic food webs. However, light penetration can be reduced by factors like water turbidity and sedimentation, affecting submerged vegetation and limiting photosynthesis (Lehtiniemi et al., 2005). Reduced light availability not only affects photosynthetic organisms but also impacts predator-prey interactions by diminishing visibility, especially for predators relying on visual cues (Meager & Batty, 2007). Eutrophication, driven by excessive nutrient input such as nitrogen and phosphorus, exacerbates this issue by promoting dense algal blooms. These blooms reduce water clarity, limiting light penetration and further restricting photosynthetic activity. Human-induced eutrophication, or cultural eutrophication, leads to widespread consequences, including algal blooms, degraded water quality, and hypoxic conditions harmful to aquatic ecosystems (Carpenter et al., 1998; Schindler, 2006; Bhat et al., 2017). As algal blooms block light, they harm the growth of aquatic plants in littoral zones and reduce predator efficiency in locating prey, compounding the detrimental effects of reduced light availability (Lehtiniemi et al., 2005).

1.3. Predators and parasites in aquatic ecosystem

Biotic factors, such as predation pressure and competition for food, can profoundly affect the morphology and behavior of organisms. In scale-eating cichlids like *Perissodus microlepis* from Lake Tanganyika, food competition has led to morphological adaptations, notably craniofacial asymmetry. This asymmetry improves feeding efficiency by expanding the contact area between the predator’s teeth and the prey’s scales. Over time, successful predation has also been associated with the development of behavioral lateralization, where individual fish exhibit a preference for attacking prey from a specific side, either left or right, thereby improving foraging efficiency in a resource-scarce environment (Takeuchi et al., 2016).

Additionally, species of *Roeboides* consume scales selectively during the low-water season when competition for insect prey is intense (Peterson and Winemiller, 1997; Peterson and McIntyre, 1998). This suggests that heightened food competition may also increase aggression and promote lepidophagy. These morphological and behavioral changes illustrate how biotic factors like food competition shape predator-prey interactions, driving evolutionary adaptations.

Parasites play a crucial role in aquatic ecosystems, representing one of the most successful modes of life in nature (Poulin and Morand, 2000). They have independently evolved across multiple phyla, forming extensive host-parasite relationships that impact ecosystem dynamics. Parasitism is widespread, constituting a significant proportion of global biodiversity and influencing biomass, abundance, and productivity in various ecosystems (Dobson et al., 2008; Hechinger et al., 2011). These relationships are shaped by antagonistic coevolution, where parasites adapt to exploit specific biological traits of their hosts, ensuring successful transmission and survival. Consequently, parasites impact host organisms at multiple levels, from gene expression to population dynamics, influencing host morphology, reproduction, behavior, and overall survival (Marcogliese, 2004). Aquatic ecosystems, in particular, are vulnerable to parasitic infections due to the strong interconnectivity between organisms in the food web. Fish parasites, a major component of aquatic biodiversity, often become involved in the host's ecological processes, serving as bioindicators of environmental changes. Parasites can provide valuable insights into the feeding, migration, and population structure of their hosts. Furthermore, parasites are sensitive to environmental stressors such as pollutants and eutrophication, which makes them useful tools for monitoring ecosystem health (H.W. Palm, 2011). As global warming alters aquatic environments, parasites, along with their hosts, are subjected to changing conditions. These shifts can either increase or decrease parasitic burdens depending on the species and environmental factors involved, further demonstrating the importance of assessing parasites' role in ecosystems (Marcogliese, D. J., 2001; Cable et al., 2017).

Parasites impose significant energetic costs on their hosts, which can lead to pathological, immunological, and physiological effects. In fish, parasitic infections can cause oxidative stress, impair immune function, and alter essential behaviors such as predator avoidance and feeding. For example, studies have shown that parasites can reduce host fitness by disrupting normal physiological processes, manipulating host behavior, and increasing host vulnerability to predation (Sures et al., 2001; Münderle et al., 2004; Gérard et al., 2016). This can have cascading effects on fish populations and the broader aquatic community. Fish heavily infected with parasites may experience a reduction in fecundity, altered reproductive cycles, and increased mortality rates, particularly if the parasites cause significant pathological damage or act as endocrine disruptors (Sokolowski and Dove, 2006; Trubiroha et al., 2010). Additionally, parasites that alter host behavior to increase susceptibility to predation can drastically affect predator-prey dynamics within an ecosystem (Lafferty and Morris, 1996). One of the critical impacts of parasites on fish physiology is their role in inducing oxidative stress. Oxidative stress occurs when the balance between reactive oxygen species (ROS) production and antioxidant defenses is disrupted, leading to cellular damage. Parasites, like other environmental stressors, can elevate ROS levels in fish, resulting in lipid peroxidation, protein oxidation, and DNA damage (Belló et al., 2000; Sures et al., 2017). This oxidative stress can severely impact fish health by weakening their immune defenses and impairing nutrient absorption (Kiron, 2012). The response of fish to oxidative stress involves a range of antioxidant defense mechanisms, including enzymatic systems such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Di Giulio and Meyer, 2008; Folgueira et al., 2019). These enzymes play a crucial role in neutralizing ROS and preventing damage to cellular macromolecules. For instance, SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide, which is then converted to water by catalase, thereby protecting cells from oxidative damage (Folgueira et al., 2019).

In addition to oxidative stress, parasitic infections can interfere with neurotransmitter functions and stress responses in fish. Acetylcholinesterase (AChE), an enzyme responsible for breaking down the neurotransmitter acetylcholine, is often used as a biomarker to assess neurotoxic effects in fish (Adams, 2001). Parasite-induced disruptions to AChE activity can affect locomotion, balance, and predator-avoidance behaviors, leaving fish more vulnerable to predation and other environmental stressors (Bretaud et al., 2000). Such disruptions in normal physiological responses highlight the far-reaching effects of parasitic infections on fish health and survival, especially in ecosystems where environmental stressors like pollution exacerbate these impacts. The cumulative effects of parasitic infections on fish health underscore the importance of understanding how parasitic infections impact fish physiology and behavior is crucial for managing aquatic ecosystems, particularly as environmental changes continue to exacerbate stressors. The role of parasites as both stressors and bioindicators in aquatic environments makes them a critical area of study, with significant implications for biodiversity conservation (Lushchak, V. I., 2011;Valavanidis et al., 2006; Chowdhury et al., 2020)

1.4. Pollution in aquatic ecosystem

Aquatic pollution has emerged as a major global issue, significantly affecting the health and sustainability of aquatic ecosystems. These ecosystems, which include both marine and freshwater environments, are crucial for maintaining biodiversity and providing essential ecosystem services such as food, water, and economic resources (Barange et al., 2010). Unfortunately, anthropogenic activities, such as industrialization, agriculture, and urbanization, have led to the introduction of numerous pollutants into these systems. These pollutants, including agrochemicals, heavy metals, industrial solvents, and household waste, pose serious threats to aquatic organisms and disrupt ecosystem functions (Verhougstraete et al., 2015; Beiras, 2018). Polluted water bodies not only endanger the supply of clean drinking water but also degrade ecosystem processes, impacting the health of aquatic organisms and reducing biodiversity (Hampel et al., 2015). The sources of aquatic pollution are varied, with agriculture, industrial effluents, and urban runoff being the main contributors. Agriculture, responsible for 70% of global water usage, is a significant contributor to aquatic pollution through the discharge of fertilizers, pesticides, and organic matter into water bodies (Mateo-Sagasta et al., 2017). These pollutants degrade water quality and pose risks to both aquatic life and human health. For instance, pesticides such as insecticides, herbicides, and fungicides are washed into aquatic systems, where they accumulate in organisms, leading to toxic effects that propagate through the food chain and may eventually impact human populations (Mateo-Sagasta et al., 2017). Industrial waste, including heavy metals like cadmium, mercury, and lead, as well as volatile organic compounds, is another major source of contamination. These chemicals are often discharged into water bodies without adequate treatment, resulting in the bioaccumulation of toxic substances in aquatic organisms, which can lead to long-term environmental damage (Demirak et al., 2006; Hampel et al., 2015).

Sewage is one of the largest contributors to aquatic pollution and has a profound impact on freshwater ecosystems. It is estimated that 58% of urban wastewater and 81%

of industrial waste is discharged into water bodies without proper treatment, leading to the contamination of approximately 73% of water bodies globally (Vargas-Gonzalez et al., 2014). Sewage contains a mix of organic matter, heavy metals, nutrients, pharmaceuticals, and personal care products that contribute to eutrophication and oxygen depletion in water bodies (Akpor & Muchie, 2011; Bhat et al., 2017). The high organic load of sewage increases the biological oxygen demand (BOD), which in turn lowers dissolved oxygen (DO) levels in aquatic systems. Low DO levels, often below 5 mg/L, can impair the functioning of fish and other aquatic organisms, leading to population declines and altering ecosystem dynamics (Momba et al., 2006). Additionally, eutrophication caused by excessive nutrient input from sewage and agricultural runoff results in harmful algal blooms, further reducing water quality and biodiversity (Schindler, 2006). The impact of pollutants on aquatic organisms is multifaceted, affecting their physiology, behavior, and overall survival. For instance, heavy metals can accumulate in fish and other organisms, causing oxidative stress, cellular damage, and reproductive dysfunction (Bhat et al., 2017). Pesticides may induce neurotoxic effects, impairing the ability of fish to navigate, avoid predators, or find food (Schreinemachers & Tipraqsa, 2012). Sewage-induced eutrophication disrupts the base of aquatic food webs by reducing light availability and oxygen levels, which directly affect photosynthetic organisms such as phytoplankton (Carpenter et al., 1998). These changes have cascading effects throughout the food web, impacting higher trophic levels such as zooplankton, fish, and marine mammals, ultimately threatening the stability and functioning of aquatic ecosystems (Bhat et al., 2017).

In recent years, plastic pollution has become an increasingly critical concern for aquatic environments, particularly microplastics. These small plastic particles, resulting from the breakdown of larger debris, are ubiquitous in both marine and freshwater systems (Hampel et al., 2015). Microplastics are ingested by various aquatic organisms, leading to physical harm, reduced feeding efficiency, and behavioral changes. Moreover, plastics can adsorb and transport toxic chemicals, such as heavy metals and persistent organic pollutants, further exacerbating their detrimental effects on aquatic organisms (Mateo-Sagasta et al., 2017). Plastic pollution presents a growing challenge that requires urgent attention, as its long-term impact on aquatic ecosystems remains largely uncharted.

Plastic pollution has become one of the most significant environmental challenges for aquatic ecosystems, with microplastics and plastic additives posing serious threats to marine and freshwater organisms. Plastics, while beneficial to society due to their durability and versatility, accumulate extensively in natural habitats as a result of unsustainable use and poor waste management (Andrady & Neal, 2009; Barnes et al., 2009). Plastic debris has permeated nearly every part of the aquatic environment, including oceans, rivers, lakes, and even the deep sea (Cole et al., 2011; Van Cauwenberghe et al., 2013). Rivers play a critical role in transporting large amounts of plastics from inland sources to marine environments, significantly contributing to marine plastic pollution (Moore et al., 2005; Lechner et al., 2014). Once in aquatic environments, larger plastic items break down into microplastics (MPs), which are smaller than 5 mm in diameter and are considered an emerging global environmental issue (Sutherland et al., 2010; Depledge et al., 2013; GESAMP, 2010; UNEP, 2011). Microplastics are widely distributed across various ecosystems, including marine, freshwater, sediment, and even polar regions (Andrady, 2011; Auta et al., 2017). Numerous aquatic organisms ingest microplastics, ranging from zooplankton (Jemec et al., 2016) and shellfish (Su et al., 2016) to fish (Jabeen et al., 2017; Zhang et al., 2017) and marine mammals (Hutton et al., 2008). The ingestion of microplastics leads to several adverse outcomes for aquatic species, including mechanical injuries, reduced growth rates, decreased reproductive success, and increased morbidity (Jackson et al., 2000; Cannon et al., 2016; Nadal et al., 2016). For example, microplastics can cause blockages in the digestive systems of fish, affecting their ability to feed properly and reducing their overall fitness. In addition to physical damage, microplastics can leach toxic chemicals used as additives during plastic production, such as plasticizers, and adsorb pollutants from the surrounding environment, which can further harm aquatic organisms (Ward & Kach, 2009).

Plasticizers, which are chemicals added to plastics to increase flexibility and durability, pose another significant threat to aquatic ecosystems. These substances, particularly phthalates like di-(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), and butyl benzyl phthalate (BBP), are known to disrupt endocrine functions in aquatic organisms (Paluselli et al., 2018). Phthalates interfere with hormone regulation, leading to reproductive issues and developmental problems in fish and other species. For example, fish exposed to phthalates experience impaired reproduction, altered sex ratios, and reduced fertility, threatening the stability of fish populations (Paluselli et al., 2018). Due to their harmful effects, the use of certain phthalates, such as DEHP and DBP, has been regulated or banned in regions such as the European Union, the United States, and Japan (European Union, 2007). In addition to phthalates, other plastic additives like polyethylene (PE)-based plasticizers are widespread pollutants in aquatic environments. PEs, which are added to products such as food packaging, medical devices, and personal care items, have a global production exceeding 6 million tons annually (Wang et al., 2018; Seyoum & Pradhan, 2019). These plasticizers are not chemically bound to the polymers they are mixed with, allowing them to easily leach into aquatic ecosystems during production, use, or disposal (Vats et al., 2013; Henkel et al., 2019). Once in the water, PEs can bioaccumulate in aquatic organisms and move up the food chain, potentially impacting humans as well (Arambourou et al., 2019). The widespread contamination of aquatic ecosystems with plasticizers has been documented in various environments, including rivers, lakes, wetlands, and marine environments (Salaudeen et al., 2018; Zhang et al., 2021).

Plastic pollution also affects the behavior and physiology of aquatic organisms. Fish exposed to microplastics and plasticizers exhibit altered behaviors, such as reduced predator avoidance and impaired foraging abilities (Jabeen et al., 2017). These behavioral changes increase the vulnerability of fish to predation and reduce their overall fitness. Furthermore, plastic pollution induces oxidative stress in aquatic organisms, leading to cellular damage and reduced immune function (Koniecki et al., 2011; Chi & Gao, 2015). The breakdown of plastics in the environment accelerates the release of harmful plastic additives, exacerbating their impact on aquatic life (Gao & Wen, 2016). The ingestion of microplastics, combined with the leaching of toxic additives like phthalates and PEs, leads to physiological and behavioral disruptions in aquatic organisms, reducing their growth, reproduction, and survival rates. Given the widespread distribution of plastic pollutants, urgent action is required to mitigate their impact on aquatic ecosystems through further research on the long-term effects of plastic pollution (Carnevali et al., 2010; Forner-Piquer et al., 2019).

1.5. Biological invasions in aquatic environment

Biological invasions pose a significant threat to aquatic ecosystems globally. The introduction of non-native species into freshwater and marine environments, often facilitated by human activities such as long-distance trade and habitat modifications, disrupts ecological balance by introducing new functional components and triggering trophic cascades (Sala et al., 2000; Kolar & Lodge, 2000). While many introduced species fail to establish, those that succeed often have profound ecological impacts, altering community structure, increasing competition, and introducing predation pressures on native species (Ricciardi & Rasmussen, 1999). Invasive species can cause direct interactions with resident organisms, such as competition for resources or predation, and indirect changes in habitat conditions, such as increased turbidity or altered habitat structure (Crooks, 2002). For example, the introduction of Peacock Bass (Cichla spp.) in Lake Gatun, Panama, significantly simplified the food web, reducing mosquitofish (Gambusia affinis) populations and indirectly leading to an increase in mosquitoes and mosquito-borne illnesses (Zaret & Paine, 1973). Similarly, the introduction of zebra mussels (Dreissena polymorpha) has drastically reduced phytoplankton and small zooplankton populations, causing disruptions at higher trophic levels (Ward & Ricciardi, 2007). Invasive species have profound effects on native fish populations by disrupting predator-prey relationships, introducing new competition, and altering the food web. For instance, the introduction of zooplanktivorous fish like Rutilus rutilus and Alburnus alburnus into Spanish reservoirs led to a decline in large-bodied zooplankton populations, which subsequently released phytoplankton from grazing pressure, resulting in an increase in primary production (Ordoñez et al., 2010). Such shifts have cascading effects on the ecosystem, including the survival and reproduction of native species. Filter feeders like zebra mussels can further disrupt ecosystems by filtering out key primary producers, leading to bottom-up disruptions in food availability for higher trophic levels (Ward & Ricciardi, 2007). These changes underscore the need for early detection and rapid response to invasive species before they can cause irreversible damage to biodiversity and ecosystem functioning.

Early detection of invasive species is critical for preventing widespread ecological damage and enabling timely management interventions. The sooner invasive species are identified, the more effective the management response can be, whether through physical removal, habitat restoration, or containment measures (Anderson, 2005). Delayed detection allows invasive species to establish and spread, making eradication efforts significantly more challenging and costly. As a result, modern techniques such as environmental DNA (eDNA) analysis have emerged as highly effective tools for early detection of aquatic invasive species (Jerde et al., 2011; Goldberg et al., 2013). Traditional methods like visual surveys or physical captures are labor-intensive, costly, and often fail to detect species at low densities, making eDNA a more efficient and sensitive approach

(Ficetola et al., 2008). eDNA technology has revolutionized the detection and monitoring of invasive species, particularly in aquatic environments where conventional methods may fail to detect elusive or low-density populations. eDNA sampling involves collecting water samples and filtering them to capture DNA shed by organisms, such as from skin cells or excretions (Ficetola et al., 2008). After the DNA is collected, quantitative real-time polymerase chain reaction (qPCR) analysis can be used to detect specific invasive species (Goldberg et al., 2013). This method is highly sensitive and allows for the early detection of invasive species, even before they are visually or physically detectable (Jerde et al., 2011).

The primary advantage of eDNA is its ability to detect invasive species with minimal environmental disruption and reduced labor compared to traditional methods like netting or electrofishing. Additionally, eDNA sampling can cover large geographic areas, making it a valuable tool for widespread monitoring efforts. However, one of the limitations of traditional qPCR analysis is the time delay involved, as samples are often processed in laboratory settings, which can take days or weeks (Egan et al., 2015). Despite this, advancements in field-based eDNA tools and real-time qPCR technologies are improving the method's efficiency, enabling faster detection and immediate management responses. As these technologies continue to evolve, they offer the potential to revolutionize invasive species management, allowing for more proactive and cost-effective strategies to protect aquatic biodiversity (Thomas et al., 2020).

Aquatic ecosystems are increasingly under threat from human-induced environmental changes, including pollution, habitat destruction, and the introduction of invasive species. These pressures disrupt the natural balance, leading to altered behaviors, physiological stress, and even species declines, which in turn affect the entire ecosystem's health and biodiversity. The importance of this study lies in its holistic approach to understanding the multifaceted impacts of environmental characteristics and human activities on aquatic fauna. By investigating key areas such as micro-adaptations in fish in response to environmental pressures, the physiological effects of parasites, the behavioral and physiological changes induced by pollutants like plastics and plasticizers, and the ecological consequences of biological invasions, this research offers comprehensive insights into how aquatic animals adapt and respond to various stressors. Given the increasing anthropogenic pressures on aquatic ecosystems, such as pollution, habitat alteration, and species invasions, this study is critical for informing conservation strategies and management practices. It highlights the urgent need for targeted interventions to preserve biodiversity, maintain ecosystem balance, and ensure the health and sustainability of aquatic ecosystems in the face of ongoing environmental change.

Chapter 2

Hypothesis and Objectives

Hypothesis

1. Particular anthropogenic activities affect the morphology, behaviour and physiology of aquatic animals.
2. Particular environmental factor plays an important role in shaping the morphology of aquatic organisms.

Objectives

1. Study of micro adaptations in fishes and its correlation with environmental factors.
2. Evaluation of effects of parasites on the behavious and physiology of aquatic fauna.
3. Evaluation of effects of pollutants on the physiology of aquatic fauna.
4. Assesment of biological invasion on native aquatic organism.
5. Application of eDNA method for early detection of living threats to aquatic life.

Chapter 3

Micro adaptations in fish: correlation with environmental factors

This chapter investigates the role of micro-adaptations in fishes, focusing on the correlation between these adaptations and environmental factors, such as competition for food. Predation is an influential biotic factor in aquatic ecosystems, shaping the evolutionary trajectories of both predators and prey. Predators exert direct pressure on prey populations by consuming them, while prey evolve specific survival mechanisms to evade capture. However, in some instances, the reverse can occur, where predators adapt in response to prey due to competition for food. These adaptations, both morphological and behavioural, help predators improve their efficiency. For example, scale-eating cichlids have developed craniofacial asymmetry, which enhances their feeding abilities (Takeuchi et al., 2016). Furthermore, predator species often modify their behaviour to increase hunting success, such as the lateralized behaviour seen in some fish, which improves their foraging effectiveness (Peterson & Winemiller, 1997). These evolutionary interactions highlight the critical role of predation in shaping specialized traits within fish populations.

The present study focuses explicitly on *Pachypterus khavalchor*, a lepidophagous (scale-eating) catfish from the Horabagridae family, native to the Western Ghats of India. Through a detailed osteological analysis, using clearing and double-staining methods, *P. khavalchor* was examined. The study revealed several key adaptations that enable this species to efficiently feed on the scales of other fish. These included a straight dorsal roof to the cranium, a long premaxilla equipped with outward-facing villiform teeth, and a spoon-shaped lower jaw also bearing villiform teeth. A unique feature of this species is the presence of five long, ossified ceratobranchials, with the fifth containing a set of 80-90 conical teeth, which likely facilitate scale consumption. This outward-facing arrangement of teeth, distinct from the inward-facing teeth seen in most catfish species, suggests a specialized adaptation for lepidophagy. These findings not only shed light on the evolutionary pathway that has driven lepidophagy in this species but also provide valuable baseline data for further research into the Horabagridae family.

In conclusion, the micro-adaptations observed in *P. khavalchor*, notably the specialized dental and jaw structures, illustrate a clear example of how environmental pressures, such as competition, drive evolutionary changes. The detailed anatomical study presented here contributes significantly to understanding the interplay between morphology and ecology in fish species and lays the groundwork for future taxonomic and ecological studies within the family.

3.1. Osteological description of Indian lepidophagous catfish *Pachypterus khavalchor* (Siluriformes: Horabagridae) from the Western Ghats of India

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RESEARCH PAPER

Osteological description of Indian lepidophagous catfish *Pachypterus khavalchor* (Siluriformes: Horabagridae) from the Western Ghats of India

Manoj PISE[1†], Sachin M. GOSAVI[2†], Pankaj A. GORULE[3], Chandani R. VERMA[1*], Sanjay S. KHARAT[4], Lukáš KALOUS[1] and Pradeep KUMKAR[1]

[1] *Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Czech Republic; e-mail: verma@af.czu.cz, kalous@af.czu.cz, kumkar@af.czu.cz, pise@af.czu.cz*

[2] *Department of Zoology, Maharashtra College of Arts, Science and Commerce, Mumbai Central, India; e-mail: schn.gosavi@gmail.com*

[3] *Department of Life and Environmental Sciences, University of Cagliari, Cagliari, Italy; e-mail: pankaja.gorule@unica.it*

[4] *Department of Zoology, Modern College of Arts, Science and Commerce, Ganeshkhind, Maharashtra, India; e-mail: kharat.sanjay@gmail.com*

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Abstract. The present study provides a comprehensive osteological description of *Pachypterus khavalchor* from the family Horabagridae. Nine individuals of *P. khavalchor* representing both males and females collected from the type locality were cleared and double-stained to provide a description of osteological characteristics. The presence of an almost straight dorsal roof to the cranium, a long and protruding premaxilla with numerous rows of tiny, villiform teeth, a spoon-like lower jaw with villiform teeth projecting outward, and five long and ossified ceratobranchials, with the 5th ceratobranchial containing a set of 80 to 90 conical teeth, sheds light on the ecomorphological adaptation in *P. khavalchor* that may have led to the evolution of lepidophagy. Furthermore, a slight difference in the structure of the complex hypurapophysis was observed between males and females. The information on the osteology of the Khavalchor catfish forms a baseline for taxonomic research of the entire Horabagridae family comprising four genera with ten species distributed in Asia.

Key words: skeletal morphology, lepidophagy, sexual dimorphism, systematics

Introduction

Despite the widespread sequencing of DNA and subsequent reconstruction of phylogenetic relations based on molecular data osteology remains an important source of data for fish systematics because of the structural and functional nature of evolutionary information (Keivany & Nelson 2004, Katwate et al. 2013, Britz et al. 2020). Osteological traits of fishes not only give vital information on taxonomy and evolutionary relationships of particular species with other taxonomic groups but also shed light on certain key biological and ecological aspects of fish life (Katwate et al. 2013, Gosavi et al. 2018, Britz et al. 2020).

The Khavalchor catfish *Pachypterus khavalchor* (Kulkarni, 1952) (Horabagridae: Siluriformes) is well-known for its feeding behaviour; eating scales

* *Corresponding Author*

† *These authors contributed equally to this work*

Fig. 1. *Pachypterus khavalchor* (a) in life, (b) cleared and double stained male, 63.6 mm (lateral view).

of other fishes which is recognised as lepidophagy (Kulkarni 1952, Gosavi et al. 2018). This specific strategy is evolutionarily conserved and expressed by only selected fishes belonging to five freshwater and seven marine families (Sazima 1983).

There exist various specific behaviours related to lepidophagy, of which many are related to particular morphological structures (Peterson & Winemiller 1997). Some species, such as the cichlid fish *Perissodus microlepis*, stealthily approach and suddenly tear off the scales of their prey as it swims past (Nshombo et al. 1985); for that purpose it has evolved dental and craniofacial asymmetries (Stewart & Albertson 2010). In contrast, *P. khavalchor* represents an aggressive type of scale eating behaviour. It chases and attacks prey, dislodging scales, which are eaten when they fall to the substrate (Gosavi et al. 2018).

Although information on several aspects of *P. khavalchor* is available, such as feeding habits (Gosavi et al. 2018), population dynamics (Gosavi et al. 2019a), digestive physiology (Gosavi et al. 2019b), reproductive biology (Gosavi et al. 2020), a detailed osteological description is scarce.

In addition to information on skeletal adaptations in the Khavalchor catfish reflecting its lepidophagy, the use of osteological data also has broader value. These data are essential for comparative studies among representatives of the entire family Horabagridae. Although some attention has recently been given to this neglected family (Ng & Vidthayanon 2011), osteological studies, other than X-ray images of *Horabagrus brachysoma* (Ali et al. 2014), are completely lacking.

For reference, we list the genera in the family Horabagridae, including the number of species in parentheses to clarify to which genera the missing information refers. The family currently consists of ten species *Pseudotropius* (4), *Horabagarus* (2), *Pachypterus* (3) and *Platytropius* (1) (Fricke et al. 2022). The three-species genus of *Pachypterus* has been represented recently by *Pachypterus atherinoides* (Bloch, 1794), *Pachypterus acutirostris* (Day, 1870) and *P. khavalchor*.

This osteological study aims to address the evolution of complex phenomena such as lepidophagy in fishes and provide baseline data for future taxonomic investigation of the family Horabagridae.

Table 1. Morphometric data of *Pachypterus khavalchor* used for osteological description.

Code	BNHS FWF 338	BNHS FWF 339	BNHS FWF 340	BNHS FWF 341	MCZPK1	MCZPK2	MCZPK3	MCZPK4	MCZPK5
Gender	Female	Male	Female	Male	Female	Male	Male	Female	Female
Total length	92.46	89.89	92.37	82.79	80.55	90.12	94.5	92.01	92.35
Standard length	72.7	70.75	72.35	64.17	70.64	75.53	76.95	73.52	74.44
Length of caudal fin	20.33	19.22	20.69	18.01	11.27	17.03	19.7	15.77	18.9
Predorsal length	27.76	24.18	27.56	38.09	24.14	26.98	28.51	28.55	28.56
Pre anal length	42.34	39.23	43.63	39.13	41.45	41.44	42.7	39.02	41.51
Prepelvic length	32.85	31.72	34.46	31.52	32.33	32.24	33.2	31.89	32.43
Prepectoral length	16.89	16.98	15.41		15.92	15.95	16.57	16.37	17.1
Length of head	18.23	17.12	17.15	17.4	17.13	16.9	17.88	17.76	15.49
Length of snout	6.2	6.03	6.65	5.82	6.07	5.52	7.18	6.79	7.17
Eye diameter	4.73	3.74	4.86	4.64	4.46	4.4	4.12	4.89	4.77
Interorbital width	7.2	6.15	6.54	6.54	5.71	5.47	6.8	5.41	6.08
Gape width	7.79	6.53	7.54	6.5	6.21	6.1	6.89	5.51	6.19
Nasal barbel length	8.57	14.37	8.41	13.1	14.1	10.13	12.12	10.21	10.1
Maxillary barbel length	20.54	26.16	18.56	18.65	17.12	25.64	26.03	26.04	26.19
Inner mandibular barbel length	9.15	11.47	10.93	8.19	7.76	11.17	11.29	10.23	10.27
Outer mandibular barbel length	16.3	17.75	12.46	9.89	9.04	16.99	17.26	13.06	17.39
Depth of body at anus	14.2	13.91	14.26	13.99	12.93	14.29	15	15.01	12.61
Length of dorsal fin base	6.69	7.83	7.19	7.95	7.53	7.53	7.9	7.35	7.5
Length of adipose fin base	3.39	2.16	3.03	3.2	3.1	2.17	2.72	2.92	3.12
Post adipose distance	11.49	12.81	14.5	13.08	12.33	15.66	16.51	15.76	17.37
Dorsal to adipose distance (origin both)	30.88	31.42	31.15	33.29	30.88	32.4	31.52	30.82	33.21
Length of caudal peduncle	6.92	13.19	12.93	11.17	7.3	12.69	13.7	13.69	13.6
Depth of caudal peduncle	6.2	6.88	6.38	6.17	5.87	7.06	7.03	7.14	5.86

Material and Methods

Nine individuals of *P. khavalchor* (Fig. 1a) including both males (n = 4) and females (n = 5) were collected using a cast net from the River Panchaganga (type locality; 16.681° N, 74.394° E and 535 m a.s.l.) at Kolhapur, Maharashtra, India with the help of local fishermen. After capture, fish were immediately anesthetised, preserved in 10% buffered formalin, and transported to the laboratory. Morphometric measurements were taken using a digital calliper (Mitutoyo, Japan) and expressed to the nearest 0.1 mm. Morphometric methods follow Keskar et al. (2015). The digestive system of all specimens was removed and the gut contents were examined under a stereomicroscope. All specimens were cleared and stained differentially for the bone and cartilage following the methodology defined by Potthoff (1984). The osteological description was taken from cleared and stained specimens using a stereomicroscope (Magnus, India). The terminology for skeletal description followed Bockmann & Miquelarena (2008). Four specimens were deposited in the Bombay Natural History Society (BNHS) museum collection, Mumbai, Maharashtra, India, with accession numbers BNHS FWF 338-341 and the others in the collection of the Department of Zoology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, India MCZPK 1-5. Scanning Electron Microscopy was used to examine the oral cavity of *P. khavalchor*, following the methodology established by Fishelson et al. (2014). At 10 kV accelerating voltage, samples were inspected and photographed using an analytical scanning electron microscope (JEOL JSM-6360A; JEOL, Akishima, Japan).

Anatomical abbreviations

ac – anterior ceratohyal; acp – articular cleithral process; af – anterior fontanel; ap – autopalatine; an – antorbital; bb2, 3 and 4 – basibranchials 2 to 4; bo – basioccipital; br – branchiostegal rays; bs – basipterygium; cb1-5 – ceratobranchials 1 to 5; cle – cleithrum; cWV – compound Weberian vertebra; de – dentary; dh – dorsal hypohyal; eap – external anterior process (or anterolateral arm); eb1-5 – epibranchials 1 to 5; ep – epioccipital; epu – epural; es – extrascapular; ex – exoccipital; fr – frontal; ha+has – complex hypurapophysis (hypurapophysis and secondary hypurapophysis fused); hb1-3 – hypobranchials 1 to 3; hu1+hu2 – ventral hypural plate formed by coossification of hypurals 1 and 2; hu3+hu4+hu5 – dorsal hypural plate formed by co-ossification of hypurals 3, 4 and 5; hy – hyomandibular; iap – internal anterior process (or anteromedial arm); ic – interceratohyal cartilage; ih – interhyal; i1 – infraorbital sensory branch 1; i3-6 – infraorbital sensory branches 3 to 6; io – interopercle; le – lateral ethmoid; lp – lateral process; ma – mesocoracoid arch; me – mesethmoid; mx – maxilla; na – nasal; of – optic foramen; op – opercle; os – orbitosphenoid; pa – parasphenoid; pb1, 2, 3, 4 – pharyngobranchials 1, 2, 3 and 4; pcb – posterior complex bone of pectoral girdle (coracoid, mesocoracoid, and scapula fused); pcp – postcleithral process; pc – posterior ceratohyal; pf – posterior fontanel; ph – parhypural; pop – preopercle; ppr – posterior process; pre – premaxilla; pro – prootic; ps – pterosphenoid; pt – pterotic; pu1+u1 – complex centrum composed of preural centrum 1 and ural centrum 1; pu2 – preural centrum; qu – quadrate; rpr1 – rigid part of pectoral-fin ray 1; so – suspensorium; soc – supraoccipital; sp – sphenotic; spr1 – soft part of pectoral-fin ray 1; st1-4 – suborbital tubules 1 to 4; tf – trigeminofacial foramen; tp5 – transverse process 5; tp6 – transverse process 6; trp – tripus; uh – urohyal; ur – uroneural; vh – ventral hypohyal; vo – vomer; W adap – Weberian complex anterodorsal accessory process; W tp4a – anterior limb of Weberian complex transverse process 4; Wtp4b – posterior limb of Weberian complex transverse process 4.

Results

Details of the morphometric measurements are provided in Table 1. Gut content observations of all studied specimens show the presence of multiple stacked scales in the stomach. Insect body parts and larvae were also detected in the digestive system of *P. khavalchor*. No noticeable sexual dimorphism was found except for a slight difference in the structure of the complex hypurapophysis. The representative image of a clear and stained specimen of *P. khavalchor* is shown in Fig. 1b, and detailed osteological representations are shown in Figs. 2 and 5.

Cranium (Fig. 2a, b)

The dorsal roof of the cranium is almost straight, with no apparent fossae/crests and several tiny bones articulated with distinct boundaries. There are two elongated conspicuous cranial fontanels (anterior and posterior) separated by an epiphyseal bar. For ease of description, the neurocranium is divided into rostral, optic and otic regions.

The rostral region is divided into several subregions, including the pre-maxilla and maxilla, mesethmoid, autopalatine, nasals, and vomer. The premaxilla is long and protruding, with numerous rows of tiny, villiform teeth anteriorly directed. Teeth present on

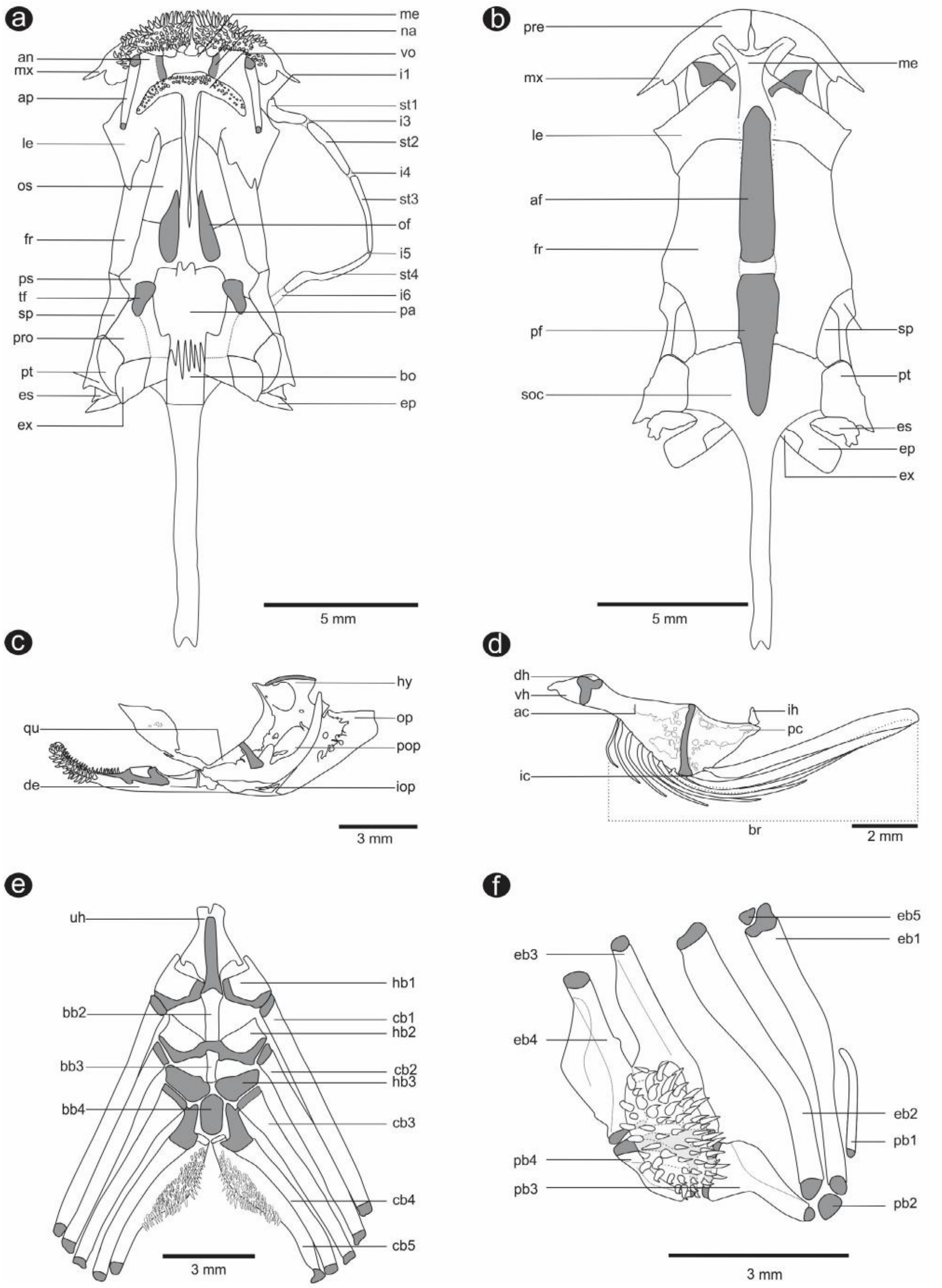

Fig. 2. Osteology of *Pachypterus khavalchor*, specimen BNHS 6032, (a) cranium, ventral view, (b) cranium, dorsal view, (c) lower jaw, (d) hyoid skeleton, (e) gill arch, (f) dorsal gill arch.

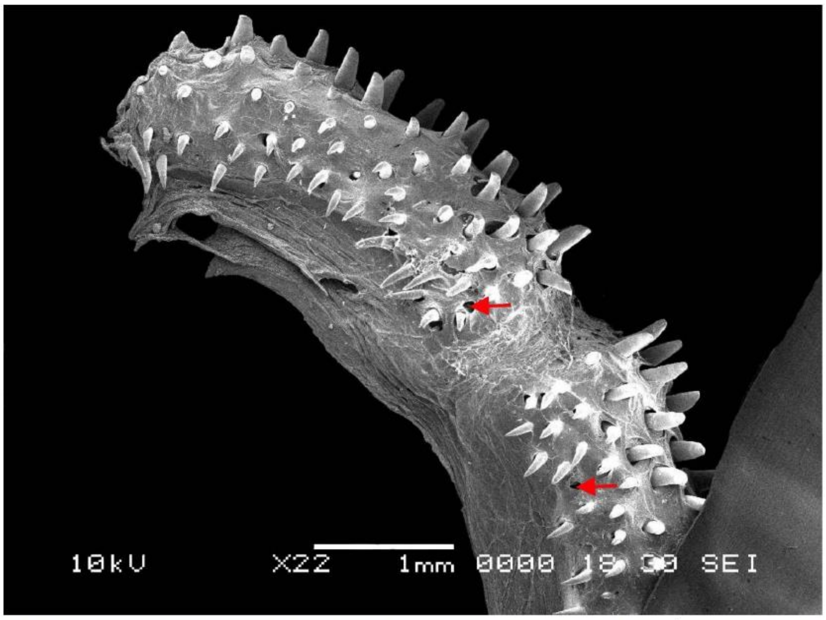

Fig. 3. Scanning electron micrograph of lower jaw of *Pachypterus khavalchor*, dorsal view (arrow heads shows cavities due to dislodged teeth).

the posterior side of the premaxilla are shorter than the anterior, with a few broken or dislodged (Fig. 3). The maxilla is small, entirely ossified, and located distally to the premaxilla. The mesethmoid is a "Y" shaped structure with short, thick, and blunt cornua anterolaterally directed. The autopalatines are paired, tubular, and laterally situated, with tiny, rounded cartilages at their ends. The nasals are ossified and short, with entrance and exit sites for the supraorbital laterosensory canal and antorbital. The vomer is sagittiform in shape, centrally located, partially ossified with laterally curved arms and possessing tiny villiform teeth on the front.

The orbital region is clearly visible in the dorsal view, delimited anteriorly by the lateral ethmoid, laterally by the frontal, and posteriorly by the sphenotic. The inter-orbital width accounts for around 40% of the entire length of the cranium. Lateral ethmoids are paired, entirely ossified, roughly rectangular, bordered anteriorly by autopalatines and located on each side of the vomer. It comprises the posterior section of the olfactory organ and connects to the orbitosphenoid through an unsutured articulation. The frontal bone, the largest in the skull, is entirely ossified and is bordered anteriorly by the lateral ethmoid and posteriorly by the supraoccipital. The sphenotic bones are paired, completely ossified, approximately triangular, laterally positioned, and bordered anteriorly by frontal, anterolaterally by parasphenoid, and posterolaterally by prootic. It has three laterosensory canal openings: the supraorbital laterosensory canal (anteromesial opening), the infraorbital laterosensory canal (lateral opening) and the otic laterosensory canal (posterior opening). The infraorbital laterosensory canal has six branches (i1-6) connected with suborbital tubules (st1-4). The supraorbital laterosensory canal has eight branches (s1-8). The optic foramen is elongated, roughly twice the length of the trigeminofacial nerves, and is surrounded by orbitosphenoids (anterodorsally and anteroventally), pterosphenoids (posterodorsally), and parasphenoids (posteroventrally). The trigeminofacial foramen is smaller, oval, and surrounded by pterosphenoid (anteriorly and dorsally) and prootic (posteriorly and posteroventrally).

Prootic, pterotics, supraoccipital, parasphenoid, epiotic, extrascapular, basioccipital, pterosphenoid, and exoccipital bones articulate in the otic area. Pterotics are completely ossified, located posterolaterally to the prootics (on the ventral side), and meet the supraoccipital on the dorsal side, whereas the epiotic, which is elongated and forms the posterior-most border, meets on the posteromedial side. All of their connections are sutured. The extrascapular is ossified and slightly triangular in the posterolateral corners of the skull. The supraoccipital bone is completely ossified and is long, straight, and forked distally. The width of the pointed tip of the supraoccipital process is constant until its posterior end. The parasphenoid is fully ossified and square in shape, anteriorly bifurcated and merges with the posterior portion of the vomer, with a zig-zag sutured pattern posteriorly. The basioccipital is broad, positioned posterior to the parasphenoid, joins the exoccipital laterally, and tapers posteriorly and articulated with the first abdominal vertebra.

Lower jaw, suspensorium and associated structures (Fig. 2c)

The lower jaw resembles a spoon, having a broad base and a tapering dentigerous area. The dentary is completely ossified and elongated, with several irregular rows of small villiform teeth on the anterior margin and dorsal surface projecting outward and inward respectively. The preopercle is triangular, strong and slightly concave, firmly attached to the hyomandibula. The preopercle is connected to the quadrate anteriorly. Its anterior section extends up to the posterior edge of the dentary and firmly articulates with the preopercle. The inter-opercular is approximately triangular, posteriorly close to the opercle boundary. The hyomandibula bone is roughly rectangular and joins with the quadrate via a cartilaginous sheet. Posteroventrally, the hyomandibula connects the preopercle and sub-triangular opercle.

Hyoid arches (Fig. 2d)

The dorsal and ventral hypohyals differ in size and are divided by a tiny irregular cartilage band. The anterior ceratohyal is longer than the posterior ceratohyal and is divided by a thin cartilaginous strip called the inter-ceratohyal. It is narrower in the centre and broader towards the tip, providing a surface for hypohyal articulation. The posterior ceratohyal, on the other hand, is broader at the base and narrower towards the tip. The nodular and ossified interhyal is located at the tip of the posterior ceratohyal. There are nine branchiostegal rays in all. The first seven connect to the anterior ceratohyal ray, the eighth to the inter-ceratohyal ray, and the ninth to the posterior ceratohyal ray. The length and breadth of the branchiostegal rays increase anteroposteriorly.

Gill arch (Fig. 2e, f)

Basibranchial: basibranchial 1 is absent. Ossified basibranchial 2 and 3 are fused, forming a long rod. Basibranchial 2 is anteriorly positioned on the dorsal side of the urohyal, whereas the posterior tip of basibranchial 3 is located in the intermediate area of hypobranchial 3. Basibranchial 4 is entirely cartilaginous and bordered anteriorly by hypobranchial 3, laterally by the cartilaginous heads of ceratobranchial 4 and posteriorly by the ossified head of ceratobranchial 5, respectively.

Hypobranchials: three hypobranchials are present. Hypobranchial 1 has an uncinate process on its anterodistal side. Hypobranchials 1 and 2 are entirely ossified, with a cartilage sheet running along their entire posterior border. Hypobranchial 3 is approximately rectangular and totally cartilaginous. Hypobranchial 4 is absent.

Ceratobranchials: there are five long and ossified ceratobranchials. Ceratobranchials 1 to 4 have cartilage at their extremities and are noticeably wider than the middle portion and form a rod-like shape. Ceratobranchial 5 has a set of 80-90 conical teeth of almost the same size. The basal head of ceratobranchial 5 is cartilaginous with its tip ossified.

Epibranchials: there are five epibranchials. Epibranchials 1 to 4 are elongated and completely ossified with cartilage at their extremities. Epibranchial 5 is tiny, knob-like and completely cartilaginous.

Pharyngobranchials: four pharyngobranchials are present. Pharyngobranchial 1 is an ossified rod-like structure with cartilage at the tip of one end. Pharyngobranchial 2 is nodular and completely cartilaginous. Pharyngobranchial 3 is irregular, broad and entirely ossified, except for the presence of cartilage at their extremities. Pharyngobranchial 4 is rod-like, completely ossified with a cartilage sheet at both ends.

Tooth plate: a globular tooth plate is located on the posterior side of pharyngobranchial 3, pharyngobranchial 4, and the terminal ends of epibranchial 3 and 4, bears around sixty-four teeth on each tooth plate.

Urohyal: the urohyal dorsal view is triangular, ossified, and has a greater dorsal keel than the horizontal position. Its posterior boundary approaches the anterior boundary of the basibranchials.

Weberian apparatus (Fig. 4)

The anterior four vertebrae contribute to the Weberian apparatus. The tripus is well developed with hook-

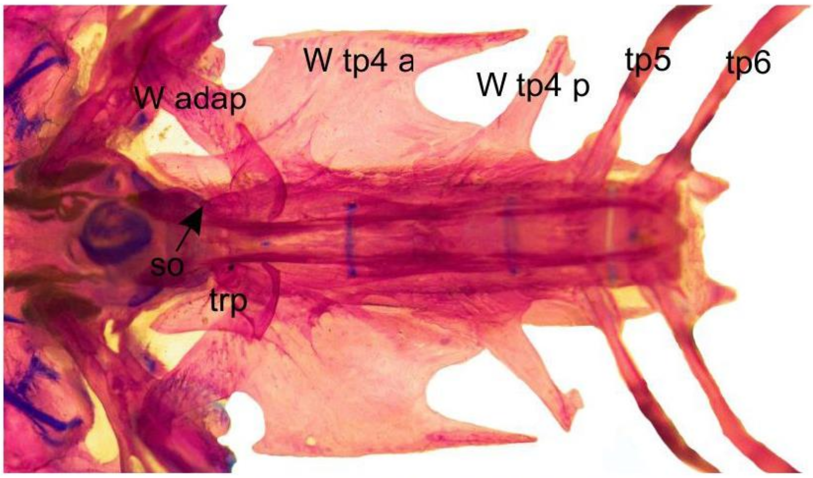

Fig. 4. Weberian apparatus attached to neurocranium in *Pachypterus khavalchor*, ventral view.

like transformator process at the posterior end. The suspensorium is nodule-like in shape and loosely attached to transverse process 4. A broad anterior limb and a rod-like posterior limb of transverse process 4 divides the fourth transverse process, giving a deep U shape. The transverse process of vertebrae 5 and 6 are longer than the posterior limb of the fourth transverse process. The accessory the anterodorsal process of the compound Weberian centrum is fused with anterior limb of transverse process 4 and lies close to the exoccipital.

Vertebral column (Fig. 1b)

There are 39 vertebrae in total, including 13 abdominal (including Weberian vertebrae 1-4) and 26 caudal vertebrae. The distal ends of the ribs are narrow. The neural and haemal spines of the caudal vertebrae are mostly straight, with no apparent projection. The first dorsal fin is supported by seven distal-proximal pterygiophores, one unbranched dorsal fin ray, and seven-branched soft rays are present. The anal fin has three anal spines and the remaining twenty-two are branched soft rays and is supported by the distal-proximal pterygiophores.

Pectoral girdle (Fig. 5a)

The pectoral girdle is formed by the ossification of two long bones, the cleithrum. These two bones are fused, forming the posterior complex bone ventrally, and are joined by four interlocking sutures. The post-cleithral process is pointed and narrow. The anterior cleithral process is curved and about half the length of the post-cleithral process. The mesacoracoid arch is pointed, located about above the anterior cleithral process. The rigid pectoral ray is ossified and bears serrations, whereas the soft pectoral ray is short, branched, and totally cartilaginous.

Pelvic girdle (Fig. 5b)

The basipterygium has a slightly arched and excavated dorsal and ventral surface. External and internal anterior processes (anterolateral and anteromedial arms, respectively) are very long anteriorly oriented processes. The basal arm of the anterolateral is consistently thick but narrow at the tip and devoid of cartilage. The basal portion of the anteromedial arm is wider than the tip that meets its counterparts. The anteromedial arm is slightly shorter than the anterolateral arm. The basipterygium has a slight lateral process and a prominent posterior process. Both the lateral and posterior basipterygium cartilages are clearly separated from one another. The bony section of the posterior process is curved and shorter than the majority of the basipterygium. Each branched cartilaginous part of the posterior process is longer than the bony portion of the posterior process. The cartilaginous middle portion of basipterygium is separated from each other. The basipterygium has six segmented pelvic fin-rays. One ray is unbranched, while the following five are branched.

Caudal skeleton (Fig. 5c)

The ossified hypural 1 and 2 are merged, resulting in a single ventral caudal plate. The parhypural runs near to the ventral caudal plate, distinctly separated from the margin of the ventral caudal plate. The rod-like, entirely ossified uroneural, is located above the dorsal hypural plate.

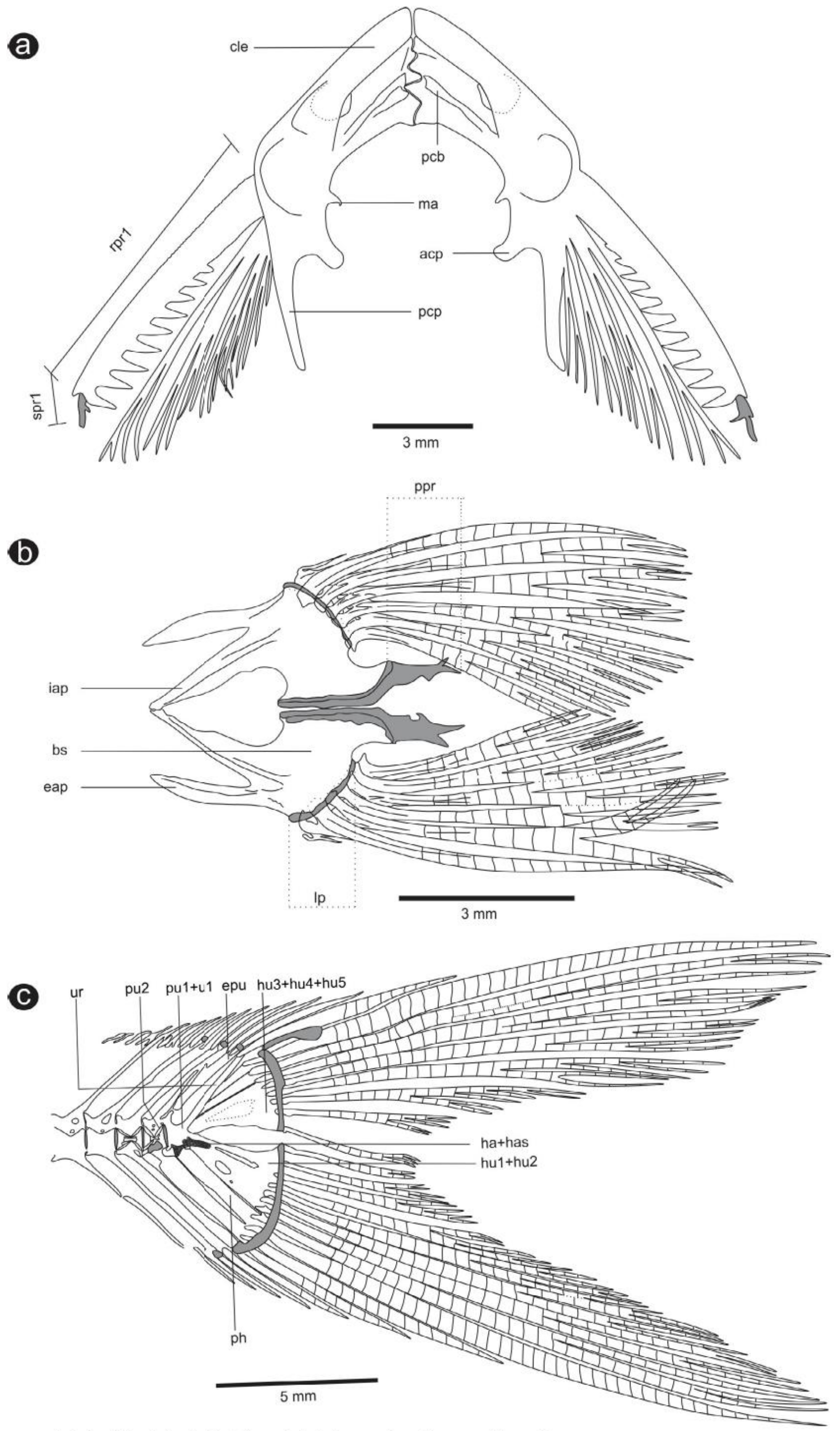

Fig. 5. (a) pectoral girdle, (b) pelvic girdle, (c) caudal skeleton of *Pachypterus khavalchor*.

The dorsal and ventral hypural plates are distinct from one another except for their base. The hypural three, four, and five plates are merged and ossified to create a single dorsal, caudal plate. The hypurapophysis and secondary hypurapophysis are fused, forming a complex hypurapophysis, a wide horizontal plate, that reaches the base of hypural 2 (hypurapophysis "type C" of Lundberg & Baskin 1969). There is a slight difference in the structure of the complex hypurapophysis between males and females. The hypurapophysis and secondary hypurapophysis are more robust and pointed in females than males. The caudal artery foramen is located on the ventral caudal plate. The dorsal, caudal plate comprises a uroneural and complex dorsal hypural plate (including hypurals 3, 4 and 5), bears seven branched rays and two unbranched rays with one dorsal most attached to the uroneural. The ventral caudal plate is made up of a parhypural and complex ventral hypural plate (including hypurals 1 and 2) that bears eight branched and two unbranched rays. The epural is a broad region located near the neural spine of preural centrum 2. The intricate centrum is made up of two parts: preural centrum 1 and ural centrum 1, which is made up of the haemal spine and neural spine.

Discussion

We present a thorough osteological description of *P. khavalchor* in this work. However, due to the lack of information about the osteological aspects of the Horabagridae family, our comparative analysis is limited. Thus, the osteology of *P. khavalchor* was compared to that of *H. brachysoma* (family Horabagridae) and its close relatives of the family Bagridae (Kaatz et al. 2010) as well as other fishes belonging to the order Siluriformes.

Lepidophagy in fish is known to have evolved as a derived, highly specialised habit supported by behavioural and morphological modifications (Sazima 1980, 1983). Gosavi et al. (2018) provided empirical evidence for scale-eating behaviour in the catfish *P. khavalchor*, as well as limited data on the significance of the oral anatomy in the lepidophagous behaviour of this species. The peculiar form and arrangement of teeth in the upper and lower jaws together with behaviour and digestive physiology have been reported to assist *P. khavalchor* in efficiently using the scales of live fish as food (Gosavi et al. 2018).

According to Gosavi et al. (2018), the pre-maxilla of *P. khavalchor* is protruding, with many rows of tiny, molariform teeth extending outward, indicating that the mouth cavity is suited for hard food, such as scales. However, our study shows that the teeth present on the pre-maxilla are directed outward but villiform in shape. Similar villiform shaped pre-maxillary teeth were shown in carnivorous catfishes such as *Bagrus bajad*, *Pseudobagrus aurantiacus* (*Tachysurus aurantiacus*) and *Pseudobagrus tokiensis* (*Tachysurus tokiensis*) (Watanabe & Maeda 1995, Eshra & El Asely 2014).

Generally, premaxillary teeth are directed inward in catfishes, such as *Pangasius macronema*, *Pareiorhaphis miranda*, *Hypostomus pantherinus* and *Horaglanis krishnai* (Mercy & Pillai 1985, Diogo & Diogo 2007, Pereira & Zanata 2014, Zawadzki et al. 2021), but our investigation shows the orientation of the premaxillary teeth is outward, which was also supported by Gosavi et al. (2018). Orientation of teeth observed in *P. khavalchor* may be among the most important adaptations in support of lepidophagous behaviour.

The dietary patterns of *H. brachysoma* are unspecialised and opportunistic (reviewed in Raghavan et al. 2016). There have been no reports of lepidophagy in this species, suggesting it is likely to have generic oral morphology, as no specific oral structural description is available to make concrete statements.

Both the upper and lower pharyngeal jaws have conical pharyngeal teeth, which could be utilized for crushing hard food materials, such as scales, insect body parts and molluscs. Other non-lepidophagous Siluriformes fishes, such as *Rita rita*, *Kryptoglanis shajii* and *Erethistes pussilus*, have a similar arrangement and structure of the phranyngeal teeth (Khanna 1962, Gauba 1967, Lundberg et al. 2014). This observation demonstrates that the structure of pharyngeal teeth is not designed for lepidophagy, but rather for crushing hard objects.

The anal fin ray count of *P. khavalchor* is three unbranched rays and the remaining twenty-two are branched soft rays, while *H. brachysoma* has three unbranched rays and twenty-nine branched fin rays in some specimens. There is one unbranched dorsal fin ray and seven-branched soft rays in *P. khavalchor*, and one unbranched dorsal-fin ray, and six to seven branched rays in *H. brachysoma* (Ali et al. 2014). The supraoccipital process is long and distally forked in *P. khavalchor*, which is identical to *Pseudobagrus adiposalis* valid as *Tachysurus adiposalis*, while it is short and distally not forked in *Pseudobagrus gracilis* valid as *Tachysurus gracilis*, and *Pseudobagrus brachyrhabdion* valid as *Tachysurus brachyrhabdion* (Li et al. 2005, Cheng et al. 2008). *Tachysurus lani* has a slender supraoccipital process with uniformly converging sides and a forked apex (Cheng et al. 2021). The pterotics are completely ossified and are positioned postero-laterally to the prootics (on the ventral side) and medially to the supraoccipital on the dorsal side. In *P. khavalchor*, all connections between them are sutured, but in *B. bajad*, the pterotics constitute the posterolateral portion of the otic capsule and are sutured with parieto-supraoccipital.

The osteology described in this study serves as a reference for further study of lepidophagous behaviour in catfishes, as well as a species-specific osteological investigation.

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Author Contributions

Conceptualisation: M. Pise, S.M. Gosavi, P. Kumar; fieldwork: S.M. Gosavi, P. Kumar; laboratory work: M. Pise, C.R. Verma, P. Kumar, P.A. Gorule; MS preparation: all authors contributed to MS preparation.

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Chapter 4

Effect of parasites on fish physiology

This chapter explores the physiological impacts of parasitic infections on aquatic fauna, particularly fish. Parasites are among the most successful life forms, influencing a wide range of host organisms across various ecosystems (Poulin & Morand, 2000; Dobson et al., 2008). Parasites can exert profound effects on their hosts, including altered morphology, reproduction, behaviour, and survival (Marcogliese, 2004). In aquatic environments, fish parasites are a significant part of biodiversity, often serving as biological indicators of ecosystem health, as they reflect environmental conditions and anthropogenic changes such as pollution (Palm, 2011). Fish parasites can cause lethal and sublethal effects, with the latter notably altering host behavior and physiology, thereby influencing population dynamics and ecosystem structure. For example, parasites often manipulate their host's behaviour to make them more susceptible to predation, thus completing their complex life cycles (Lafferty & Morris, 1996). This manipulation can affect predator-prey interactions, as parasitized fish exhibit altered foraging and locomotor behaviours, making them easier prey for predators (Barber et al., 2000).

The study presented in this chapter investigates the detection and effects of nematode parasites, specifically from the genus *Contracaecum* (family Anisakidae), on hillstream loaches (families Cobitidae and Nemacheilidae) in the northern Western Ghats of India. These nematodes use fish as intermediate hosts, infecting fish-eating birds and mammals through the food chain. The study utilized modern methods to identify the parasites. Results revealed the presence of *Contracaecum* in the muscles of infected loaches, leading to oxidative stress in the host fish. This oxidative stress, driven by elevated levels of reactive oxygen species (ROS), caused membrane damage in the infected fish tissues, affecting their physiological homeostasis. Such stress could be linked to increasing pollution and habitat degradation, suggesting that environmental factors have contributed to the rising incidence of parasitic infections in the region over the past decade. The chapter also addresses the potential health risks for local tribal communities, who often consume loaches without proper gutting or cooking, increasing their susceptibility to parasitic infections. Molecular analysis of the nematodes suggests that the Indian isolate could represent a new, undescribed strain, although further research is needed to confirm this. In conclusion, parasitic infections have significant physiological effects on aquatic fauna, impacting individual fish and broader ecosystem dynamics. This study underscores the importance of understanding the link between parasitic infections and environmental factors, particularly in light of anthropogenic stressors like pollution.

4.1 *Contracaecum* nematode parasites in hillstream loaches of the Western Ghats, India

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Contracaecum nematode parasites in hillstream loaches of the Western Ghats, India

Chandani R. Verma[1] | Pradeep Kumkar[1] | Tushar Khare[2] | Manoj Pise[1] | Lukáš Kalous[1] | Neelesh Dahanukar[3]

[1]Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

[2]Department of Environmental Science, Savitribai Phule Pune University, Pune, India

[3]Department of Life Sciences, School of Natural Sciences, Shiv Nadar University, Delhi, India

Correspondence
Neelesh Dahanukar, Department of Life Sciences, School of Natural Sciences, Shiv Nadar University, Delhi-NCR, NH – 91, Tehsil Dadri, Gautam Buddha Nagar, Uttar Pradesh 201314, India.
Email: neelesh.dahanukar@snu.edu.in

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Abstract

Nematode parasites of the family Anisakidae infect definitive hosts, such as fish-eating birds and mammals, through primary intermediate hosts like copepods and secondary intermediate hosts like fishes. However, consumption of raw or undercooked fish can lead to nematode infection called anisakidosis in humans. We observed the presence of nematode infection in hillstream loaches of families Cobitidae and Nemacheilidae available for human consumption in the local markets in the northern parts of Western Ghats, India. Scanning electron micrograph and genetic identification employing mitochondrial *cytochrome oxidase subunit II*, identified the nematode to the genus *Contracaecum*. Histology of infected host revealed the presence of the parasite in muscles. Antioxidant enzyme analysis of host liver suggested that infection leads to oxidative stress in the fish. We suspect that a gradual increase in parasite infection of the loaches in the last decade could be attributed to various anthropogenic stressors that are altering riverine habitats. Since loaches are consumed by tribal people who often prepare the fish without degutting and possibly undercooked, there is a potential threat of human infection.

KEYWORDS
Anisakidae, Cobitidae, human health, Nemacheilidae, wildlife health

1 | INTRODUCTION

Anisakid nematodes of the family Anisakidae Railliet & Henry, 1912 and parasitic genus *Contracaecum* Railliet & Henry, 1912 infect terrestrial and aquatic animals of both marine and freshwater origin (Fagerholm & Overstreet, 2008; Shamsi, 2019). Genus *Contracaecum* is considered the most diverse group within the family Anisakidae, having a wide geographical distribution, and a host range spanning from invertebrates to vertebrates (Shamsi, 2019). Piscivorous birds and mammals associated with freshwater, brackishwater and marine environments (such as cormorants, pelicans, penguins, and seals) are infected after consuming infested fish, and become definitive hosts of *Contracaecum* (Borges et al., 2014; D'amelio et al., 2012; Nascetti et al., 1993; Sardella et al., 2020). Definitive hosts shed the parasite eggs through their excrements which are ingested by invertebrates, such as gastropods, cephalopods, polychaetes, copepods, amphipods, and decapods, and serve as the first intermediate hosts for nematodes (Huizinga, 1967; Køie & Fagerholm, 1995; Shamsi, 2019). Teleost fishes act as the second intermediate, or paratenic, hosts and gain the parasite through ingestion of infected copepods (Garbin et al., 2013; Shamsi et al., 2011; Szostakowska & Fagerholm, 2007; Valtonen et al., 1988). Although some levels of parasitic infection can co-exist with the host in the normal conditions, various anthropogenic stressors can elevate the parasite loads (Marcogliese & Cone, 2001; Schludermann et al., 2003; Williams & MacKenzie, 2003), adversely affecting both the definitive and

Chandani R. Verma and Pradeep Kumkar are joint first authors.

intermediate hosts with *Contracaecum* infection (Buchmann & Mehrdana, 2016; Kumar et al., 2019; Olivero-Verbel et al., 2006). Further, ingestion of raw or undercooked fish carrying third-stage larvae of *Contracaecum* can cause a painful condition called anisakiasis or anisakidosis in humans (Buchmann & Mehrdana, 2016; Dei-Cas et al., 1986; Im et al., 1995; Nagasawa, 2012; Schaum & Müller, 1967; Shamsi & Butcher, 2011). As a result, *Contracaecum* parasite influences the health of both wildlife and humans.

In India, *Contracaecum* has been isolated from a number of bird species, including pelicans, cormorants, storks, ducks, darters, herons, bitterns, egrets, buzzards, vultures, eagles and kites (Chaturvedi & Kansal, 1977; Inglis, 1954; Islam & Talukdar, 2009; Kumar et al., 2019; Pazhanivel et al., 2017; Soota, 1981; Sreedevi et al., 2017). *Contracaecum* parasites have also been isolated from marine, brackishwater and freshwater fish (Chaturvedi & Kansal, 1977; Sood, 2017). However, studies on this parasite are currently restricted to occasional veterinary reports, morpho-taxonomy and inclusion in checklists. Up today, there is neither any genetic data on the *Contracaecum* found in India nor any studies that discuss the effect of the parasite on the host health.

Our freshwater fish surveys in the northern parts of the Western Ghats, a global biodiversity hotspot, revealed the presence of *Contracaecum* infections in hillstream loaches of the families Cobitidae and Nemacheilidae. These infections started appearing in early 2010 and has gradually increased over recent years. In this paper, we provide baseline information on the morphology and genetics of *Contracaecum* and provide preliminary insights on their physiological effects on fish host. Based on our findings, we argue that since these fish are extensively caught and eaten by tribals, these communities are vulnerable to parasitic infections.

2 | MATERIALS AND METHODS

2.1 | Study site and specimen collection

Hillstream loaches of the families Cobitidae and Nemacheilidae with visible signs of nematode infection were purchased from local fish markets and fishers (Table 1) of Maharashtra, India, during September–October 2020. Specimens from Lonavla and Urawade were specifically used for isolating third-stage larvae, histology of infected host, genetic identification of the parasite, and examining oxidative stress on the host. Few individuals without visible signs of infection were also purchased and used as controls for the study of hepatic antioxidant machinery.

2.2 | Histology

One specimen each of *Lepidocephalichthys thermalis* and *Indoreonectes evezardi* were used for histopathological study of nematode cysts. Immediately after dissecting the fish, infected region of the body was fixed for 24h using Bouin's fixative. Upon fixation, the samples were processed for a regular histological procedure of dehydration, clearing, infiltration, and embedding (using paraffin wax with melting point of 58–60°C). Manual rotary microtome (Leica RM 2125 RTS, Leica Ltd) was used to obtain histological sections of 7 μm thickness. Histological sections were mounted on the glass slides using Mayer's albumin and stained using the standard haematoxylin–eosin procedure (Bancroft & Gamble, 2008).

2.3 | Morphology and scanning electron microscopy

One specimen each of *Lepidocephalichthys thermalis*, *Indoreonectes evezardi*, and *Schistura denisoni* were dissected to isolate live parasites in their third larval state. The surface morphology of isolated parasitic nematode was studied using stereo-zoom microscope (Olympus SZ61TR) and scanning electron microscopy (SEM, Nova NanoSEM NPEP303) following the protocol described by Fishelson and Delarea (2014). Samples were examined and photographed using an analytical SEM at an accelerating voltage of 3–5 kV. Parasitic nematode larvae were identified to genus level based on Cannon (1977) and Shamsi et al. (2011).

2.4 | Genetic analysis

Isolated parasites (three from *Lepidocephalichthys thermalis* and one from *Indoreonectes evezardi*) were preserved in ethanol from which DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) following the manufacturer's protocol. Mitochondrial *cytochrome oxidase subunit* II (*cox2*) gene was amplified using PCR with primer pair 211F (5′ – TTT TCT AGT TAT ATA GAT TGR TTY AT – 3′) and 210R (5′ – CAC CAA CTC TTA AAA TTA TC – 3′) (Nadler & Hudspeth, 2000), with an annealing temperature of 45°C. Gene amplification, purification, and sequencing protocols follow Suranse et al. (2017).

The raw chromatograms were checked using free software FinchTV 1.4.0 (Geospiza, Inc.; http://www.geospiza.com), and BLAST (Altschul et al., 1990) was used to examine the similarity to the listed sequences in the NCBI database. Gene sequences generated in the study are deposited in GenBank under the accession numbers OM160985–OM160988. Additional comparative sequences were retrieved from NCBI GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using MUSCLE (Edgar, 2004), implemented in MEGA 11 (Tamura et al., 2021). Sequence data were partitioned into three codon positions, and Partition analysis (Chernomor et al., 2016) and ModelFinder (Kalyaanamoorthy et al., 2017) were used to find the right partitioning scheme, and nucleotide substitution model for the partition scheme employing minimum Bayesian Information Criterion (BIC; Schwarz, 1978). Maximum likelihood (ML) analysis was performed in IQ-TREE 2.1.3 (Minh et al., 2020) with best partition scheme and ultrafast bootstrap support for 1000 iterations

TABLE 1 Location details for *Contracaecum* infected loaches collected in the present study

Family/species	River	Location	Latitude (°N)	Longitude (°E)
Cobitidae				
Lepidocephalichthys thermalis	Indrayani	Lonavla	18.746	73.449
Lepidocephalichthys thermalis	Mula	Urawade	18.484	73.672
Nemacheilidae				
Indoreonectes evezardi	Mula	Urawade	18.484	73.672
Schistura denisoni	Indrayani	Lonavla	18.746	73.449
Nemacheilus anguilla	Koyna	Patan	17.369	73.904
Nemachilichthys ruppelli	Koyna	Patan	17.369	73.904
Paracanthocobitis mooreh	Mutha	Panshet	18.386	73.637

(Hoang et al., 2018). Phylogenetic tree was edited in FigTree v1.4.4 (Rambaut, 2018). Raw genetic distance between sequences was estimated in MEGA 11 (Tamura et al., 2021).

2.5 | Antioxidant enzyme activities and lipid peroxidation levels

To evaluate the status of hepatic antioxidant machinery, the liver tissue from five non-infected (control) and five infected specimens each of *Lepidocephalichthys thermalis* and *Indoreonectes evezardi* was isolated and homogenized in phosphate buffer (100mM, pH 7.5; containing 0.5mM EDTA). The homogenate was centrifuged at 15,000rpm for 20min at 4°C. Supernatant was collected as a crude extract for measuring the activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione-S-transferase (GST), and lipid peroxidation levels. All the steps were carried out between 0–4°C. Absorbance values for assays were recorded using UV–VIS spectrophotometer (UV-1800; Shimadzu Corp.). The SOD activity was determined by measuring the reduction in absorbance of blue coloured formazan produced by superoxide ($O_2^{\bullet -}$) and nitro-blue tetrazolium (NBT) by enzyme at 560nm (Dhindsa et al., 1981). Catalase activity was measured by quantifying the rate of decomposition of H_2O_2 at 240nm by the enzyme in crude extract (Aebi, 1984). Peroxidase activity was determined as rate of formation of tetraguaiacol at 470nm by the enzyme (Castillo et al., 1984). The GST activity was calculated by measuring the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 340nm (Habig et al., 1974). Malondialdehyde (MDA) was quantified as an end-product of the lipid peroxidation; via thiobarbituric acid method described by Hodges et al. (1999). The total protein content of the crude extract was determined by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as standard and was further used to calculate the enzyme activities. Significant difference in the enzyme activity between uninfected and infected hosts was tested using t test. Since multiple tests were performed, Bonferroni corrected alpha was used for statistical analysis.

3 | RESULTS

3.1 | Parasitic infection in hillstream loaches

During our freshwater fish surveys in the northern Western Ghats of India since 1998, infected loaches started appearing in early 2010. Five species of hillstream loaches, namely *Lepidocephalichthys thermalis* (family Cobitidae), *Indoreonectes evezardi*, *Nemacheilus anguilla*, *Nemachilichthys ruppelli*, *Paracanthocobitis mooreh*, and *Schistura denisoni* (family Nemacheilidae) were observed to have subcutaneous infections (Figure 1). In addition to the localities mentioned in Table 1, from where the specimens were collected, infected individuals were also observed from Nira River (Bhor:18.155°N, 73.844°E; Sarola: 18.178°N, 73.952°E), Mutha River (Bahuli: 18.415°N, 73.664°E; Warje: 18.474°N, 73.809°E), and Mula River (Paud: 18.529°N, 73.610°E; Aundh: 18.568°N, 73.810°E) in Pune District; Koyna River (Karad: 17.276°N, 74.173°E) in Satara District; and Hiranyakeshi River (Gavse: 16.128°N, 74.123°E) in Sindhudurg District, all in the state of Maharashtra, India. In one individual each of *Lepidocephalichthys thermalis* (Figure 1a), *Schistura denisoni* (Figure 1e), and *Indoreonectes evezardi*, we observed third-stage larva in distended subcutaneous regions. Dissection of these infected lesions revealed presence of parasitic nematodes (Figure 1b,c). In the remaining individuals, the cysts appeared as dark coloured subcutaneous structures (Figure 1d–g). Histological cross section (Figure 2) of an individual each of *Lepidocephalichthys thermalis* and *Indoreonectes evezardi* revealed that the parasitic nematode cysts infected muscles.

3.2 | Morphological and genetic characterization of nematode

Scanning electron micrograph (Figure 3) of the isolated nematode showed structural characteristics of *Contracaecum* but they could not be identified to the species level. Best partitioning scheme and appropriate model for the partition was estimated as TN+F+I+G4

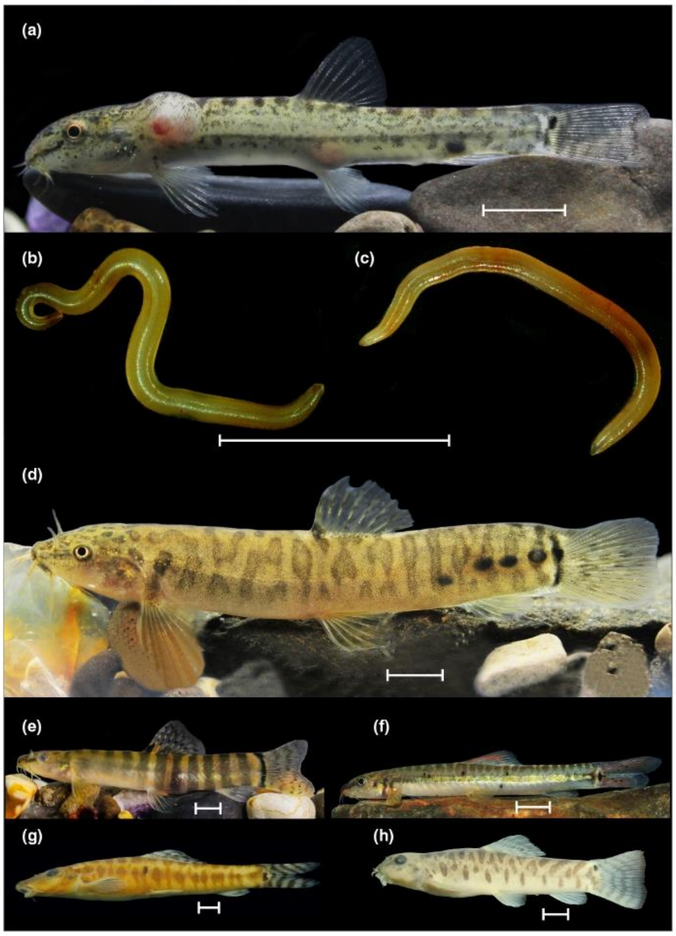

FIGURE 1 Infected loaches and the *Contracaecum* parasite from the northern Western Ghats of India. (a) Infected host *Lepidocephalichthys thermalis* from Lonavla showing subcutaneous cysts. (b, c) *Contracaecum* parasites isolated from *L. thermalis*. Other loach hosts infected by parasite include (d) *Indoreonectes evezardi* from Urawade, (e) *Schistura denisoni* from Lonavla, (f) *Nemacheilus anguilla* from Patan, (g) *Nemachilichthys ruppelli* from Patan, and (h) *Paracanthocobitis mooreh* from Panshet. Scale bar 5 mm

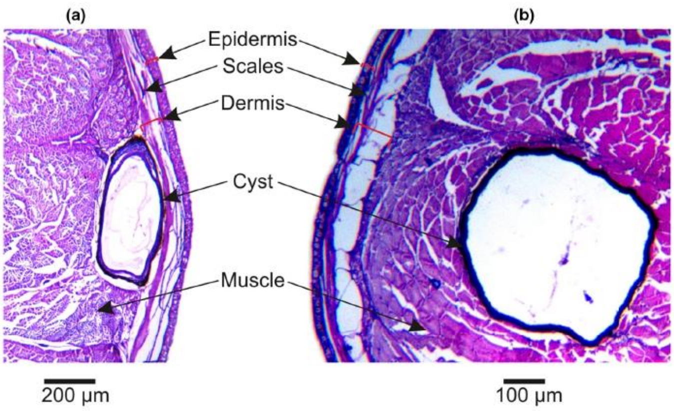

FIGURE 2 Histological cross sections of infected hosts (a) *Lepidocephalichthys thermalis* and (b) *Indoreonectes evezardi* showing the presence of cysts in the muscles

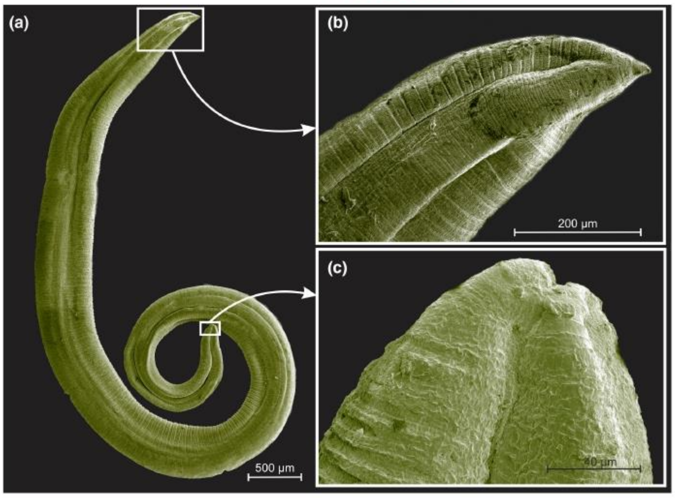

FIGURE 3 Scanning electron micrograph displaying the complete structure of *Contracaecum* species and its posterior and anterior ends. (a) Complete female specimen, (b) posterior end, and (c) anterior end

for combined partition of first two codon positions and HKY+F+G4 for the third codon position of *cox2* gene (BIC = 11818.895, lnL = −5182.707, *df* = 226). Maximum likelihood tree (Figure 4) based on best partition scheme revealed that the isolated nematode was nested within the larger clade of *Contracaecum*. Nematode worms isolated from *Lepidocephalichthys thermalis* (OM160985, 86 and 88) and *Indoreonectes evezardi* (OM160987) were genetically similar and formed a well-supported (bootstrap value 100) monophyletic clade. *Contracaecum* species from India was genetically distinct from available sequences of the genus sampled from a large geographical area (Figure 4) and differed with a raw genetic distance of 10.8%–16.8%.

3.3 | Influence of infection on the antioxidant status of the liver

All hepatic antioxidant parameters analysed displayed statistically significant elevated levels in infected, compared with non-infected (control) samples (Figure 5). The SOD activity in the enzyme extract from infected *L. thermalis* and *I. evezardi* was 1.6-fold higher than the same in non-infected samples indicating the parallel impacts on both the species (Figure 5a). CAT activity also followed the increased patterns in infected samples of *L. thermalis* (1.9-fold higher) and *I. evezardi* (1.6-fold higher) (Figure 5b). Similarly, POX activity was 3.1- to 5.1-fold higher in infected *L. thermalis* and *I. evezardi*, respectively (Figure 5c). The GST activity was 2.2-fold higher in the infected livers of *L. thermalis* than their non-infected counterparts, whereas the GST activity was 1.8-fold higher in infected *I. evezardi* than the non-infected samples (Figure 5d). The lipid peroxidation levels measured in terms of MDA content was found to be higher in infected livers of *L. thermalis* (2.5-fold higher) and *I. evezardi* (3.2-fold higher) (Figure 5e). Overall, the enzyme activities and lipid peroxidation levels of the liver samples from infected fishes suggested the elevated antioxidant status compared with their non-infected (control) counterparts.

4 | DISCUSSION

Our fish surveys in the northern Western Ghats of India spanning more than two decades, has resulted in the observations of incidence of nematode infections in loaches since 2010. Kumar et al. (2019) also suggested an increase in the incidence of *Contracaecum* infection in pelicans of southern India since 2011. It has been suggested that the increase in parasitic events are often the results of biotic or abiotic changes in the environment due to heavy metals, hydrocarbons and organic enrichment of sediments by domestic sewage (Marcogliese & Cone, 2001; Schludermann et al., 2003; Williams & MacKenzie, 2003). Both, heavy metal and organic waste in the aquatic habitats have been documented from the study area (Abhyankar et al., 2020; Gosavi et al., 2020; Nawani et al., 2016) and may have contributed in part to the increase in the incidence of parasitic nematodes in loaches.

We provide the first molecular data on parasitic genus *Contracaecum* from the Indian subcontinent. Although the parasite could not be identified at the species level, Indian isolate formed a distinct clade nested within the larger clade of the genus *Contracaecum* and had a raw genetic distance of 10.8%–16.8% from other sequenced *Contracaecum* available in GenBank. We suspect that the Indian *Contracaecum* could be an 'undescribed' endemic strain. However, further taxonomic studies are essential to validate this claim.

Parasites are known to affect the fish host adversely through oxidative stress, tissue damage, immunosuppression, and endocrine disruption (Jobling & Tyler, 2003; Marcogliese et al., 2005; Marnis et al., 2019; Olivero-Verbel et al., 2006), which can sometimes lead

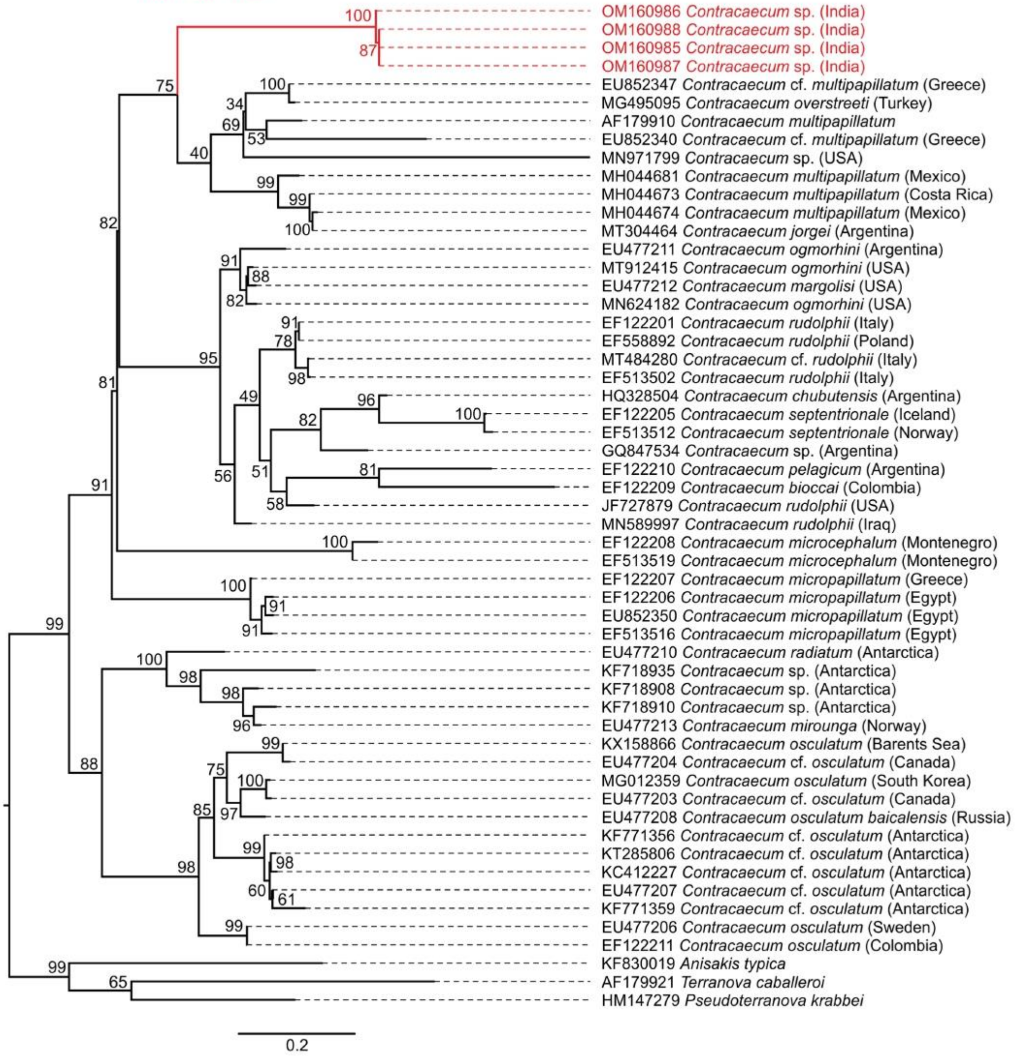

FIGURE 4 Maximum likelihood tree based on mitochondrial *cytochrome oxidase subunit 2* gene sequence of parasitic nematode *Contracaecum* sp. isolated from host *Lepidocephalichthys thermalis* (OM160985, 86 and 88) and *Indoreonectes evezardi* (OM160987). Species of *Anisakis*, *Terranova* and *Pseudoterranova* are used as outgroup taxa. Values along the nodes are bootstraps based on 1000 iterations.

to the mass mortality of infested hosts. Although mass mortality due to parasitic infection has not been reported for fishes in India, there are reports of mass mortality of fish-eating birds, namely pelicans (Kumar et al., 2019). Nevertheless, our study on the antioxidant status of the liver suggested that the enzyme activities and lipid peroxidation levels of the liver samples from infected fish was higher than their non-infected (control) counterparts—indicating that the fish host could be under oxidative stress. Further, histological studies showed that the parasite occupied muscles of loaches, which might affect the streamline structure of the fish body hampering its movement. It has been shown that excretions from another Anisakid worm, *Pseudoterranova decipiens* contain several pentanols and pentanons which act as local anaesthetics in the cod muscle during worm penetration, which affect muscle contractility (Ackman & Gjelstad, 1975). Although similar mechanisms are not known in *Contracaecum*, muscle damage may still reduce the swimming abilities of a fish which

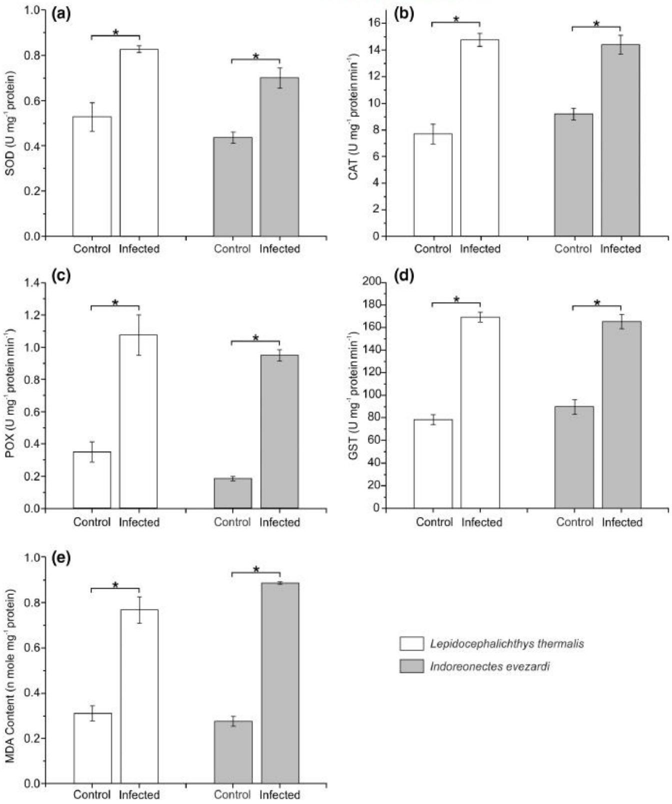

FIGURE 5 Oxidative stress in *Contracaecum* infected and uninfected individuals of *Lepidocephalichthys thermalis* and *Indoreonectes evezardi*. (a) Superoxide dismutase (SOD), (b) catalase (CAT), (c) peroxidase (POX), (d) glutathione-S-transferase (GST), and (e) lipid peroxidation levels in the liver of control (uninfected) and infected *L. thermalis* and *I. evezardi*. Results are represented as the mean ± standard error among the three replicates. Asterisk indicates significant differences between control and infected samples after Bonferroni correction. One unit of SOD represents the enzyme required for 50% reduction in absorbance at 560 nm mg^{-1} protein, one unit of CAT represents mM H_2O_2 decomposed mg^{-1} protein min^{-1}, one unit of POX represents μM tetraguaiacol formed mg^{-1} protein min^{-1} and one unit of GST represents μM S-(2,4-dinitrophenyl) glutathione formed mg^{-1} protein min^{-1}. Lipid peroxidation levels are represented as malondialdehyde (MDA) content mg^{-1} protein

can lead to higher risk of predation. Further, most of these loach species, especially *Indoreonectes evezardi*, are altitudinal migrants and show upstream migration during breeding season. As a result, parasitic infection in the muscle has a potential to affect the breeding behaviour of these species.

From their secondary intermediate fish hosts, *Contracaecum* has been isolated from a number of species originating from marine (Berland, 1961; Chaturvedi & Kansal, 1977; Guardone et al., 2020; James & Srivastava, 1967; Sood, 2017), brackishwater (Chaturvedi & Kansal, 1977; Motamedi et al., 2019; Sood, 2017), and freshwater (Aydogdu et al., 2011; Chaturvedi & Kansal, 1977; Gholami et al., 2014; Motamedi et al., 2019; Sardella et al., 2020; Sood, 2017) sources. In India, *Contracaecum* has been isolated from several commercial marine and freshwater fishes, including freshwater catfishes *Mystus* sp. and *Pangasius pangasius* (Chaturvedi & Kansal, 1977; Sood, 2017). *Contracaecum* has been identified in Cypriniformes fishes of family Cyprinidae (Aydogdu et al., 2011; Gholami et al., 2014; Sood, 2017) and Cobitidae (Shamsi et al., 2019; Zhang et al., 2021); however, ours is the first report of *Contracaecum* from the loach family Nemacheilidae.

Loaches consume several invertebrate taxa, such as gastropodes, oligochaetes, copepods and ostracods (Sauvonsaari, 1971), which are the first intermediate hosts of *Contracaecum* nematodes (Fagerholm & Overstreet, 2008; Garbin et al., 2013; Shamsi, 2019). This could be the reason for the incidence of *Contracecum* infection in loaches. However, the definitive hosts of these *Contracaecum* nematodes is not clear, and there is need to study the birds that prey on hillstream loaches to investigate this further.

Several studies have shown that ingestion of raw or undercooked fish carrying third-stage larvae of *Contracaecum* can cause anisakidosis in humans (Buchmann & Mehrdana, 2016; Dei-Cas et al., 1986; Im et al., 1995; Nagasawa, 2012; Schaum & Müller, 1967; Shamsi & Butcher, 2011), which causes a very painful condition. Buchmann and Mehrdana (2016) suggested that relatively few human cases of *Contracaecum* larvae causing anisakidosis is likely to be under-reported due to misidentification of the causative agent. This problem could be even more elevated in Indian scenario because the helminth parasite might continue to remain undiagnosed, especially in the rural areas where the hillstream loaches are consumed by poor people.

In Maharashtra, where we discovered *Contracaecum* parasites and their third-stage larvae, loaches are caught and sold in local markets, as well as consumed by members of the tribal community called Katkari (Keskar et al., 2017). The cooking method, which involves use of complete fish without degutting, might also include only partial cooking due to limited resources available to the tribal community. As a result, there is a high likelihood that *Contracaecum* parasite can gain entry into the human body. Unfortunately, poverty, as well as lack of medical attention specifically to detect nematode parasites, might be the reasons for absence of any documented reports of *Contracaecum* in human communities of these areas.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/, reference number OM160985-OM160988.

ORCID

Neelesh Dahanukar https://orcid.org/0000-0001-7162-9023

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Chapter 5

Effects of pollutants on behaviour and physiology of aquatic fauna

This chapter addresses the impact of pollutants like microplastics and associated pollutants and nano particles on the behavior and physiology of aquatic organisms, particularly fish, planktons, and flatworms, in freshwater, estuarine, and marine environments. Aquatic ecosystems face increasing threats from pollutants like microplastics, plasticizers, and nanoparticles, which affect aquatic organisms in various ways. Microplastics, formed from the degradation of larger plastic materials, are particularly concerning due to their widespread presence and detrimental effects on aquatic species. These pollutants are ingested by organisms, leading to physiological stress, reduced growth, reproductive issues, and changes in behavior. In this chapter, we examine how these pollutants impact the survival mechanisms, stress responses, and habitat colonization patterns of aquatic fauna in different environments.

In the Ulhas River, a heavily polluted waterway in India, microplastic concentrations ranging from 40 to 600 particles per kilogram of sediment were observed, with contaminants such as low-density polyethylene (LDPE), high-density polyethylene (HDPE), polypropylene, and polystyrene. These microplastics, coupled with biofilm formations, highlight the advanced degradation of plastic debris and its growing impact on environmental health. Further downstream, in the coastal waters and estuaries of Maharashtra, significant contamination from microplastics, plasticizers, and pharmaceuticals was detected. Over 70% of these areas posed ecological risks, especially for fish and crustaceans. The presence of plastispheres—biofilm-covered microplastic particles—demonstrates how microplastics serve as new habitats for microorganisms like fungi and diatoms, further disrupting ecosystem balance by influencing habitat colonization and microbial interactions.

The impact of microplastics and plasticizers, such as diethyl phthalate (DEP), on aquatic organisms is profound. Fish species like the common spiny loach displayed impaired predator recognition abilities after exposure to DEP, leading to reduced anti-predator responses and lower survival rates. These behavioral disruptions compromise essential survival mechanisms and reduce overall fitness. Similarly, freshwater flatworms suffered from neurotoxic effects, including reduced locomotion and impaired regeneration, further threatening biodiversity and ecosystem stability. In addition, mudskippers exposed to microplastics in the Ulhas River estuary experienced physiological burdens, such as liver damage and reduced body condition, while microplastics acted as vectors for heavy metals, compounding the health risks for aquatic fauna. Due to microplastic pollution, many aquatic organisms are using these particles to adhere and colonize, forming what is known as the plastisphere. This phenomenon has led to the creation of novel microhabitats, where biofilm-covered microplastics provide surfaces for microbial colonization, altering natural habitat selection and further complicating ecological dynamics.

Alongside these chemical pollutants, carbon nanofibers have emerged as a new class of contaminants affecting bottom-dwelling species like the spiny loach. Exposure to carbon nanofibers increased oxidative stress markers, causing hepatic damage and impairing detoxification processes. Additionally, these fibers disrupted predator-recognition abilities, leaving fish more vulnerable to predation. This emerging pollutant poses a serious risk to aquatic species, adding to the complex and multifaceted threats posed by environmental contaminants in aquatic ecosystems.

5.1. Microplastic contamination in Ulhas River flowing through india's most populous metropolitan area

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Microplastic Contamination in Ulhas River Flowing Through India's Most Populous Metropolitan Area

Chandani R. Verma · Manoj Pise · Pradeep Kumkar · Sachin M. Gosavi · Lukáš Kalous

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Abstract In addition to providing drinking water and a means of transportation, urban rivers also support inland fisheries, agriculture, and industry. Urban rivers, however, are continually being influenced by anthropogenic stressors, such as plastic pollution, and are thus predicted to play a significant role in the worldwide ocean plastic pollution problem. The management of urban rivers remains one of the major challenges due to the lack of knowledge on the degree of riverine microplastics (MPs), particularly in nations like India. Therefore, the current study investigates on MP pollution in the Ulhas River, which runs through Mumbai, India's most populous metropolis, and contributes significantly to MPs entering the Arabian Sea. MPs were extracted from sediment samples collected across the Ulhas River basin and then identified using FTIR-ATR, Raman spectroscopy, and SEM–EDX. MP particles were detected in every sediment sample taken from the Ulhas River, and their concentration ranged from 40 to 600 particles kg^{-1} of sediment. LDPE, HDPE, polypropylene, polystyrene, polyethylene, polyester, and nylon were found to be the primary polymers. When combined with demographic estimates and land use patterns, the estimated pollution load index (PLI), polymeric risk (H), and pollution risk index (PRI) show that five of the nine sub-basins are expected to represent significant ecological risk and are therefore referred to as "hotspots" of MP pollution. The majority of MPs in the Ulhas River originate from secondary sources. Examining MPs using SEM–EDX indicates their ageing, disintegration, and association with biofilm. The information obtained from this study's data is useful for establishing water quality standards, monitoring and controlling pollution efficiently, and serving as a foundation for further research.

Chandani R. Verma and Manoj Pise contributed equally to this work

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C. R. Verma · M. Pise · P. Kumkar · L. Kalous (✉)
Department of Zoology and Fisheries, Faculty of Agrobiology, Food, and Natural Resources, Czech University of Life Sciences, Prague, Czech Republic
e-mail: kalous@af.czu.cz

S. M. Gosavi (✉)
Department of Zoology, Maharashtra College of Arts, Science and Commerce, Mumbai, India
e-mail: schn.gosavi@gmail.com

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1 Introduction

Plastic contamination is a worldwide environmental issue. Concerns about the harmful effects of plastic waste on living beings proliferated once it was discovered that large-sized plastic could be broken down

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into micro-sized particles (less than 5 mm in size), called "microplastics". Because of their small size, microplastics (MPs) can enter living organisms via food, water, and air, contaminating the whole food chain (McCormick et al., 2014; Ory et al., 2018; Payton et al., 2020; Peters et al., 2017). MP particles are often mistaken as prey by lower trophic level organisms, which can eventually reach higher trophic level organisms via trophic transfer (Kumkar et al., 2021). Once entered into living organisms such as fishes, amphibians, and birds, MPs have been known to cause several deleterious effects (Besseling et al., 2013, 2015; Naidoo & Glassom, 2019; Ory et al., 2018; Wright et al., 2013). Recent studies have also shown that MP function as a carrier for heavy metals and other pollutants in aquatic ecosystems (Shruti et al., 2019), thereby amplifying their detrimental effects such as endocrine disruption (Rochman et al., 2014), damage to the reproductive system (Sussarelle et al., 2016), and liver (Rochman et al., 2013), which eventually affects species growth, fitness, and survival (Naidoo & Glassom, 2019). As a result, there has been an enormous growth in the published literature on MP pollution in coastal and freshwater aquatic bodies around the world. Nevertheless, riverine MP pollution is still understudied (Meijer et al., 2021), particularly in countries like India, despite the fact that Indian rivers are predicted to contribute substantially to global plastic pollution in the ocean.

India is the second-largest producer of plastic trash and contributes considerably to global plastic pollution, with relatively small but severely polluted urban rivers carrying 80% of this plastic trash to the ocean (Meijer et al., 2021). Urban rivers, however, are continually being influenced by anthropogenic stressors, such as plastic pollution, and are thus predicted to play a significant role in the worldwide ocean plastic pollution problem. The management of urban rivers remains one of the major challenges due to the lack of knowledge on the degree of riverine MPs. There is a significant data gap concerning riverine MP investigations in India, with only a few studies on major river systems such as the Ganga River (Sarkar et al., 2019), Netravati River (Amrutha & Warrier, 2020), Sabarmati River (Patel et al., 2020), and Brahmaputra and Indus Rivers (Tsering et al., 2021). Therefore, for the current investigation, we chose the Ulhas River in Mumbai, Maharashtra, India.

The Ulhas River (Fig. 1A) is a west-flowing urban river with a basin having an area of 4733 km^2, a length of 111 km, a perimeter of 498 km, and a drainage density of 1.06 km/km^2 (Das & Pardeshi, 2018). It starts on the northern outskirts of the Western Ghats mountain range and flows through Mumbai metropolitan region, the second-most populous metropolis in India, where Mumbai is the eighth-most populous city in the world. It spans a diverse area such as mountain forest, agricultural, industrial, suburban, and urban residential zones before flowing into the Arabian Sea at Vasai. Despite having a high conservation value (105 estuarine and 40 freshwater fish species; Lal et al., 2020), the Ulhas River is extremely significant since it is one of the rivers that provides drinking water to millions of people, the only source of freshwater fish for most urban residents, and the sole source of income for artisanal fishers. However, as Mumbai is the financial hub of India, it draws immigrants from all over the country, increasing its population and encouraging a wide range of anthropogenic activities in the Ulhas River basin, which have a number of detrimental effects on the river (Rathod, 2020) and have experienced rapid urbanisation and industrialization in recent years. The middle zone of Ulhas River has Asia's largest industrial cluster (Thane-Belapur industrial belt), with hundreds of industries, such as dyeing, pharmaceutical, plastic, petroleum, and paint industries (Menon & Mahajan, 2011; Rathod, 2020; Zingde & Govindan, 2001). In recent years, fish species and catches in the river have consistently decreased to unprofitable levels, and it has been speculated that one of the causes may be an increase in plastic pollution (Rathod, 2016, 2020). However, till date, the precise estimates on MP abundance in the Ulhas River are not available and thus deserve attention.

Furthermore, the river is heavily polluted with heavy metals like chromium, lead, nickel, copper, cadmium, zinc, and mercury (Raut et al., 2019). The capacity of MPs to transport heavy metals, organic and inorganic pollutants, and radionuclides has been demonstrated in a number of earlier studies (Goday et al., 2019; Ioannidis et al., 2022; Kinigopoulou et al., 2022). Since heavy metals and MPs are known to be present in fish gathered from the Ulhas River (Kumkar et al., 2021; Menon & Mahajan, 2011), the possibility that these heavy metals enter fish via MP

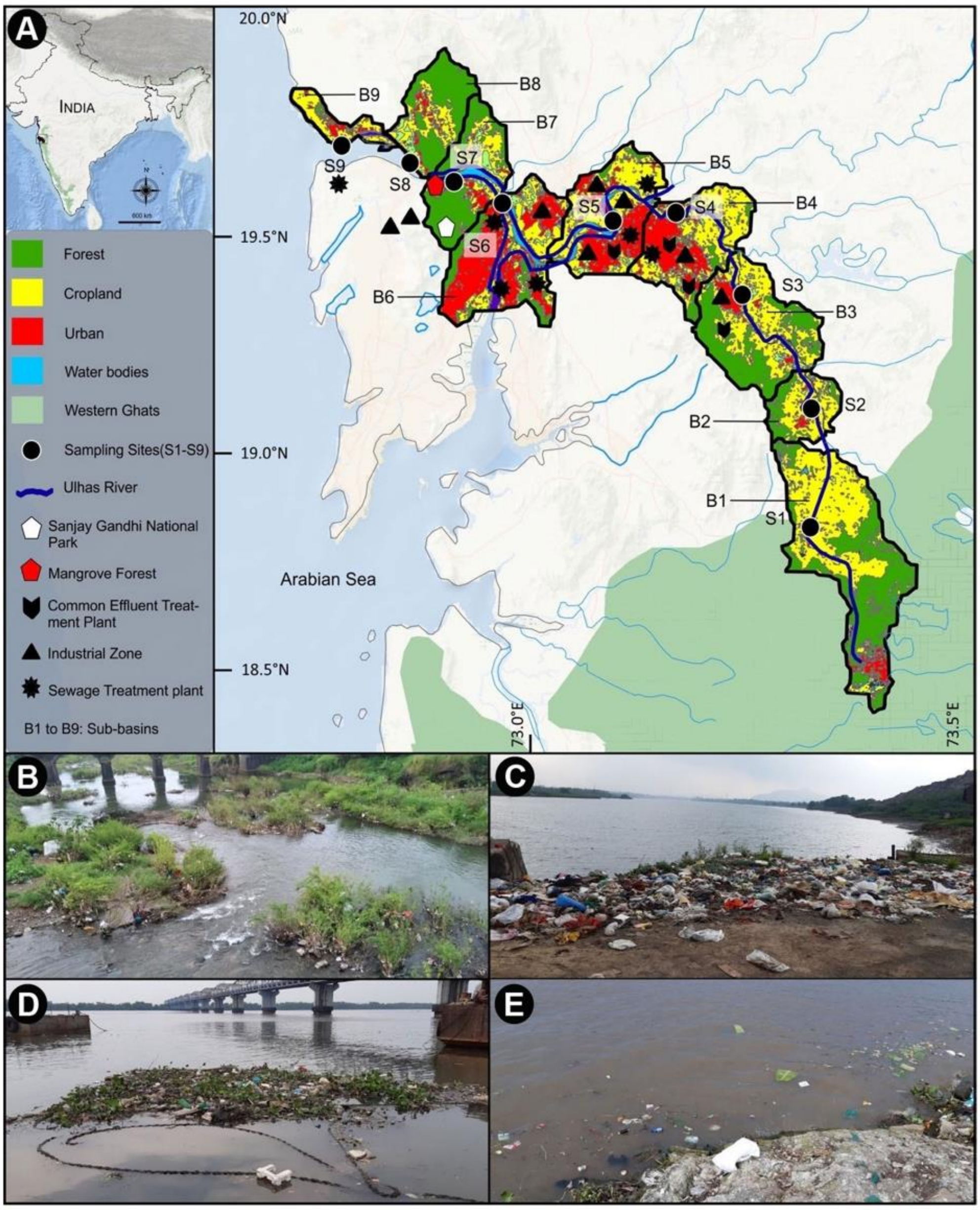


Fig. 1 **A** Study area with sub-basins, B1 to B9, and sediment collection sites, S1 to S9, in the Ulhas River basin, Maharashtra, India; **B** to **E** representative photographs of the plastic pollution at study sites

intake cannot be overlooked. However, supporting empirical evidence is still lacking. Therefore, MPs have highlighted severe concerns about the health of this dependent community and about a harm to the ichthyofauna. The precise estimations of the MP contamination would undoubtedly aid in the formulation of plans for the conservation of a diversity of fish species as well as the establishment of priority management zones for efficient pollution monitoring.

Therefore, we quantified MP pollution in the entire Ulhas River with the following fundamental objectives: (1) to collect, quantify, and characterize MPs in sediment; (2) to determine various sources of MPs; (3) to determine potential role of MPs to function as heavy metal carriers; and (4) to determine MP hotspots in the Ulhas River.

2 Materials and Methods

2.1 River Demography and Land Use Pattern

The details of the demographic estimates and land use characteristics of the Ulhas River sub-basins are provided in Table 1. The land use in the Ulhas River basin is heterogeneous, with the upper river stretch primarily dominated by forest and agricultural land with dispersed villages, the middle river section principally hosting a combination of industry and suburban residential zones, while the lower stretches comprising the estuarine zone are primarily an urban residential area (Fig. 1A). HydroSHEDS (Hydrological data and maps based on SHuttle Elevation Derivatives at multiple Scales; http://www.hydrosheds.org) was used to define the river's sub-basin area (Lehner & Grill, 2013). Data on land use for forestry, agricultural purpose, and residential development (village, semi-urban, and urban) were retrieved from the Copernicus database (Buchhorn et al., 2020), and the percent contribution of each land use parameter was estimated using QGIS 3.10. The Worldclim version 2.1 program (https://www.worldclim.org/data/worldclim21.html) and ArcGIS online were used to compute sub-basin-specific precipitation and demographic parameters (population and population density), respectively.

2.2 Sediment Sampling and Microplastic Extraction

In December 2020, sediment samples from nine sampling stations were collected in triplicates using a shovel (5 cm depth) and a stainless-steel container while maintaining a distance of 100 m between any two sampling points to ensure the precision of the sampling (Nel & Froneman, 2015; IlonaSekudewicz et al., 2021; Wen et al., 2018). We focused on the top 5 cm of the sediment since past studies have shown that MP abundance is the highest in this layer and falls dramatically with depth (Filho & Monteiro, 2019; Nguyen et al., 2020; Turra et al., 2014; Yu et al., 2016). While sampling, precautions were made to ensure that most of the rivers' sub-basins were covered. MPs were isolated from sediment samples according to a well-established method (Amrutha & Warrier, 2020; Shruti et al., 2019). Briefly, wet sediment samples were dried in a hot-air oven (at

Table 1 Details of the Ulhas River demographic characteristics and land use pattern and MP abundance during the study period

Sub-basin	Area (km^2)	Population (no. of individuals)	Population density (persons/km^2)	Total households (no. of houses)	% forest	% crop	% urban	Annual mean precipitation (mm)	MP abundance (M ± SE)
B1	248.20	61,302	479.60	27,223	43.11	46.74	4.83	328.56	40.0 ± 5.77
B2	57.61	19,468	663.20	8822	29.51	59.02	5.21	335.81	96.7 ± 31.8
B3	160.34	150,885	1801.30	73,305	41.16	44.90	8.11	308.29	303 ± 29.6
B4	128.42	629,075	9286.10	298,800	11.68	50.62	31.93	260.34	320 ± 30.6
B5	133.13	612,980	8660.60	297,145	5.26	49.58	35.30	237.14	330 ± 92.9
B6	165.28	1,516,601	17,007.10	707,783	15.13	23.60	48.40	223.26	400 ± 80
B7	104.18	103,467	1822.60	47,488	54.71	24.00	6.72	231.67	413 ± 133
B8	87.33	57,773	1252.10	28,355	59.54	25.19	6.87	212.27	360 ± 61.1
B9	34.71	45,912	2503.70	22,534	5.76	54.74	17.29	166.35	600 ± 122

Sum of percent forest, crop, and urban land does not equal 100% as other land use accounts for the reminder

40–45 °C) for 48 h then passed through a 5 mm sieve to remove particles > 5 mm. Dried sediment (100 g) from each replicate was subjected to a process of wet peroxide oxidation with 30% H_2O_2 (250 ml) to ensure complete digestion of the organic matter present in the samples, followed by density separation with $ZnCl_2$ flotation and vacuum filtration through Whatman GF/A filter paper, 43 mm (0.45 µm). The filters containing MP particles were then carefully removed, dried in a desiccator, and stored in glass Petri dishes.

2.3 MP Analysis and Characterization

The MPs were counted, measured, and sorted by color and type using a stereomicroscope. Isolated MP particles ($n = 593$) were divided into five morphotypes based on their shape: fragments, foam, pellets/beads, film, and fibres/filaments (Karthik et al., 2018). Each morphotype was further classified according to colour as red, yellow, blue, green, black, white/colorless, and other (purple, pink, violet, grey, brown, etc.). Validation of visual MP identification was achieved using Fourier transform infrared (FTIR) spectrometry with an attenuated total reflectance (ATR) attachment (Thermo Scientific Nicolet™ iS20 FTIR with Smart™ iTX Diamond ATR; wavelength range = 4000 to 500 cm^{-1}, spectral resolution = 4 cm^{-1}, and number of sample scans = 64). Owing to the limitation of size, which is capable of identifying several polymer types by FTIR, we also used Raman spectroscopy (Witec Alpha 300 R). In total, 190 MP particles (an average of 21.11% of particles from each sampling station) were characterized for their polymer type. The polymer type was determined by comparing them to the reference library.

2.4 Surface Texture and Surface Elemental Analysis

Scanning electron microscopy (SEM) coupled with energy-dispersive X-ray analysis (EDX) is a widely accepted method (Kumkar et al., 2021; Shruti et al., 2019) for determining the surface texture/topography of MPs and identification of surface elements. This technique can provide crucial information about MP disintegration due to weathering and/or ageing and its ability to carry metal contaminants, which aquatic animals like fish can ingest. MP particles from each sampling station were sputter-coated with gold and imaged by SEM (Tescan Mira3) at 15 kV. The surface elemental composition and their distribution on MPs were determined by fully integrated Essence™ EDX analysis software (Tescan Mira3).

2.5 Pollution Indices

With some modifications, the MP pollution load index (PLI), polymeric risk index (H), and pollution risk index (PRI) were determined using the approach described by Kabir et al. (2021). These indices were employed by Kabir et al. (2021) to compare rivers, but we used them to compare basins within the river system. In a brief, the PLI is a standard approach for assessing the degree of pollution across river basins that is computed using the Ulhas River's estimated MP abundance data. The basin is considered polluted when the PLI value is greater than one (Tomlinson et al., 1980). The following formula (Eq. 1) was used to determine the PLI:

$$PLI_i = C_i / C_o \tag{1}$$

where "I" stands for a basin (B1 to B9), "n" stands for the number of basins in a river ($n = 9$), C_i stands for MP abundance in basin I, and C_o stands for the lowest baseline concentration documented in the literature. Due to a complete lack of published data in similar contexts and the analytical context of this study, the lowest MP abundance (40 particles·kg^{-1} d.w. of sediment) obtained in this study was selected as the baseline concentration. The following formula (Eq. 2) was used to determine the PLI_{river}, which is the overall MP pollution load index for the river, which is the nth root of the entire MP pollution load indices multiplied together, and PLI_i is the pollution load index for basin i:

$$PLI_{river} = \sqrt[n]{PLI_{B1} \times PLI_{B2} \times PLI_{B3} \ldots\ldots\ldots\ldots \times PLI_{Bn}} \tag{2}$$

MP pollution risks for basins and river was estimated using the formula proposed by Kabir et al. (2021). To assess MP pollution risks, the estimated abundances of MPs as well as the chemical toxicity coefficients (risk score) of MP polymers reported by Lithner et al. (2011) were used. The polymeric risk assessment was performed using Eq. 3 and 4:

$$H_i = \sum_{j=1}^{m} \{ (P_{ji}/C_i) \times S_j \} \quad (3)$$

$$H_{river} = \sqrt[n]{H_{B1} \times H_{B2} \times H_{B3} \ldots \ldots \ldots \ldots \times H_{Bn}} \quad (4)$$

where j is a polymer type (e.g. nylon, HDPE, LDPE, polyester, etc.), m is the number of polymer types identified, Pji is the number of each single MP polymer identified at basin i, and Sj is the risk score for each single MP polymer (adapted from Lithner et al., 2011) and B is the basin. The Sj values or risk score for each polymer is as follows: nylon = 50; HDPE = 11; polyethylene = 11; PET = 4; polyester = 44; polyether urethane = 7384; polypropylene = 1; polystyrene = 30; PVC = 10,551; LDPE = 11). Finally, H_i represents the sum of MP polymeric risk indices at station "i" while H_{river} represents the polymeric risk for the rivers, which is the nth root of the total polymeric risk scores multiplied together.

MP pollution risks for the basin as well as the river were calculated using Eqs. 5 and 6:

$$PRI_i = H_i \times PLI_i \quad (5)$$

$$PRI_{river} = \sqrt[n]{PRI_{B1} \times PRI_{B2} \times PRI_{B3 \ldots \ldots \ldots \ldots} \times PRI_{Bn}} \quad (6)$$

where PRI_i represents the MP pollution risk index for basin "i" and PRI_{river} represents the MP pollution risk for the river, which is the nth root of the total pollution risk scores multiplied together.

2.6 Quality Assurance/Quality Control

As airborne MPs are omnipresent, including inside laboratories, contamination with them is a recurring issue in MP research (Song et al., 2021). Therefore, procedure blanks and aerial contamination controls were used throughout the experimentation to avoid MP contamination from external sources as recommended earlier by Song et al. (2021). Briefly, to collect and store sediment samples, only steel containers with tight-fitting lids were used. To reduce background contamination, only cotton aprons and nitrile gloves were worn. All of the glassware used in the experiments was disinfected and cleaned with pre-filtered distilled water before being wrapped in aluminium foil until use. The samples were examined under a hood and all containers were covered with aluminium foil. Each batch of MPs (one per sampling station) included one procedural blank filter, for a total of nine blank filters, which were examined in the same way as the test filters. Aerial contamination was also independently assessed for each batch of MPs, by keeping a clean, pre-checked filter throughout the extraction process (Kor & Mehdina, 2020). No MPs were found on any of the nine filters, indicating that there was no MP contamination from the air.

2.7 Statistical Analysis

Before analysing the data, the Shapiro–Wilk test and Levene's test were performed to ensure a normal distribution and homoscedasticity. The abundance of MPs in sediment is reported as the number of items/particles·kg^{-1} dry weight (d.w.) of sediment. The difference in MP abundance between Ulhas River sampling stations (B1 to B9) was tested using the Kruskal–Wallis test (H test; $\alpha < 0.05$) followed by multiple-pairwise comparisons using Dunn's test. Normalized data on watershed and land use characteristics (total sub-basin area, forest cover, degree of urbanization, agricultural/cropland use, precipitation, and population density) were subjected to principal component analysis (PCA) to determine which of these factors had the greatest influence on MP abundance in the Ulhas River basin. Pearson's correlation matrix analysis was used to determine the exact degree of association between MPs and demographic and land use characteristics. All analyses were performed using PAST freeware (Hammer et al., 2001; Freeware, http:/folk.uio.no/ohammer/past/).

3 Results and Discussion

3.1 MP Abundance and Global Comparison

MP contamination was found in sediment samples collected from all sampling stations of the Ulhas River (Fig. 1B to E; Table 1). Differences in MP abundance between sampling stations/sub-basins were significant ($H = 16.7$; $p = 0.0328$). The abundance of MPs varied from 40 ± 5.77 particles·kg^{-1} d.w. (B1) to 600 ± 122 particles·kg^{-1} d.w. (B9) of sediment (Table 1). Several chemicals such as NaCl, $ZnCl_2$, sodium polytungstate, and sodium iodide are used for density separation during MP extraction (Shruti et al., 2019). These chemicals are known to affect the number of MPs extracted (Shruti et al., 2019). However, NaCl and $ZnCl_2$ are the most often used chemicals for MP extraction since they have specific benefits over other chemicals and their extraction results are comparable. As a result, we confined our comparisons to (1) studies that extracted MPs from riverine sediment using NaCl or $ZnCl_2$, and (2) studies that reported their findings using the same quantification unit (MP particles.kg^{-1} of sediment). A comparative analysis of riverine MP abundance in sediment across the world (Fig. 2) is provided in Supplement 1. The mean MP abundance (318.15 ± 36.98 particles.kg^{-1} d.w. of sediment) in the Ulhas River recorded in the present study was higher than those reported for the Netravati River in India (253 particles·kg^{-1} of sediment; Amrutha & Warrier, 2020), Yangtze River in China (25–340 particles·kg^{-1} of sediment; Zhao et al., 2014) which is reasonable given the polluted status of the Ulhas River basin.

3.2 MP Morphotypes and Possible Sources

All five MP morphotypes, viz., fragments (72.85%), beads (8.43%), filaments (8.43%), film (7.08%), and foam (3.20%), were detected in samples from the Ulhas River basin (Fig. 3A). Details of the site-specific abundance of each morphotype are provided in Supplement 2. MP morphotypes can provide valuable information on their possible sources (Amrutha & Warrier, 2020; Kabir et al., 2021; Karthik et al., 2018). Breakdown of plastic carry bags, packing materials, and plastic containers, as well as home plastic objects, are the principal sources of MP fragments (Amrutha & Warrier, 2020; Shim et al., 2018); hence, the higher abundance of MP fragments in heavily inhabited B3, B4, B5, and B9 sub-basins is entirely reasonable. This clearly shows that the MPs detected in the Ulhas River are largely secondary in origin. Beads comprised the second-highest category of MPs in the Ulhas River basin, particularly in the lower reaches (B6 to B8), which is reasonable given that these are highly developed urban areas with greater use of personal care items and cosmetics, dominated by small-scale industries that use plastic pellets as feedstock for manufacturing plastic objects (Kumkar et al., 2021). On the contrary, B1, B2, and B3 sub-basins are dominated by MP films, possibly because of the greater use of plastic carry bags. Although single-use plastic bags have been strictly prohibited in urban areas, it is still in use in rural and suburban areas, where the solid waste management is still inadequate. Artisanal fishermen,

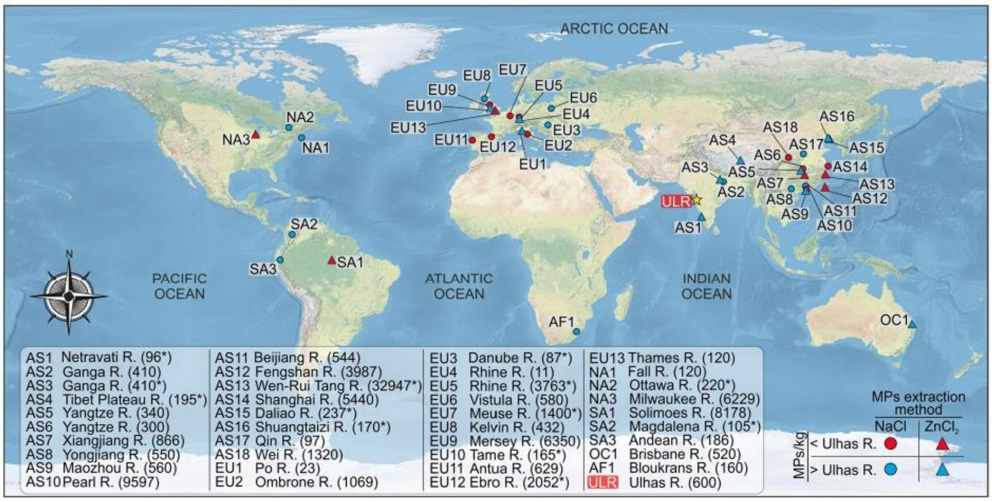


Fig. 2 Map showing comparative analysis of microplastics abundance in rivers across the globe. Note: "R" after each name is the "River". The asterisk indicates the average amount of microplastic reported for that river

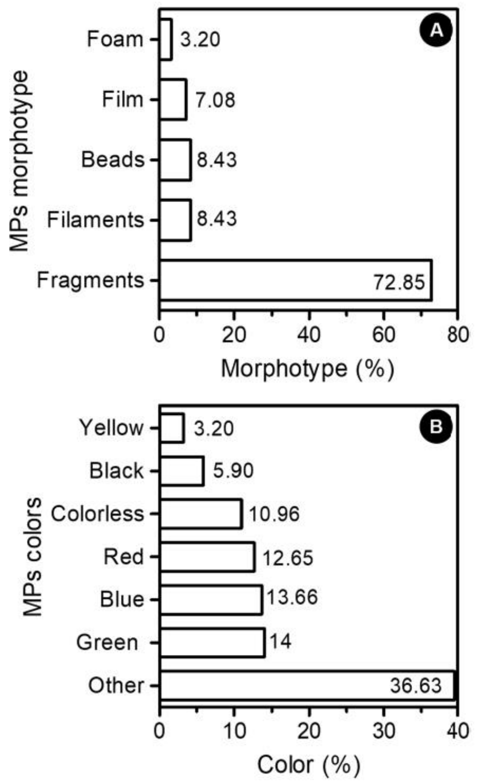


Fig. 3 Overall % distribution of microplastics in the Ulhas River; **A** morphotype-based and **B** colour-based (other includes purple, pink, violet, grey, yellow, and brown microplastic particles)

cargo ships, sand mining operations, and textile industries all use the B3 to B9 sub-basins as their primary locations, so the incidence of filaments and fibres is not surprising (Amrutha & Warrier, 2020; Su et al., 2016). It is possible that the increased prevalence of foam in B6, B7, and B9 is a result of neighbouring industries (Kumkar et al., 2021; Raut et al., 2019) that utilise foam and polystyrene fillers as packing materials, as well as furniture manufacturing firms that use foam and fillers (Rochman et al., 2019; Turner & Lau, 2016; Wang et al., 2019).

A wide range of coloured MPs was detected in the Ulhas river basin. The overall percent contribution of each colour of MPs is as follows: other (39.63%), green (14%), blue (13.66%), red (12.65%), colourless (10.96%), black (5.90%), and yellow (3.20%) (Fig. 3B; Supplement 3). The presence of a wide spectrum of coloured MPs in the Ulhas River sediments is expected, as these are extensively used in toys, clothing, medical equipment, and household packaging material (Robin et al., 2020; Wang et al., 2017). Therefore, high population density, human activities in urban and suburban zones, and a large number of diverse industries are likely to be the sources of coloured MPs. Blue, colourless, and green MPs predominate in the Ulhas River basin (Supplement 3), which is consistent with recent studies by Kumkar et al. (2021). Green, colourless, and blue MPs are commonly mistaken as prey by fish (Karthik et al., 2018; Naidoo et al., 2020; Ory et al., 2017; Wright et al., 2013), which likely to pose health problems for fish consumers. The Ulhas River basin's high ichthyofaunal diversity (Rathod et al., 2002), combined with the population's high demand for freshwater fish and known incidences of plastic ingestion of MPs by fish (Kumkar et al., 2021), clearly points to the seriousness of the public health.

3.3 MP Polymers and Link with Domestic and Industrial Sources

The results of Raman spectroscopy and FTIR-ATR analysis of MPs from sediments ($n = 190$) are as follows: low-density polyethylene (LDPE) $n = 35$, 18.42%; high-density polyethylene (HDPE) $n = 30$, 15.79%; polypropylene (PP) $n = 22$, 11.58%; polystyrene (PS) $n = 22$, 11.58%; polyethylene (PE) $n = 16$, 8.42%; polyester (POE) $n = 14$, 7.37%; polyether urethane (POU) $n = 12$, 6.32%; polyethylene terephthalate (PET) $n = 11$, 5.79%; polyvinyl chloride (PVC) $n = 11$, 5.79%; nylon (NY) $n = 4$, 2.11%; and others (cellulose, resin dispersion, sealing ring Gardena; $n = 13$, 6.83%) (Fig. 4A and 4B). Details of percent variations in MP polymer type among sub-basins are provided in Supplement 4. Chemical characterization of the polymer type in MPs has been found useful as a means of obtaining information about their probable source (Ballent et al., 2016; Galgani et al., 2015; Robin et al., 2020). The polymers identified in this study are commonly used in the manufacture of household and commercial plastic products. For example, LDPE is widely used in the manufacture of plastic bags (Dowarah & Devipriya, 2019; Lusher et al., 2017; Syakti et al., 2018), and thus it has the

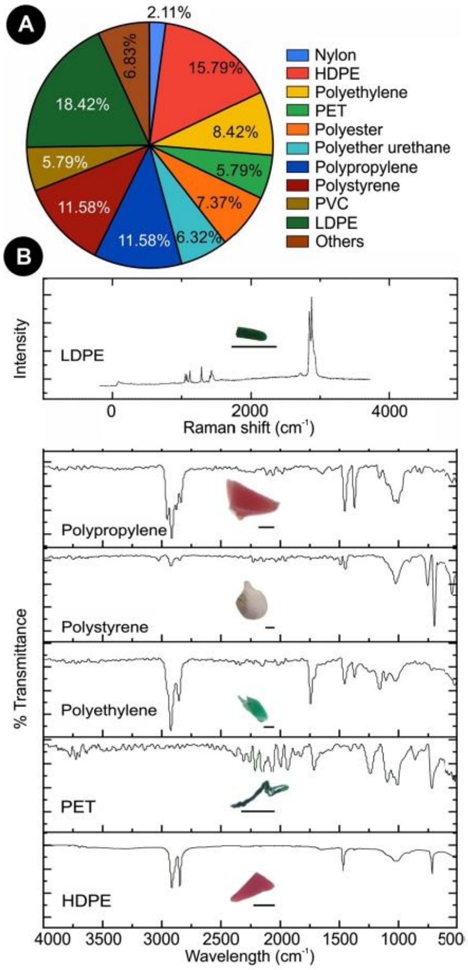
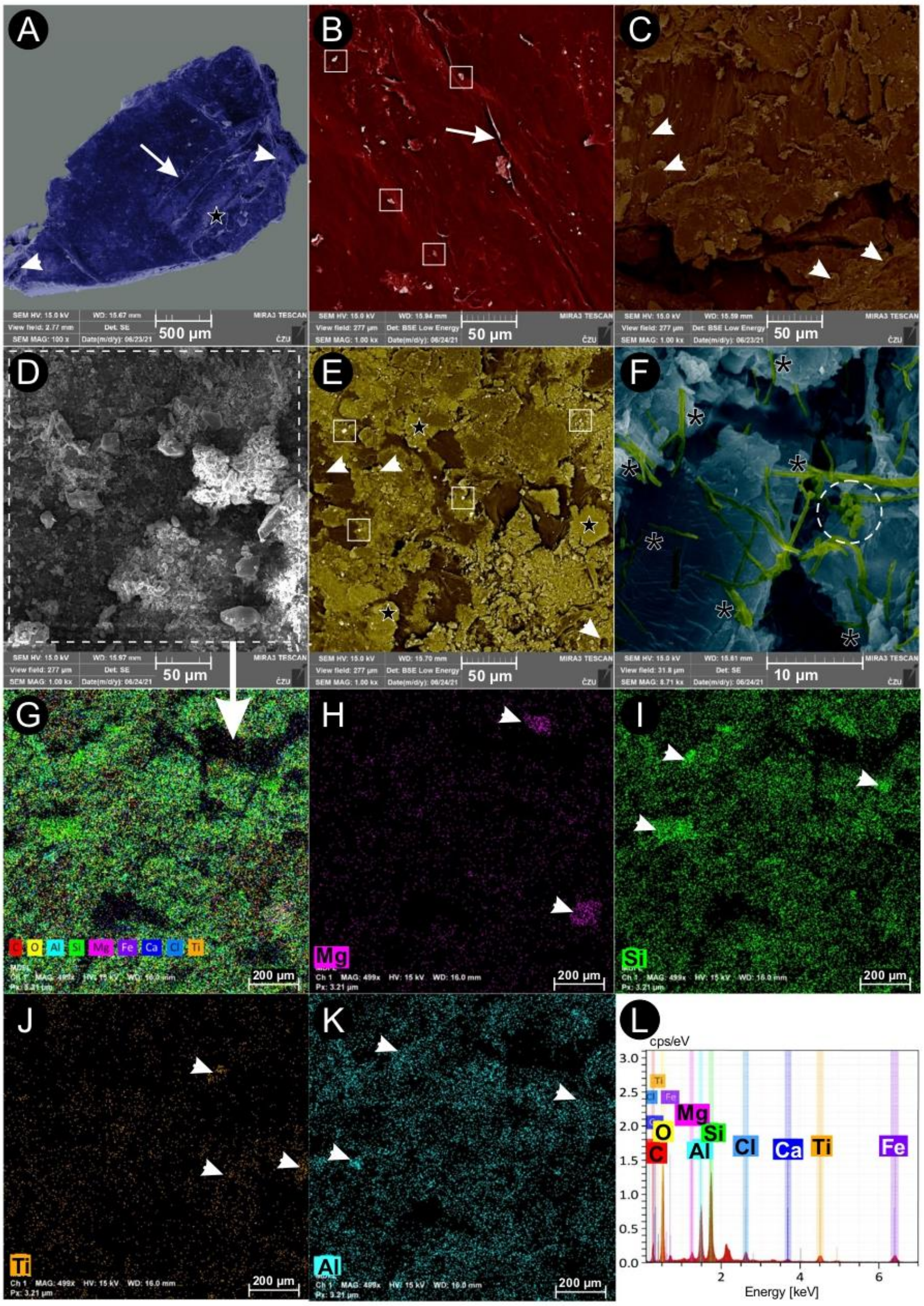


Fig. 4 **A** Pie chart showing overall distribution of microplastics by polymer type in the Ulhas River basin, India; **B** Raman and FTIR-ATR spectra of the described microplastic polymers; scale bar = 1 mm

highest occurrence (33.33%) in B1 (Supplement 4), indicating that this region's waste management system is inadequate. The greater abundance of polypropylene in B1 (25%) and PET in B2 (22.22%) is also not surprising, as these materials are extensively used for food packaging, toys, pipes, beverage bottles, fishing line, housewares, and furniture (Karthik et al., 2018; Koshti et al., 2018; Maghsodian et al., 2020). Polyester was found in abundance in the B5 sub-basin (Supplement 4), which is likely due to a large number of textile and dyeing industries in close proximity to the sub-basin and washing of synthetic clothes, which is common practice in the Ulhas River basin. Because polyether urethanes are used in the manufacture of footwear, sporting goods, outer cases of mobile and electronic devices, automotive interior parts, thermal and sound insulators, cushion and carpet, and some medical appliances, its higher occurrence in highly populated, urbanized sub-basins B4 (4%), B5 (16%), and B6 (24%) is no surprise (Supplement 4). These results suggest that the MP polymer types found in the Ulhas River basin come from both domestic and industrial sources.

3.4 Surface Topography, Elemental Composition, and Distribution Analysis

SEM imaging of the MP surface provides valuable information about their physical disintegration (Kumkar et al., 2021; Shruti et al., 2019). MP particles from the Ulhas River basin exhibited a variety of disintegration patterns, including pitting, cracking, and flaking (Fig. 5A to 5E). Our results suggest that MPs isolated from the Ulhas River are constantly disintegrating, which may transform them into other micro- or nano-forms that could pose a greater threat to aquatic biodiversity and human health. According to Oberbeckmann et al. (2015), MPs provide persistent surfaces that can be colonized by a variety of microorganisms including bacteria and fungi. We observed biofilm on the MPs that appeared structurally similar to fungal hyphae and spores (Fig. 5F). However, further analysis of these MPs colonizing microorganisms will be required for their exact identification. The EDX spectral surface analysis revealed carbon (C), oxygen (O), aluminium (Al), silicon (Si), magnesium (Mg), iron (Fe), calcium (Ca), chlorine (Cl), and titanium (Ti) (Fig. 5G and L); however, elemental mapping demonstrated a patchy or unequal distribution of these elements on the surface (Fig. 5H to K), which might be the result of the physical breakdown of the MPs. The old or eroded MPs with pits, fractures, or cracks offer locations where other contaminants could adhere (Shruti et al., 2019) and thus are likely to function as carriers of the other contaminants including heavy metals. Furthermore, biofilm development reflects the length of MP environmental


A
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500 μm
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10 μm
200 μm
Mg
Si
Ti
Al
cps/eV
Energy [keV]
C
O
Mg
Si
Al
Cl
Ca
Ti
Fe

◀**Fig. 5** SEM–EDX analysis of microplastics from the Ulhas River basin. **A** to **E** SEM images showing weathering of microplastics as indicated by pits (arrowhead), cracks (arrow), flakes (star), and attached particles (square box); **F** microplastic particle showing attached biofilm containing fungal hyphae (asterisk) and spores (circled portion); **G** to **L** representative images of surface elemental distribution and mapping. Note: elemental mapping of the square marked portion in panel **D** is provided in panels **G** to **K**

exposure; the existence of these biofilms indicates that the MP particles are old. Also, the capacity of biofilms to change hydrophobicity (Tu et al., 2020), which can enhance heavy metal adsorption on MPs, cannot be overlooked. Because the appearance of biofilm is a reflection of the duration of MP environmental exposure (Tu et al., 2020), the presence of these biofilms confirms that the MP particles are old, and thus are potential carriers of the heavy metals. Previous research in the Ulhas River estuary confirmed the presence of trace elements such as lead (Pb), nickel (Ni), chromium (Cr), copper (Cu), zinc (Zn), mercury (Hg), and cadmium (Cd) in water samples (Raut et al., 2019). Similarly, several additives and catalysts containing toxic metals (and metalloids) such as arsenic, cadmium, chromium, and lead are commonly incorporated into plastics during their production (Boyle et al., 2020; Teuten et al., 2009; Turner & Filella, 2021). Although we did not detect these elements directly associated with MP particles, this could be due to the limited number of MP particles studied. As a result, we recommend that future studies not only analyse a greater number of MP particles, but also include empirical evidence for whether harmful metals were introduced during manufacturing or accumulated from the environment. Regardless of their source, ecotoxicology and human health risks concerning the discharge of heavy metals in aquatic ecosystems must be given attention.

3.5 MP Hotspots and Associated Risk

Earlier studies have emphasized the need of delineating riverine risk zones and identifying MP hotspots in terms of pollution monitoring and management (Kabir et al., 2021). In order to determine MP hotspots, we considered the MP abundance and demographic estimates and assessed levels of risk using pollution risk indices. The details of the demographic estimates and land use characteristics of the Ulhas River sub-basins are provided in Table 1. The PCA biplot (Fig. 6A) clearly explained 91.53% of the variation in MP abundance in the Ulhas River based on the degree of urbanisation, the population density, and the total number of households. The correlation matrix (Fig. 6B) provided further support in the form of a statistically significant positive relationship between MP abundance and degree of urbanisation ($r=0.82$; $p=0.041$), and population density ($r=0.81$; $p=0.049$). A strong negative correlation ($r=-0.87$; $p=0.022$; Fig. 6B) was also found between MP abundance and precipitation. This clearly shows that demography plays important role in the distribution of MPs in the Ulhas River.

The estimated PLI values for all basins (PLI value range from 1 to 15) and the total PLI value for the Ulhas River (PLI = 6.35) also show differential pollution levels (Fig. 7A; Table 2), with the lowest pollution at B1 (PLI = 1) and the highest pollution at B9 (PLI = 15). The PLI values are organised as follows: B9 > B7 > B6 > B8 > B5 > B4 > B3 > B2 > B1 (Fig. 7A). Between river basins, the polymer risk score (*Hi*) ranged from low ($H_{B7}=0.26$) to high ($H_{B6}=164$). The B3 to B6 basins, as well as the B9 basin, were found to be at high risk for MP polymers (Fig. 7B; Table 2). Higher polymeric hazards were linked to the presence of more toxic polymers and their higher hazard scores (PVC = 10,551; polyether urethane = 7384; nylon = 50; and polyester = 44) and were high-risk indicators along the river basins (B3 to B6, and B9). In high-risk basins (B3-B6, and B9), the most hazardous polymers (PVC and polyether urethane) were found to be abundant (Fig. 7B; Supplement 4). We further observed that the basin area with higher MP abundance is unlikely to pose a greater risk of MP pollution, but the pollution risk in the basin is likely to be depending on the type of polymers released in those basins. For instance, although B3 has a lower MP abundance than B4 and B5, it poses a considerable hazard risk in terms of *Hi* value since PVC accounts for 12% of total MP abundance at B3, compared to 4% and 0% at B4 and B5, respectively (Fig. 7B; Supplement 4). B7, on the other hand, has a higher MP abundance than B1, B3, and B6, but poses a lower hazard risk by means of *Hi* value since HDPE, PE, PP, and LDPE account for 70% of total MP abundance at B7 (Fig. 7B; Supplement 4), and there are no high-risk score polymers like polyether urethane and PVC. PRIi values clearly showed

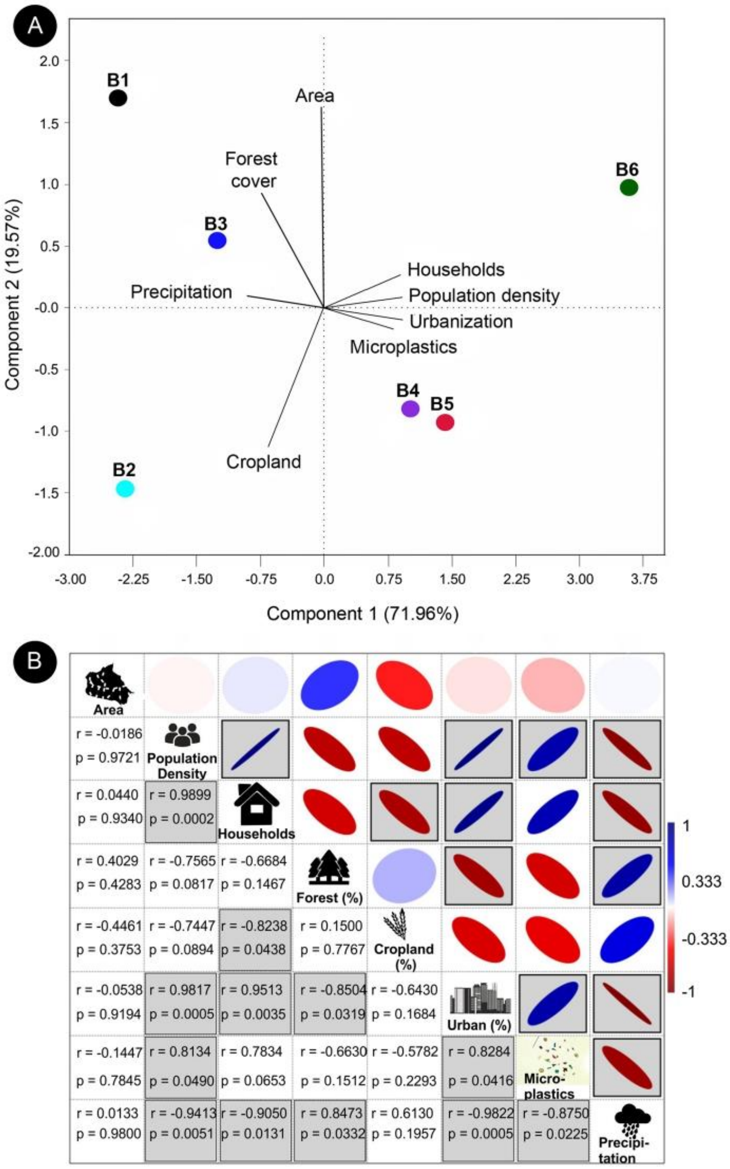


Fig. 6 **A** PCA biplot and **B** Pearson's correlation matrix for factors affecting microplastic distribution in the Ulhas River basin. Grey boxes around the scatter plots indicate significant positive (blue) or negative (red) correlations

low ($PRI_{B7,\ B8}=2.68$) to high risks ($PRI_{B6}=1640$) among the river basins (Fig. 7C; Table 2). The basins with the highest polymeric risks also had the highest pollution risks. Pearson's correlation analysis revealed a significant correlation (Pearson's $r=0.946$; $p=0.0001$) between estimated values of H and PRI (Fig. 7D), but no significant correlation (Pearson's $r=0.501$; $p=0.169$; Fig. 7E) was found between MP abundance and H, or between MP abundance and PRI (Pearson's $r=0.638$; $p=0.063$; Fig. 7F). This clearly

Table 2 Basin-specific microplastic pollution load index (PLI), polymeric risk assessment (*H*), pollution risk index (PRI), and details of risk zones, various point and non-point sources of MP and MP hotspots in the Ulhas River, Maharashtra, India

Basin	PLI score	Status	*H* score	Risk category	PRI	Risk level	Sources of MP pollution	Pollution hotspot
B1	1.00	Polluted	2.75	I	2.75	Low	Agricultural sources	No
B2	2.42	Polluted	1.27	I	3.08	Low	Anthropogenic and agricultural sources	No
B3	7.58	Polluted	105.37	III	799.05	High	Anthropogenic and agricultural sources	Yes
B4	8.00	Polluted	57.17	II	457.35	Considerable	Anthropogenic, industrial, and agricultural sources	Yes
B5	8.25	Polluted	90.60	II	747.45	High	Anthropogenic, industrial, and agricultural sources	Yes
B6	10.00	Polluted	164.00	III	1640.00	Very high	Anthropogenic and industrial sources	Yes
B7	10.33	Polluted	0.26	I	2.68	Low	Agricultural and industrial sources	No
B8	9.00	Polluted	0.30	I	2.68	Low	Agricultural sources	No
B9	15.00	Polluted	101.52	III	1522.73	Very high	Anthropogenic sources and agricultural sources	Yes

Risk categories based on the score of Kabir et al. (2021)

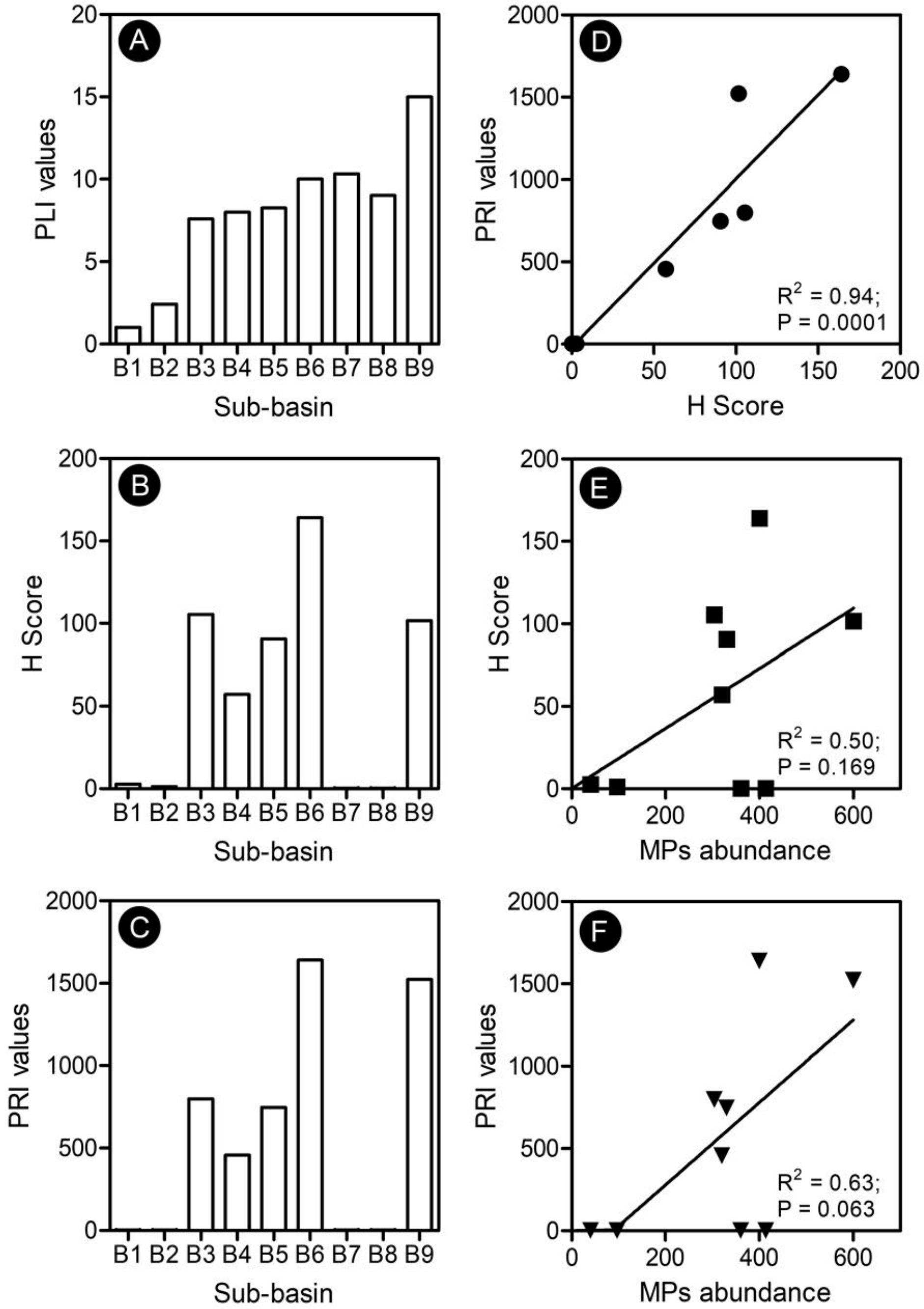
indicates that basins with similar levels of MP abundance may not pose similarly high polymeric and pollution risks.

Together, basin demography and pollution risk scores (*H* and PRI) associated with each basin of the Ulhas River can be effectively used in identifying MP hotspots in the river (Table 2). We found that agricultural land use–dominating basins (B1 and B2) and forest-dominating basins (B7 and B8) posed low risks compared to midstream B3 to B6 and downstream B9, which are dominated primarily by urban and industrial clusters, posing a high risk. Therefore, we designate sub-basins B3 to B6 and sub-basin B9 as MP hotspots in the Ulhas River.

4 Conclusions and Region-Specific Mitigation Strategies

The current study provides baseline MP concentration in the Ulhas River, Maharashtra, India, including likely sources, routes, hotspots, pollution risk and hazard score, fates, and possible mitigation strategies. The pollution hotspots identified in the present study are likely to be utilised to establish priority management zones for effective pollution monitoring and control. According to the findings of this study, the B3 to B6 and B9 sub-basins are the MP hotpots in the Ulhas River. Since MP pollution in the Ulhas River is a function of population, the collaborative efforts of the public, industries, and the government can achieve MP mitigation and long-term eco-friendly, sustainable development along the Ulhas River. For effective MP pollution management in the Ulhas River, we recommend the following mitigation strategies:

1. MPs in the Ulhas River can be mitigated to a greater extent by appropriate segregation of plastic in the B3 to B6 region, as MP pollution in Ulhas is closely related to population density. Residential and commercial buildings of these regions should have their own facilities (like dry–wet waste management systems) for segregating plastic waste. The local authority should apply measures to ensure that the separated plastic does not enter the Ulhas River.
2. Textile industries in the B3 to B6 sub-basins and fishermen and villagers in the B1 and B2 sub-basins who wash their clothes on the river banks and throw old fishing nets into the river itself all likely to contribute for the release of microfibers or filaments into the Ulhas River. As a result, policy implications must be established to construct public cloth-washing platforms with advanced technology away from river banks, as well as to upgrade waste-water treatment plants (WWTPs) in these sub-basins.


A
PLI values
20
15
10
5
0
B1 B2 B3 B4 B5 B6 B7 B8 B9
Sub-basin
D
PRI values
2000
1500
1000
500
0
0 50 100 150 200
H Score
R2 = 0.94;
P = 0.0001
B
H Score
200
150
100
50
0
B1 B2 B3 B4 B5 B6 B7 B8 B9
Sub-basin
E
H Score
200
150
100
50
0
0 200 400 600
MPs abundance
R2 = 0.50;
P = 0.169
C
PRI values
2000
1500
1000
500
0
B1 B2 B3 B4 B5 B6 B7 B8 B9
Sub-basin
F
PRI values
2000
1500
1000
500
0
0 200 400 600
MPs abundance
R2 = 0.63;
P = 0.063

◂**Fig. 7** **A** Pollution load index (PLI), **B** polymeric risk score (H), and **C** pollution risk index (PRI), and their distributions among the Ulhas River sub-basin. Correlation analysis between H score and PRI (**D**), MP abundance and H score (**E**), and MP abundance and PRI score (**F**)

3. We recommend that all microbead-containing personal care products be banned entirely in the B5 to B9 sub-basin, and rigorous awareness drives with social media should be carried out to educate the public about the need to use MP-free products.
4. Keeping a close eye on using single-use plastic in local markets in B1 and B2 sub-basins is vital. The local authority should enforce strict regulations on industries in B4 and B5 sub-basins, including furniture and carpet industries, that dump unused or waste foam close to the Ulhas River.
5. The industries along the Ulhas River basin can spend through their Corporate Social Responsibility (CSR) budget to clean up the Ulhas River, contribute to the upgradation of existing WWTPs, assist local authorities in processing plastic waste, and reduce MP pollution in the Ulhas River.
6. The current study is limited to assessing MPs in sediment; therefore, we recommend that future research focus on examining the harmful effects of MPs on aquatic biota in the Ulhas River as well as human health. Furthermore, as we only examined a subset of MP particles using SEM–EDX, more study on the interaction of MPs with heavy metals and biofilm is required.

Abbreviations *MPs*: **Microplastics;** *FTIR-ATR*: **Fourier transform infrared spectrometry–attenuated total reflectance;** *SEM-EDX*: **Scanning electron microscopy–energy-dispersive x-ray analysis;** *LDPE*: **Low-density polyethylene;** *HDPE*: **High-density polyethylene;** *PLI*: **Pollution load index;** *H*: **Polymeric risk;** *PRI*: **Pollution risk index;** *d.w.*: **Dry weight**

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Data Availability Data will be made available on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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5.2. Contaminants and their ecological risk assessment in beach sediments and water along the Maharashtra coast of India: A comprehensive approach using microplastics, heavy metal(loid)s, pharmaceuticals, personal care products and plasticisers

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Contaminants and their ecological risk assessment in beach sediments and water along the Maharashtra coast of India: A comprehensive approach using microplastics, heavy metal(loid)s, pharmaceuticals, personal care products and plasticisers

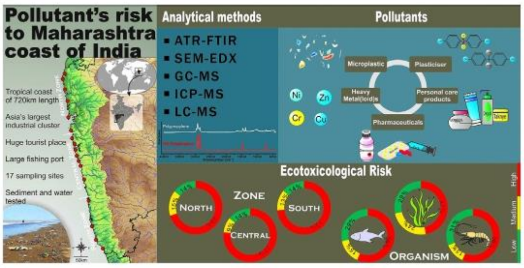
Pradeep Kumkar [a,1], Chandani R. Verma [a,1], Štěpán Hýsek [b], Manoj Pise [a], Sonia Źółtowska [b], Sachin M. Gosavi [c,*], Filip Mercl [d], Matěj Božik [e], Lukáš Praus [d], Kateřina Hanková [e], Radek Rinn [b], Pavel Klouček [e], Miloslav Petrtýl [a], Lukáš Kalous [a]

[a] *Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Czech Republic*
[b] *Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Czech Republic*
[c] *Department of Zoology, Maharashtra College of Arts, Science and Commerce, Mumbai, India*
[d] *Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic*
[e] *Department of Food Science, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Czech Republic*

HIGHLIGHTS

- The northern zone of the Maharashtra coast is identified as a MPs hotspot.
- Cu and Ni levels in coastal waters exceed permissible levels.
- MPs function as a source and a vector for contaminants in coastal water.
- Metoprolol, tramadol, venlafaxine and bisphenols are common in coastal water.
- PPCPs and HMs pose higher risk to fish and crustaceans than the algae.

GRAPHICAL ABSTRACT



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ABSTRACT

Emerging contaminants and their pervasive presence in freshwater ecosystems have been widely documented, but less is known about their prevalence and the harm they cause in marine ecosystems, particularly in developing countries. This study provides data on the prevalence and risk posed by microplastics, plasticisers, pharmaceuticals and personal care products (PPCPs), and heavy metal(loid)s (HMs) along the Maharashtra coast of India. The sediment and coastal water samples were collected from 17 sampling stations, processed, and subjected to FTIR-ATR, ICP-MS, SEM-EDX, LC-MS/MS, and GC–MS for further analysis. Higher MPs abundance, combined with the pollution load index, indicates that the northern zone is a high-impact zone with pollution concerns. Plasticisers in extracted MPs and HMs adsorption on MPs surface from surrounding waters reveal their roles as a source and vector for contaminants, respectively. The mean concentration of metoprolol (53.7–306 ng L^{-1}), tramadol (16.6–198 ng L^{-1}), venlafaxine (24.6–234 ng L^{-1}),

Abbreviations: MPs, Microplastics; HMs, Heavy Metal(loid)s; PPCPs, Pharmaceuticals and Personal Care Products; PAEs, Phthalates or Phthalate Esters; FTIR-ATR, Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; SEM-EDX, Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy; LC-MS/MS, Liquid Chromatography with Tandem Mass Spectrometry; GC-MS, Gas Chromatography–Mass Spectrometry; HQ, Hazard Quotient; PLI, Pollution Load Index.

* Corresponding author.
E-mail address: schn.gosavi@gmail.com (S.M. Gosavi).
[1] Joint first authors.

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and triclosan (211–433 ng L^{-1}) in Maharashtra's coastal waters were several folds higher than in other water systems, raising major health concerns. The hazard quotient (HQ) scores revealed that >70 % of study sites pose a high to medium (1 > HQ > 0.1) ecological risk to fish, crustaceans and algae, indicating serious concern. Fish and crustaceans (35.3 % each) show a higher level of risk than algae (29.5 %). Metoprolol and venlafaxine could represent greater ecological risks than tramadol. Similarly, HQ suggests that bisphenol A has larger ecological risks than bisphenol S along the Maharashtra coast. To the best of our knowledge, this is the first in-depth investigation into emerging pollutants in Indian coastal regions. This information is crucial for better policy formulation and coastal management in India in general, and Maharashtra in particular.

1. Introduction

Diverse lentic and lotic aquatic ecosystems, including freshwater, marine, and estuarine, offer a wide range of ecosystem services, but they are in a precarious equilibrium that easily gets impacted by anthropogenic pressure (Eid et al., 2019; Bashir et al., 2020; Vári et al., 2022; Gosavi and Phuge, 2023). Current scenario of global change, the ongoing expansion of the human population and, ultimately, anthropogenic activities, the amount and frequency of occurrence of several emerging contaminants, such as pharmaceuticals (Chakraborty et al., 2019; Kumar et al., 2023), personal care products (Chakraborty et al., 2019; Picó et al., 2020), microplastics (Li et al., 2020; Dong et al., 2020; Kumar et al., 2022; Yang et al., 2022), phthalates or phthalate esters (Prieto-Amador et al., 2021; Chakraborty et al., 2021; Kumkar et al., 2022), endocrine-disrupting chemicals (Kasonga et al., 2021; Sharma et al., 2021; Chakraborty et al., 2022), persistent organic pollutants and other micropollutants (Chakraborty et al., 2014: 2018, 2019; Picó et al., 2020), in aquatic ecosystems and biota is gradually increasing, posing a severe problem.

These emerging contaminants have been shown to pose significant threats to aquatic organisms and human health (Ramaswamy et al., 2011; Chakraborty et al., 2014; Kumar et al., 2019; Sharma et al., 2021; Kumkar et al., 2022). For instance, pharmaceuticals and personal care products (PPCPs) (Kumar et al., 2019; Sharma et al., 2019; Zhou et al., 2020a), like metoprolol (adrenergic receptor blockers), which is common in aquatic ecosystems owing to its widespread use, can inhibit growth, change gonadotropin and vitellogenin gene expression, stimulate detoxification, and cause oxidative stress in fish (Sun et al., 2015; Gröner et al., 2017). Similarly, venlafaxine (a commonly used antidepressant) has been shown to alter a variety of reproductive and non-reproductive activities in fish, including eating, shoaling, aggression, motility, fear, and anxiety (Salahinejad et al., 2022). Microplastics (MPs), another prevalent pollutant in aquatic environments, have been linked to serious consequences when consumed by living organisms (invertebrates, fish, amphibians, seabirds, etc.), such as digestive tract blockage, endocrine disruption (Wright et al., 2013), weight loss (Peters et al., 2017), growth suppression, reduced juvenile survival (Naidoo and Glassom, 2019), decreased food intake (Kumkar et al., 2021), and damage to the reproductive system and liver (Kumkar et al., 2022), which ultimately affects species growth, fitness, and survival (Naidoo and Glassom, 2019). Phthalates or phthalate esters (PAEs), are used as additives in a variety of product categories such as plastic, paints, vinyl flooring, synthetic leather, medical equipment, personal care products, and adhesive (Prieto-Amador et al., 2021; Kumkar et al., 2022) are known to cause endocrine disruption (Zhang et al., 2018a, 2018b; Zhang et al., 2021), induce oxidative stress (Ghorpade et al., 2002; Kumkar et al., 2022), alter gonadal development and growth patterns, shorten the lifespan of exposed aquatic animals (Pradhan et al., 2018), cause neurotoxicity, and even alter gene expression (Zhang et al., 2021). Similar to PPCPs, MPs, and PAEs, heavy metal(loid)s (HMs) in aquatic ecosystems are also known to have an impact on various vertebrates, invertebrates, and plants (as reviewed in Khalid et al., 2021). For instance, yellow seahorse (*Hippocampus kuda*) exhibits decreased body weight, body length, specific growth rate, and survival when exposed to Cu, Cd, and Pb in combination with high density polyethylene (Jinhui et al., 2019). When exposed to polystyrene (PS), polytetrafluorethylene, and As (III), Rice seedlings (*Oryza sativa*) display growth suppression and biomass accumulation by means of reducing root activity and RuBisCO activity (Dong et al., 2020). Furthermore, because these contaminants coexist in aquatic habitats, their affinity and carrying capacity for one another have a synergistic influence on aquatic biota (Atugoda et al., 2021; Khalid et al., 2021; Liu et al., 2021a, 2021b; Wagstaff et al., 2022). For example, it is widely known that MPs act as vectors for PPCPs and HMs, posing a two-fold risk to them (Zhou et al., 2020a, 2020b; Atugoda et al., 2021; Wagstaff et al., 2022). As a result, assessing the presence of these contaminants in aquatic ecosystems and the eco-toxicological risk they pose necessitates careful consideration of a comprehensive approach.

Several earlier studies have reported presence of microplastics (Dong et al., 2020; Li et al., 2020; Yang et al., 2022), PAEs (Selvaraj et al., 2015; Lee et al., 2019; Prieto-Amador et al., 2021), PPCPs (Chakraborty et al., 2019; Picó et al., 2020; Xu et al., 2022), endocrine-disrupting chemicals (Huang et al., 2018; Kasonga et al., 2021; Sharma et al., 2021; Chakraborty et al., 2022) and HMs (Atugoda et al., 2021; Liu et al., 2021b) in various categories of environmental matrices, such as marine, freshwater, and terrestrial ecosystems. However, there is a blatant bias in the number of investigations that have been conducted taking into account water, sediments, and/or aquatic biota, with a higher number of studies clearly considering water only (Picó et al., 2020). This underlines the dearth of holistic approach considering all environmental compartments, such as water, sediment, and biota, into account simultaneously (Shifflett and Schubauer-Berigan, 2019; Picó et al., 2020). Additionally, despite the fact that contaminants like MPs, PPCPs, PAEs, and HMs co-exist and interact with one another (Zhou et al., 2020a; Atugoda et al., 2021; Khalid et al., 2021; Liu et al., 2021b), earlier studies only focused on a small number of emerging contaminants, typically of a single kind (Bureš et al., 2021; Picó et al., 2020). Studies that take into account a variety of contaminants, including MPs, PPCPs, PAEs, and HMs, as well as any potential interactions between them and the ecological risk they entail, are thus required, especially in developing countries like India where such data is severely lacking. Given the limitations discussed previously, as well as the need for studies in developing countries, the primary goal of this study was to improve our current understanding of contaminants such as MPs, PPCPs, PAEs, and HMs along the Indian coastline (particularly in the Maharashtra region), with a focus on determining their abundance, distribution, potential interactions, and eco-toxicological effects on aquatic organisms.

We selected the Maharashtra coast for the following reasons: (1) In India, earlier research on PPCPs (Ramaswamy et al., 2011; Kumar et al., 2019; Sharma et al., 2019), PAEs (Selvaraj et al., 2015; Chakraborty et al., 2019; Mukhopadhyay and Chakraborty, 2021) and HMs (Singh et al., 2005; Gowd and Govil, 2008) is largely focused on freshwater ecosystems; (2) Despite extensive research on MPs in coastal sediments in India (Karthik et al., 2018; Robin et al., 2020; Patchaiyappan et al., 2020, 2021; Rabari et al., 2022), a thorough assessment of certain emerging contaminants like MPs along the Maharashtra coast is still lacking; (3) investigations on the eco-toxicological risk posed by these micropollutants (PPCPs, PAEs, and HMs) are not yet available and, (4) Evaluation and analysis of MPs and micropollutants (PPCPs, PAEs, and HMs) will also aid in better understanding the link between MPs and micropollutants, particularly in terms of possible interactions between them and the function of MPs as a source and vector of these micropollutants. We anticipated significant variations in abundance and distribution of these contaminants in sediment and water along the Maharashtra coast due to the highly diverse population zones (urban, semi-urban, and villages), anthropogenic activities (tourism, fishing, industries, etc.), and differential land-use patterns (highly populated, less populated, cropland dominated, and forest dominated). Additionally, the largest industrial cluster in Asia that borders the coastal regions of Maharashtra consists of a variety of industries, including those that produce paint, dye, plastic, petroleum and petroleum products, food packaging, and chlor-alkali (Menon and Mahajan, 2011). These industries are likely to have a significant impact on the PAEs, PPCPs and HMs contamination in the Maharashtra coastal regions (Raut et al., 2019). Therefore, a comprehensive analysis of the distribution of various contaminants throughout the Maharashtra coast is essential.

In this study, by covering the entire Maharashtra coast and using a comprehensive approach considering MPs, HMs, PPCPs, and PAEs as emerging contaminants, we addressed the following objectives: (1) to collect, quantify, and characterise MPs in sediment and water; (2) to confirm the presence of PPCPs and HMs in coastal waters and to assess the interaction between HMs, PPCPs and MPs; (3) to confirm the role of MPs as a source of PAEs; (4) to assess ecotoxicological risk to fish, crustaceans, and algae caused by the detected contaminants; and (5) to identify pollution hotspots along the Maharashtra coast to establish priority management zones for efficient pollution monitoring and control.

2. Materials and methods

2.1. Study area

Maharashtra is the third-largest state in India in terms of geographical area (3.08×10^5 sq. km) and the second-largest state in terms of population (Naidu et al., 2022). The state is bounded on one side by the Arabian Sea and on the other by the Western Ghats mountain ranges. The five coastal districts of Maharashtra are Thane, Raigad, Mumbai, Ratnagiri, and Sindhudurg. Maharashtra State alone contributes approximately 25 % of the countries' industrial production and 23.2 % of the GDP, as reported by the 2011 India Census. Urban areas, industries, fish landing areas, and tourism are some of the leading human activities widespread along the Maharashtra coast. The coastal districts are dominated by the oil and pharmaceutical industries, as well as thermal power plants and the fertiliser industry (Yadav et al., 2015; Naidu et al., 2022). Apart from being popular tourist destinations, the central and southern coastal areas of Maharashtra also include major fishing grounds and ports (Mumbai, Ratnagiri and Sindhudurg) that support local people's livelihoods.

2.2. Sampling stations and sampling

The sampling locations ($n = 17$; Fig. 1) were distributed throughout the five coastal districts spanning the entire Maharashtra coastline. Based on various anthropogenic activities and geographical factors such as urbanisation, industrialisation, tourism, fishing, shipping, and the presence of ecologically sensitive zones, the coastline was divided into three geographical zones (Supplement 1). The details of the zones are as follows: 1) North zone [$n = 6$ stations: from Dahanu (N1) to Alibaug (N6)], which is influenced by urbanisation, sewage and effluent inputs from highly polluted rivers, tourism, transport, and fishing; 2) Central zone [$n = 7$ stations: from Kashid (C1) to Guhagar (C7)], which is impacted by tourism, fishing, inputs from industrial effluents, and suburban activities; and 3) South zone [$n = 4$ stations: from Bhate (S1) to Vengurla (S4)], which is influenced by tourism and fishing. In August 2021, beach sediment and coastal water samples were collected from 17 locations along the Maharashtra coast. The sampling methodology described by Karthik et al. (2018) and Robin et al. (2020) was followed. Using a stainless-steel scoop, we collected the top 5 cm of beach sediment from each quadrant (30 cm × 30 cm). Sediment samples were immediately placed in a paper bag and transferred to the laboratory.

2.3. Extraction and determination

2.3.1. Microplastics

In laboratory, the sediment samples were dried in a hot-air oven at 70 °C for 24 h. A 100 g sub-sample from the bulk sample was then sieved through a 5-mm sieve to remove particles >5 mm. Large-sized MPs (4–5 mm) were manually separated. The sub-sample was then mixed with 100 mL of saturated NaCl solution and allowed to settle for 30 min, after which the supernatant was transferred into another beaker. This procedure was repeated two more times to ensure complete isolation of MPs. To remove the organic matter in the samples, isolated MPs were treated with 150 mL of 30 % H_2O_2 and allowed to settle for 24 h. The clear supernatant was then filtered using Whatman GF/A filter paper (43 mm diameter). The filters containing MPs were then carefully removed, dried, and stored in glass petri dishes wrapped in aluminium foil.

Coastal water samples were collected using a well-established bottle-sampling method (Crew et al., 2020) with minor modifications (Kumkar et al., 2021). In each case, we collected 100 L of water at a depth of 0–25 cm below the surface using a 2 L stainless-steel vessel, which was then filtered through a stainless-steel sieve with a mesh size of 100 μm. A sub-sample of filtered water (5 mL) was collected in PP cryogenic tubes, frozen, and stored for analysis of other contaminants like HMs and PPCPs (the procedures further described in Sections 2.3.3 and 2.3.4). Each strainer was then covered with aluminium foil and brought to the laboratory. Each strainer was washed three times in 1 L of distilled water before being vacuum-filtered in the same manner as the sediment sample. The strainers were thoroughly examined under a stereo-zoom microscope (Olympus SZ61) for attached MPs particles to ensure complete MP isolation. A blank test was also performed to determine whether the filtered distilled water used was devoid of MP particles.

A stereomicroscope (Olympus SZ61) was used to examine the MP-containing filters. MPs were counted, measured, and categorised by size (0–1 mm, 1–2 mm, 2–3 mm, 3–4 mm, and 4–5 mm), colour (red, green, blue, white, yellow, transparent, colourless, and others), and morphotype (fragments, foam, beads, film, and fibres/filaments). The chemical composition of the MPs was determined using ATR-FTIR (Thermo Scientific Nicolet™ iS20 FTIR with Smart iTX Diamond ATR). The polymer type was identified by comparing the spectral data with the OMNiC reference library. Scanning electron microscopy (SEM) coupled with energy dispersive X-ray analysis (EDX) is a widely accepted method (Shruti et al., 2019; Kumkar et al., 2021) for determining the surface texture/topography of MPs and identification of surface elements. This technique can provide crucial information about MP disintegration due to weathering and/or ageing as well as its ability to carry metal contaminants, which aquatic animals such as fish can ingest. MPs from the beach sediment and water from each sampling station were sputter-coated with gold and imaged using SEM (Tescan Mira3, Tescan Orsay Holding, Brno, Czech Republic). The surface elemental compositions and their distribution on the MPs were determined using an energy dispersive spectroscopy system (Bruker XFlash X-ray detector, Karlsruhe, Germany, and ESPRIT 2 software).

2.3.2. Phthalate esters (PAEs)

We used the well-established methodology described by Gao et al. (2019) and Kumkar et al. (2022), and used the gas chromatography–mass spectrometry (GC–MS) approach, for the qualitative identification of the PAEs associated with the isolated microplastics. Briefly, all processes were carried out in glass, and the lids were covered with an aluminium foil. The glassware was cleaned with hot water and an extraction solvent, and rinsed with distilled water. The glassware and aluminium foil were heated in a muffle furnace at 350 °C for 60 min. The MP samples were transferred to glass tubes to which 5 mL of the extraction mixture were added [n-hexane: dichloromethane (1:1, v:v)], vigorously vortexed, sonicated in a heated bath for 30 min, and left for 24 h on a shaker. The extraction was performed twice. A rotary evaporator was used to evaporate the solvent. The tubes were capped with aluminium foil. Analyses were performed using an Agilent 7890A GC coupled to an Agilent 5975C MSD (Agilent Technologies, Palo Alto, CA, USA) with a DB-5MS column (30 m × 0.25 mm, 0.25 μm film thickness). Sample extracts were resuspended in 100 μL of n-hexane and transferred to a GC vial with a 200 μL glass insert. A total of 1 μL per sample was injected into the inlet in splitless mode maintained at 300 °C with an automated liquid sampler (GC-ALS). At an electron ionisation energy of 70 eV, the source temperature was 325 °C.

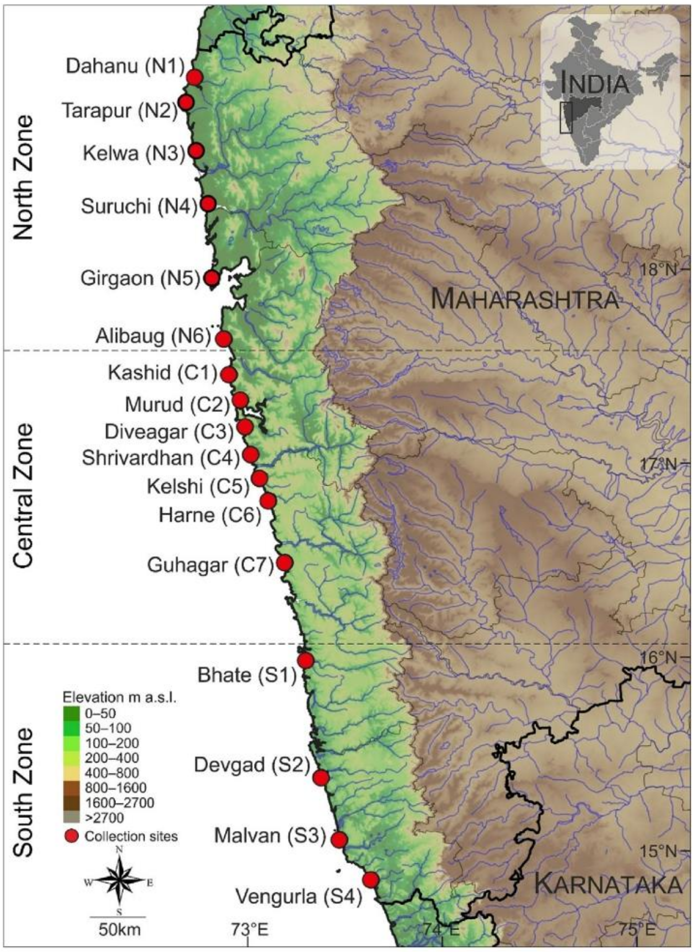

Fig. 1. Map of the study coastline with collection sites (n = 17) and three different zones. North zone with six (N1 to N6), central zone with seven (C1 to C7), and south zone with four (S1 to S4) sampling stations.

The samples were measured in SCAN mode (mass range 59–400 m/z) and SIM mode (ions 163 and 149 m/z were selected). Starting with 80 °C for 30 s, the oven temperature was increased at a rate of 10 °C/min to a maximum of 325 °C. The retention times in the standard solution, EPA Phthalate Esters Mix (CRM48805, Supelco), were used to reference the retention times of the analytes in the sample solutions.

2.3.3. Heavy metal(loid)s (HMs)

Coastal water samples collected from all sampling stations were divided into two aliquots. One aliquot was 10× diluted and acidified using nitric acid, and subjected to Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; Agilent 7700×) for analysis of the following HMs: arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), nickel (Ni), vanadium (V), and zinc (Zn). ICP-MS was operated in He mode. The determination was based on partially matrix-matched external standards (3.5 g L^{-1} NaCl in 1 % HNO_3), with Ge, In, Rh, and Lu serving as internal standards. For quality assurance of the analytical results, certified reference material trace elements in natural water (SRM-1640a, NIST) were spiked into the aqueous matrix.

2.3.4. PPCPs and bisphenols

Due to the limited amount, the other aliquot of each water sample was filtered through a syringe filter (regenerated cellulose, 0.22 μm pore size) and directly analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Samples were first screened for the presence of 108 compounds from pharmaceuticals, residues of personal care products, and bisphenols. The list of targeted compounds, detection limits, and other details are shown in Supplement 2. Out of 108 targeted compounds, 6 analytes were detected in at least one sample. According to these detected analytes, corresponding internal standard (IS) solution (MeOH) was prepared where isotopically labelled analogues were selected. The samples were then spiked with IS solution using an automated analytical syringe (eVol XR, Trajan (SGE), Australia) and analysed again by LC-MS/MS. The detected analytes were now quantified by the isotope-dilution method.

The compounds used as internal standards (IS) are shown in Supplement 3. Matrix-matched calibrations were performed using artificial seawater (29.75 g L^{-1} NaCl and 5.25 g L^{-1} $MgSO_4$), MilliQ water was used for procedural blanks. To comply with quality-control procedures, blanks, blank solvents, procedural blanks, and control spiked samples were included in each run. Each calibration point consisted of 3 individual injections performed at the beginning, in the middle, and at the end of each run. The chromatographic separation of measured compounds was performed with a UHPLC system (1290 Infinity II, Agilent) using 0.005 % HCOOH in H_2O (mobile phase A) and MeOH (mobile phase B) in positive ionisation mode (ESI+), and 0.5 mM NH_4F in H_2O (mobile phase A) and MeCN (mobile phase B) in negative ionisation mode (ESI-). Gradient elution started at 5 % B and was held for 5 min, and then was ramped to 100 % over 13 min and maintained for 8 min. The separation was performed in a C18 column (Poroshell 120, EC-C18, 3.0 × 100 mm, 2.7 μm, Agilent) fitted with a pre-column (Poroshell 120, EC-C18, 3 mm, Agilent) and in-line pre-filter (0.3 μm, Agilent) and in-line trap column (Poroshell 120, EC-C18, 4.6 × 50 mm, 2.7 μm, Agilent). The analytical column was maintained at 40 °C and the mobile phase flow rate was 0.56 mL min^{-1}. The injection volume was 2 μL. The UHPLC system was coupled to a triple quadrupole mass spectrometer (6495 B, Agilent) equipped with an electrospray ionisation (ESI) source. Detailed ESI and mass spectrometry (MS) parameters can be found in Mercl et al. (2021). The mass spectrometer was operated in the dynamic multiple reaction monitoring (dMRM) mode.

2.4. Pollution levels and risk assessment

2.4.1. Pollution load index

The pollution load index (PLI) is frequently used to estimate the extent of pollution levels in different areas of the water body (Kabir et al., 2021; Verma et al., 2022). The estimated MP abundance were used to calculate the PLI in sediment and coastal water from Maharashtra coast. The PLI represents the pollution level across the sampling stations (Tomlinson et al., 1980). The PLI was computed using the following formula:

$$CFi = \frac{Ci}{Co}$$

$$\mathrm{PLI} = \sqrt{\mathrm{CF_i}}$$

$$\mathrm{PLI}_{\mathrm{Sediment/coastal\ water}} = \sqrt[n]{\mathrm{PLI}_1 \times \mathrm{PLI}_2 \times \ldots\ldots\ldots \times \mathrm{PLI}_n}$$

where 'i' denotes a sampling station, 'n' presents the number of sampling stations, 'Ci' represents MPs abundance at i^{th} station, and 'Co' is the minimum baseline concentration in the zone. Because of the lack of prior data in the same environments, we considered the minimum MPs abundance recorded in this study as the minimal baseline concentration. Sampling stations are considered polluted if PLI value > 1 (Tomlinson et al., 1980; Kabir et al., 2021; Verma et al., 2022).

2.4.2. Ecotoxicological risk assessment

Ecotoxicological risk was estimated by calculating the hazard quotient (HQ) based on the United States Environmental Protection Agency (USEPA) guidelines (USEPA, 1998). The lowest relevant acute effect concentration (LC_{50}, EC_{50}) or the lowest no observed effect concentration (NOEC) in the chronic toxicity test values were obtained from the literature, mainly from the ECOTOXicology Knowledgebase (USEPA, 2022). The predicted no-effect concentration (pNEC) values for Tramadol, Metoprolol, and Venlafaxine were obtained from Mheidli et al. (2022), while other pNECs were derived by dividing the lowest relevant acute effect concentration (LC_{50}, EC_{50}) or the lowest no-observed-effect concentration (NOEC) in chronic toxicity tests by an assessment factor (AF). AFs of 1000 and 10 were used to divide the acute and chronic toxicity concentrations, respectively (Chakraborty et al., 2019). The pNEC values for each compound are provided in Supplement 4. The hazard Quotient (HQ) of the estimated pore water at each site was calculated using the following formula:

$$HQ = \frac{MEC}{pNEC}$$

where MEC is the measured environmental concentration. In this case, it is the converted pore-water equivalent concentration. HQ was calculated for every site and compound. Criteria for interpreting the HQ are given as follows: HQ < 0.1, "low risk"; HQ ranging from 0.1 to 1, "medium risk"; and HQ > 1, "high risk".

2.5. Validation and quality assurance

For microplastics, external contamination was strictly checked during the onsite collection and laboratory processing of the samples. Only steel equipment (scoop and strainers) and containers with tight-fitting lids were used to collect and store the samples. Before use, all glassware was sterilised, cleaned with pre-filtered distilled water, and covered with aluminium foil. After processing each sample, the work space was cleaned with acetone, and the flasks and beakers were covered with aluminium foil throughout the experiment. and the flasks and beakers were concealed with aluminium foil throughout the experiment. Procedural blanks and aerial contamination controls were used throughout the MP-extraction process to check for plastic particle contamination from external sources. The results of the procedural blanks were negative.

2.6. Statistical programs and analysis

Normality and homoscedasticity of data were tested using Shapiro–Wilks and Levene's tests respectively. The abundance of MPs in beach sediment and coastal water is expressed as the number of MPs particles/kg and MPs particles/L, respectively. Pearson's correlation analysis was used to investigate the relationship between the MPs abundance in beach sediments and coastal waters. We used two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparisons to test the effects of sample (sediment and coastal water) and zones (northern, central, and southern) on the abundance of MPs. Likewise, two-way ANOVA followed by Bonferroni post-hoc comparisons were used to test the effects of sample (sediment and coastal water) and zones (northern, central, and southern) on the pollution load index (PLI). The difference in the concentrations of dissolved HMs in coastal waters between the different sampling stations was visualised using a heat map. The difference in the concentrations of dissolved HMs in the coastal waters between different zones (northern, central, and southern) was analysed using one-way ANOVA followed by Tukey's multiple comparison test. Pearson's correlation analysis was used to investigate the relationship between the MP abundance in water and HMs. Similarly, the difference in the concentration of PPCPs and bisphenol distribution in water along the three different coastal zones (northern, central, and southern) was analysed using one-way ANOVA followed by Tukey's multiple comparison test. Pearson's correlation analysis was used to investigate the relationships between MP abundance and the concentration of PPCPs and bisphenol in the coastal waters. Statistical analyses were conducted using GraphPad Prism and the freeware 'PAST' (Hammer et al., 2001; http://folk.uio.no/ohammer/past/).

3. Results and discussion

3.1. Microplastics (MPs)

3.1.1. MPs abundance

MP contamination was recorded in all 17 sediment samples and 15 coastal water samples. The average MP abundance in sediments was 108.24 ± 28.19 particles/kg. Sediment samples collected from Bhate Beach (S1) had the lowest MPs abundance, whereas Suruchi Beach (N4) had the highest abundance (Fig. 2a). Similarly, the average MP abundance in water was 49.41 ± 12.77 particles/100 L. MP abundance was lowest in the Kashid beach (C1) water samples and highest in the Suruchi beach (N4) samples (Fig. 2b). A comparative analysis of MP abundance in sediments and water across various Indian coasts is presented in Supplement 5. The average MP abundance in the beach sediment of the Maharashtra coast is lower than in Puducherry (720.30 ± 191.60 MPs/kg; Dowarah and Devipriya, 2019), the Andaman Islands (414.35 ± 87.4 MPs/kg; Patchaiyappan et al., 2020), and Odisha (258.7 ± 90.0 MPs/kg; Patchaiyappan et al., 2021). In contrast, we recorded higher average MP abundances in the Maharashtra coast sediments than in Tamil Nadu (45 ± 12 MPs/kg; Tiwari et al., 2019) and Gujrat (1.4–26 MPs/kg; Rabari et al., 2022). Although the average MP abundance along the Maharashtra coast was less than that of most Indian coasts, we noticed substantial spatial heterogeneity between beaches. For instance, we found 460 MP particles/kg in sediment collected from Suruchi Beach and only 10 MP particles/kg in sediment collected from Bhate Beach. Such heterogeneous MP abundances highlight critical information on the region's waste disposal system and inputs from possible sources. The maximum MP abundance observed in the Suruchi Beach sediment could be attributed to its proximity to the mouth of the Ulhas River. The Ulhas River is one of the most polluted rivers in Maharashtra State and carries enormous amounts of residential and industrial wastes (Raut et al., 2019; Kumkar et al., 2021; Verma et al., 2022). A recent study by Verma et al. (2022) recorded the highest MPs abundance (600 ± 122 particles/kg of sediment) in the Ulhas River sub-basin located in close proximity to Suruchi beach. In addition, Suruchi Beach is a popular tourist destination, where tourists discard a

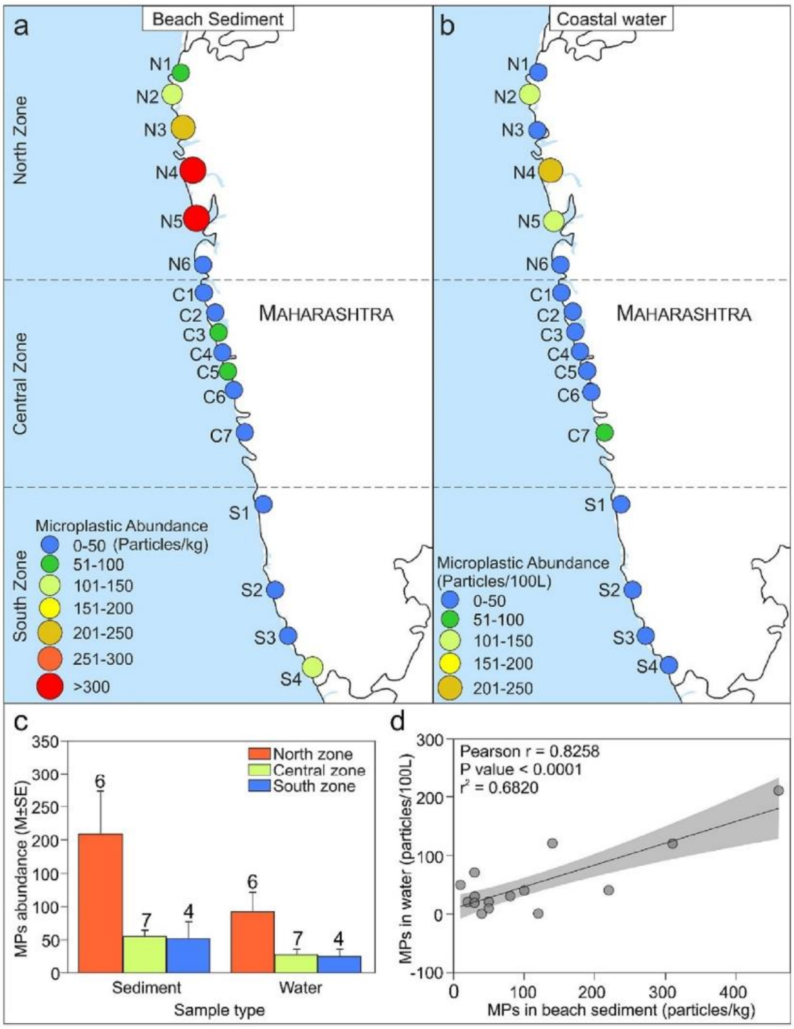

Fig. 2. Distribution of MPs along the Maharashtra coast: MPs abundance (a) in beach sediment and (b) in coastal water; (c) Differential MP abundance in three different impact zones; (d) Correlation between MPs in beach sediment and coastal water. Note: Numbers of the top of bars in panel "c" indicates number of sampling stations.

significant amount of plastic trash on beaches owing to a limited waste-management system. Therefore, land-based sources and riverine transport likely to account for the majority of the MP abundance at Suruchi Beach. On the other hand, Bhate Beach is a popular tourist destination as well. Still, we found less MPs on Bhate Beach, which may be related to better waste disposal by local authorities and more stringent implementation of plastic control policies. Our findings are significant because they provide comprehensive details on differential MP abundance and distribution over the extensive Maharashtra coast, in contrast to other studies. The differential MP distributions in beach sediments and coastal waters along Maharashtra's 720 km coastline highlight the fact that the entire coastline cannot be managed uniformly, necessitating the consideration of source and region-specific policies and microplastic mitigation strategies.

3.1.2. Pollution load index (PLI)

Two-way ANOVA showed a significant effect of zone (F = 7.866; P = 0.001) and sample type (F = 4.390; P = 0.045) on MP abundance along the Maharashtra Coast (Fig. 2c). Furthermore, a significant positive correlation (Pearson r = 0.8258; P < 0.0001; r^2 = 0.682) was observed between MP abundance in the sediment and water (Fig. 2d). Likewise, two-way ANOVA showed a significant effect of zone (F = 5.589; P = 0.009) and sample type (F = 4.609; P = 0.041) on PLI along the Maharashtra Coast (Supplement 6). The overall PLI for Maharashtra coast sediment and water was 2.59 and 1.84, respectively. A PLI > 1 for all sediment and coastal water samples analysed indicates significant MPs pollution along the Maharashtra coast. The $PLI_{Sediment}$ varied from 1.0 (Bhate Beach) to 6.78 (Suruchi Beach). Likewise, the PLI_{Water} varied from 1.0 (Kashid Beach) to 4.03 (Suruchi Beach). Furthermore, the difference in the PLI among three zones was noticeable. Therefore, we propose grouping the Maharashtra coastline into three effect zones (north, central, and south) for better management of plastic pollution. Details of the number of beaches, average MP abundance in sediment and water, PLI values and possible sources of MPs in the respective zones are provided in Supplement 1. MPs abundance was nearly four times greater in the northern zone than in the central and southern zones (Fig. 2c), which could be attributed to urbanisation, sewage and industrial inputs from highly polluted rivers, recreational and religious activities, transportation, and fishing. Our observations of higher MP abundance on the northern-zone beaches are comparable with those of earlier studies (Jayasiri et al., 2013; Tiwari et al., 2019; Naidu et al., 2022). Therefore, based on MP abundance, estimated PLI values and anthropogenic stressors data from current and previous studies (Supplement 1), we designated the northern zone as a “high impact zone”, the central zone as a “moderately impacted zone”, and the southern zone as a “least impacted zone”. The best method to manage MP pollution along the Maharashtra Coast could be zone-specific regulations and MP mitigation measures.

3.1.3. Microplastics morphotypes

Microplastic characterisation based on shape, size, and colour often reveals important information about likely origins, chemical composition, palatability, and degradation status in the environment (Karthik et al., 2018; Robin et al., 2020). Details of the site-specific proportions of each MP morphotype, colour, and size are provided in Supplement 7. We recorded all five MP morphotypes (fragments, filaments, beads, film, and foam) of varying sizes and colours (Supplement 8). The overall proportion of MP morphotypes in sediment was as follows: fragments (78.80 %), filaments (7.61 %), beads (7.07 %), film (3.80 %), and foam (2.72 %). Likewise, the overall proportion of MP morphotypes in water was as follows: fragments (77.38 %), filaments (9.52 %), film (7.15 %), beads (4.76 %), and foam (1.19 %). Fragments and filaments dominated both the sediment and water samples (Fig. 3a and b). Other studies conducted along the Indian coastline also report the dominance of fragment and filament morphotypes (Jayasiri et al., 2013; Karthik et al., 2018; Dowarah and Devipriya, 2019; Tiwari et al., 2019; Robin et al., 2020; Rabari et al., 2022). Fragments are mainly formed through the breakdown or disintegration of larger plastic products as well as abrasion and/or weathering (Karthik et al., 2018; Shruti et al., 2019; Robin et al., 2020). Filaments/fibres/lines are formed from abandoned fishing nets and ropes used by vessels (Karthik et al., 2018; Robin et al., 2020; Rabari et al., 2022) and sewage sources, such as microfibres from clothing and household textiles (Henry et al., 2019). We observed a large amount of plastic refuse, particularly on beaches in the northern and central zones (Fig. 4a–d). The presence of filament morphotypes could be related to both fishing activities (Fig. 4e–j) and domestic and industrial inputs (Fig. 4c). We found abandoned or discarded fishing nets (Fig. 4e–j) and religious debris, including cloth, along various beaches throughout the Maharashtra coast, all of which add considerably to the filaments in the beach sediments and coastal waters. Additionally, there are several textile and dyeing industries as well as rope and fishing net manufacturing industries in the north and central zones of Maharashtra Coast (Kumkar et al., 2021; Naidu et al., 2022). Beads were also found in large quantities, particularly in sediments and coastal water from the north zone, which is consistent with the observation that these areas are dominated by small-scale industries that use plastic pellets as feedstock for manufacturing plastic objects (Kumkar et al., 2021). However, the transfer of beads from the north to the central and south zones due to oceanic currents cannot be ignored and requires further detailed investigation. Discarded foam and single-use plastic (Fig. 4k and l) present on several beaches along the north and south zones are likely the major sources of foam and film. These observations provide empirical evidence that the MPs observed along the Maharashtra coast are mostly of secondary origin.

In sediment, the overall size-based distribution of MPs was as follows: 0–1 mm (3.93 %), 1–2 mm (13.48 %), 2–3 mm (29.22 %), 3–4 mm (30.90 %), and 4–5 (22.47 %). Similarly, in water, the overall size-based distribution of MPs was as follows: 0–1 mm (1.23 %), 1–2 mm (12.35 %), 2–3 mm (27.16 %), 3–4 mm (39.51 %), and 4–5 (19.75 %). Although we observed spatial variation in the size-based distribution of MPs in different zones (Fig. 3c and d), it is clear that medium to large MP particles (ranging from 2 to 4 mm) dominated the beach sediments (66.67 %) and coastal waters (60.11 %). Earlier investigations by Karthik et al. (2018) along the Tamil Nadu coast of India also recorded the dominance of medium and large MPs ranging from 2.36 to 4.75 mm (>80 %), respectively. In contrast, Robin et al. (2020) and Rabari et al. (2022) reported the dominance of small MP particles along India's Kerala and Gujrat coasts respectively. According to Karthik et al. (2018), various anthropogenic and site-specific activities on beaches affect the size distribution of MPs in the marine environment. Therefore, the dominance of medium and large MPs throughout the Maharashtra coast might be linked to a single or synergistic influence of many factors.

Colourless/transparent (25 %), blue (25 %), and green (17.93 %) MPs were abundant in the sediment samples (Fig. 3e). Similarly, colourless (40.48 %), blue (23.81 %), and red (14.29 %) MPs contribute mainly in the water samples (Fig. 3f). Several earlier studies have reported the prevalence of colourless/transparent, blue, and green MPs in beach sediments and coastal waters (Karthik et al., 2018; Robin et al., 2020; Patchaiyappan et al., 2021). The specific colour of MPs plays a crucial role, as it increases the likelihood of ingestion by marine organisms (Wright et al., 2013; Auta et al., 2017; Patchaiyappan et al., 2021). Colourless/transparent, blue, and green MPs are often detected in the gastrointestinal tract of marine biota such as fish (*Coilia dussumieri*), shrimps (*Metapenaeus monoceros*, *Parapeneopsis stylifera*, and *Penaeus indicus*), and clams (*Meretrix casta*) collected from the Maharashtra coast (Karthik et al., 2018; Robin et al., 2020; Gurjar et al., 2021a, 2021b; Patchaiyappan et al., 2021; Naidu et al., 2022). The presence of coloured MPs and their occurrence in marine biota are likely to affect species fitness and pose a risk to human health, particularly in the case of small shrimp, dried fish, and bivalves, where gutting is not possible before human consumption (Gurjar et al., 2021a, 2021b).

3.1.4. Microplastic polymeric composition

Analytical approaches, such as ATR-FTIR, Raman spectroscopy, and SEM-EDX, play a vital role in determining the chemical nature of MPs

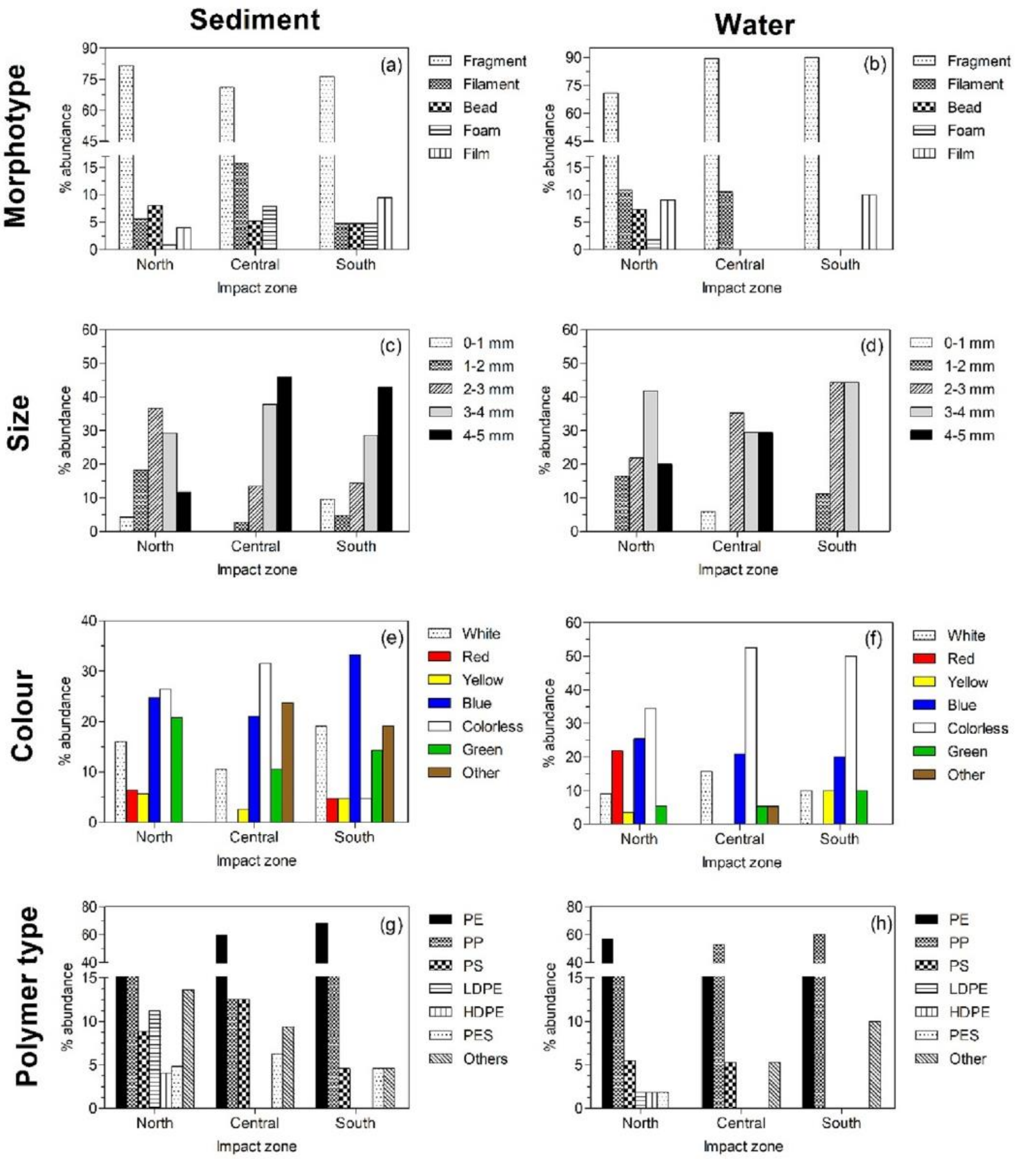

Fig. 3. Percent abundance of MPs in beach sediment and coastal water across three different zones of the Maharashtra coastline according to: (a and b) morphotype; (c and d) size; (e and f) colour; and (g and h) polymer type.

(Robin et al., 2020; Patchaiyappan et al., 2021). We used ATR-FTIR to analyse 263 of the 268 particles, and identified 12 different polymers: polyethylene (PE), polypropylene (PP), polystyrene (PS), low-density polyethylene (LDPE), high-density polyethylene (HDPE), polyester (PES), polyvinyl chloride (PVC), poly-(ethylene-*co*-propylene), poly-(divinyl benzene)-*co*-styrene, poly-(ethylene terephthalate), poly-ethylene-*co*-(vinyl acetate), and cellulose. The overall proportions of MP polymers in the beach sediments (n = 179) were as follows: PE (43.58 %), PP (20.11 %), PS (8.94 %), LDPE (7.82 %), HDPE (2.79 %), PES (5.03 %), and others (11.73 %). Likewise, the overall proportions of MP polymers in the tested coastal waters (n = 84) were as follows: PE (48.81 %), PP (40.47 %), PS (4.76 %), LDPE (1.19 %), HDPE (1.19 %), PES (1.19 %), and others (2.39 %). Representative ATR-FTIR spectra of the six types of MP particles are shown in Supplement 8. Although we observed spatial variation in the

Fig. 4. Various sources of MPs and their morphotypes along the Maharashtra coastline: (a to d) Large quantity of discarded plastic rubbish along various beaches; (e to j) Discarded fishing ropes and nets; and (k and l) Discarded foam and domestic plastic waste.

distribution of MP polymers (Fig. 3g and h; Supplement 7), it was evident that PE, PP, and PS dominate the sediment (cumulative occurrence = 72.63 %) and water (cumulative occurrence = 94.03 %) samples, followed by LDPE, polyester, and HDPE.

Polymer types found in sediments and water are found in everyday products. PE is an excellent choice for manufacturing packaging films, refuse bags, shopping bags, wire and cable insulation, agricultural mulch, containers, toys, and household items (Sruthy and Ramasamy, 2017). PE dominance across all zones is, thus, plausible, and PE in all regions may have originated from the fragmentation of hard plastics (refer to Fig. 4). The higher abundance of PE in sediment (overall = 43.58 %) and water (overall = 48.81 %) across the three zones is noticeable. Polymers typically utilised in the production of beverage bottles, stiff plastic tools, automobile interiors, textile floor coverings, carpets, fishing nets, and disposable items are polypropylene (PP) and polystyrene (PS), which contribute to PE (Patchaiyappan et al., 2021). Polystyrene blocks and boxes are commonly used as floats in fishing nets and fish transportation, respectively. Poor solid waste management (Fig. 4a–d) together with discarded nets and PS boxes (Fig. 4e–k) may contribute to the abundance of PP and PE along the Maharashtra coast. The heavily populated northern zone had the highest abundance of LDPE, and LDPE is most frequently used for producing plastic bags, which indicates that the waste management system in this zone is insufficient. India has banned the production, import, stocking, distribution, sale, and use of single-use plastic items since 1st July 2022. This ban is expected to reduce the LDPE burden in all the nation's coastal areas; however, this depends on the public's participation and strict enforcement of the rules.

HDPE is also UV-resistant and recyclable, making it an ideal material for highly disposable items, such as recycling bins, lubricating oil, conditioners, and household cleaning products (Abraham and George, 2005; Patchaiyappan et al., 2021). We found HDPE in sediment and water from beaches in the severely impacted northern zone, including Suruchi, Tarapur, and Alibaug, which are well-known tourist hotspots. As a result, the source of HDPE may be linked to tourists' improper solid waste management and littering (see Fig. 4a and b). PET and PES are members of the polyester family and are synthetic fibres that are commonly used in the plastic industry for bottles and other containers, as well as in the textile industry. This indicates that poor solid waste management caused by urbanisation, tourism, recreation, fishing, and industrial activity all likely contribute to MP pollution along the Maharashtra coast. To reduce the plastic pollution burden and preserve Maharashtra's coastal environment, it is critical to raise awareness and environmental consciousness among both residents and tourists.

3.2. Heavy metal(loid)s (HMs)

Considering the anthropogenic origin of MPs, PPCPs, bisphenols, and HMs, it is well-established fact that all these contaminants coexist in the aquatic environment (Atugoda et al., 2021; Khalid et al., 2021; Liu et al., 2021a, 2021b; Wagstaff et al., 2022). The results of the one-way ANOVA showed no significant difference between the concentrations of HMs in the various impact zones of the Maharashtra coast ($P > 0.05$, in all cases; Supplement 9), except for V ($P = 0.034$). Zinc was found to be the most abundant HM in the water samples collected from the Maharashtra coast, followed by V, Cu, As, Ni, Co, Cr, Cd, and Pb at the following concentrations: Zn (24.36 ± 9.56 μg L^{-1}), V (2.94 ± 0.49 μg L^{-1}), Cu (2.18 ± 1.18 μg L^{-1}), As (1.39 ± 0.16 μg L^{-1}), Ni (1.13 ± 0.34 μg L^{-1}), Co (0.25 ± 0.07 μg L^{-1}), Cr (0.22 ± 0.07 μg L^{-1}), Cd (0.17 ± 0.11 μg L^{-1}), and Pb (0.13 ± 0.06 μg L^{-1}). Site-specific (Fig. 5) and zone-specific HM distributions along the Maharashtra coast are provided in Supplement 9. We observed a significant positive correlation between MP abundance in coastal waters and HMs, such as Cr, Ni, Cu, Cd, and Pb (Fig. 6a). Previous research by Foshtomi et al. (2019) also found a positive correlation between the number of MPs and concentrations of 10 distinct HMs off the coast of Bandar Abbas, Iran. Overall, we observed a noticeably higher concentration of HMs (except Zn and Co) in the northern zone coastal water than in the central and southern zones (Supplement 10a). Transportation, industrial wastewater discharge, fossil fuel combustion, agricultural runoff containing pesticides, geological weathering, and household waste are the main causes of HM pollution in waterbodies (Deshpande et al., 2009; Velusamy et al., 2014; Raut et al., 2019). The higher concentrations of HMs in the north zone is likely attributed to the direct impacts of urbanisation and wastewater discharge from industries. Indeed, the Ulhas and Patalganga Rivers pass through the industrial belt and densely populated areas of the Mumbai suburban regions and carry significant amounts of sewage and industrial effluents into the northern zone of Maharashtra coast. Previous studies have reported increased concentrations of Pb, Ni, Cr, Cu, Zn, Hg, Cd, and Mn in these rivers (Yadav et al., 2015; Raut et al., 2019). In addition, numerous industries in the north-western region of the Maharashtra coast are known to release effluents directly into the Arabian Sea (Velusamy et al., 2014). This strongly indicates that the northern zone is subject to more anthropogenic influence than the central and southern zones, supporting our classification of the northern zone as a high-impact zone and our notion of zone-specific management strategies for mitigating MPs and HMs pollution. According to the National Oceanic and Atmospheric Administration (NOAA)-Screening Quick Reference Tables (SQuiRTs), Cu concentrations > 3.1 μg L^{-1} and Zn concentrations > 81 μg L^{-1} are higher than those that might potentially induce chronic toxicity in regional biota (Buchman, 2008). However, before making any definitive conclusions, comprehensive toxicological investigations utilising suitable model organisms and ecologically relevant concentrations are suggested.

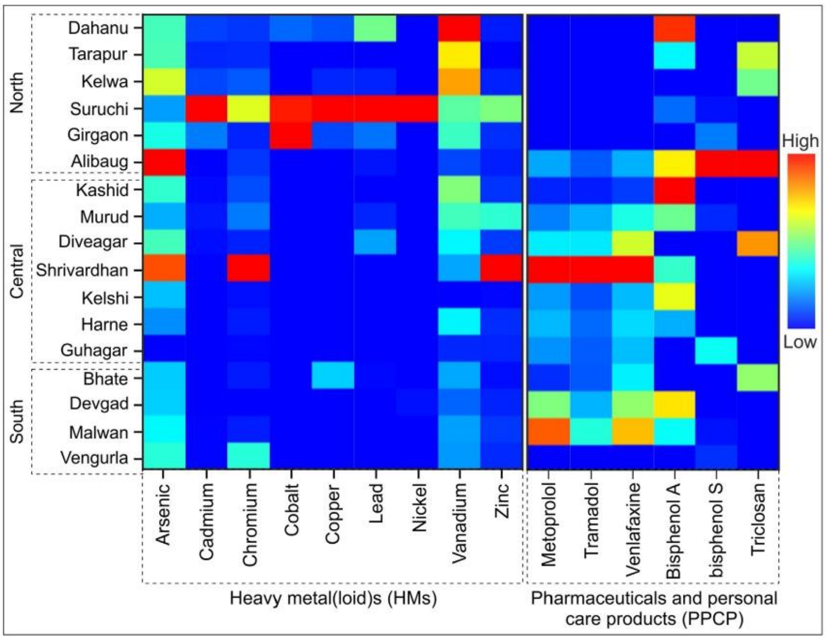

Fig. 5. Site-specific distribution of HMs and micropollutants (pharmaceuticals, personal care products, and plasticisers) in coastal water along the Maharashtra.

3.3. Pharmaceuticals and personal care products (PPCPs)

Of the 108 compounds screened in the water samples, six (metoprolol, tramadol, venlafaxine, triclosan, bisphenol A and bisphenol S) were regularly detected. Details of site specific (Fig. 5) and zone specific (northern, central and southern) distributions of PPCPs and bisphenol distributions in the water of the Maharashtra coastline are provided in Supplement 11. All three pharmaceuticals (metoprolol, tramadol, and venlafaxine) exhibited an inverse correlation with the presence of MPs (Fig. 6b). BPA and TCS showed relatively homogenous distributions across the zones studied (Supplement 10b). A statistically significant difference ($F = 3.914$; $P = 0.045$) was determined in the case of venlafaxine (Supplement 11).

3.3.1. Metoprolol

Metoprolol, one of the most popular beta-blockers, is used to treat high blood pressure. According to Maszkowska et al. (2014), the beta-blocker family of medications is one of the pharmacological classes with the largest global usage. For instance, from 2001 to 2010 there was a 57 % rise in the usage of beta-blockers in the USA (Gu et al., 2012; Godoy et al., 2015). The estimated mean concentration of metoprolol (53.7–306 ng L^{-1}; max. = 804; Supplement 11) in coastal waters of Maharashtra across three zones was considerably higher than that previously reported in seawater (8–210 ng L^{-1}) by Magnér et al. (2010) and in a Mediterranean coastal lagoon, Spain (n.d.–0.7 ng L^{-1}) by Moreno-González et al. (2015), and in lakes (n.d.–9.5 ng L^{-1}) (Daneshvar et al., 2010). The high metoprolol levels along the Maharashtra coast are an indicator that beta-blockers are not being removed from wastewater efficiently, since metoprolol is usually hard to remove from wastewater (Lara-Martín et al., 2014). This necessitates urgent upgrading of wastewater treatment facilities, particularly in southern Maharashtra, where greater metoprolol amounts were found. It is noteworthy that, metoprolol can bioaccumulate and is rather resistant to further changes (Ruan et al., 2020). In a recent study with 24 species (fish = 15, crustaceans = 5, molluscs = 4), metoprolol has been found to bioaccumulate in 22 species (<0.20–5.59 ng/g dry weight) (Ruan et al., 2020). Given that a significant portion of the population living in Maharashtra's coastal districts relies heavily on seafood, the greater concentration of metoprolol estimated in coastal water along the state's coastline is cause for concern.

3.3.2. Tramadol

Tramadol is utilised as an anaesthetic, anxiolytic, and antidepressant as well as a pain reliever because of its agonist action on opioid receptor and as an inhibitor of norepinephrine and serotonin absorption in the synaptic terminals (Edmondson et al., 2012). According to Rúa-Gómez and Püttmann (2013), acute or chronic pain is a prevalent problem, and as a result, tramadol consumption has multiplied in recent years. A good example is the 22.8 % increase in tramadol prescriptions from 2012 to 2015 in the United States (Bigal et al., 2019). The estimated mean concentration of tramadol (16.6–198 ng L^{-1}; max. 711 ng L^{-1}; Supplement 11) in coastal waters of Maharashtra across three zones was exceedingly higher than that found in Sydney estuary (<0.2–5.8 ng L^{-1}; Birch et al., 2015), Brisbane River estuary, Australia (<0.1–81.1 ng L^{-1}; Anim et al., 2020), Yarra River estuary, Australia (<0.1–8.9 ng L^{-1}; Anim et al., 2020), seawater of the Eastern Mediterranean Sea (<0.1–1.0 ng L^{-1}; Alygizakis et al., 2016) and surface water in Sri Lanka (0.085–41.7 ng L^{-1}; Guruge et al., 2019). Tramadol concentrations in the coastal waters in the southern zone were four times higher than those of the northern zone, while it was almost ten times higher in the central zone (Supplement 11). Given the extensive usage of tramadol and slow rate of elimination in sewage treatment facilities (Santos et al., 2021), the high concentrations in the central and southern zones are most likely attributed to indiscriminate use combined with the region's insufficient sewage treatment and waste disposal infrastructure. However, it is recommended that the precise concentration of tramadol in sewage water samples collected from sewage treatment plants situated in these

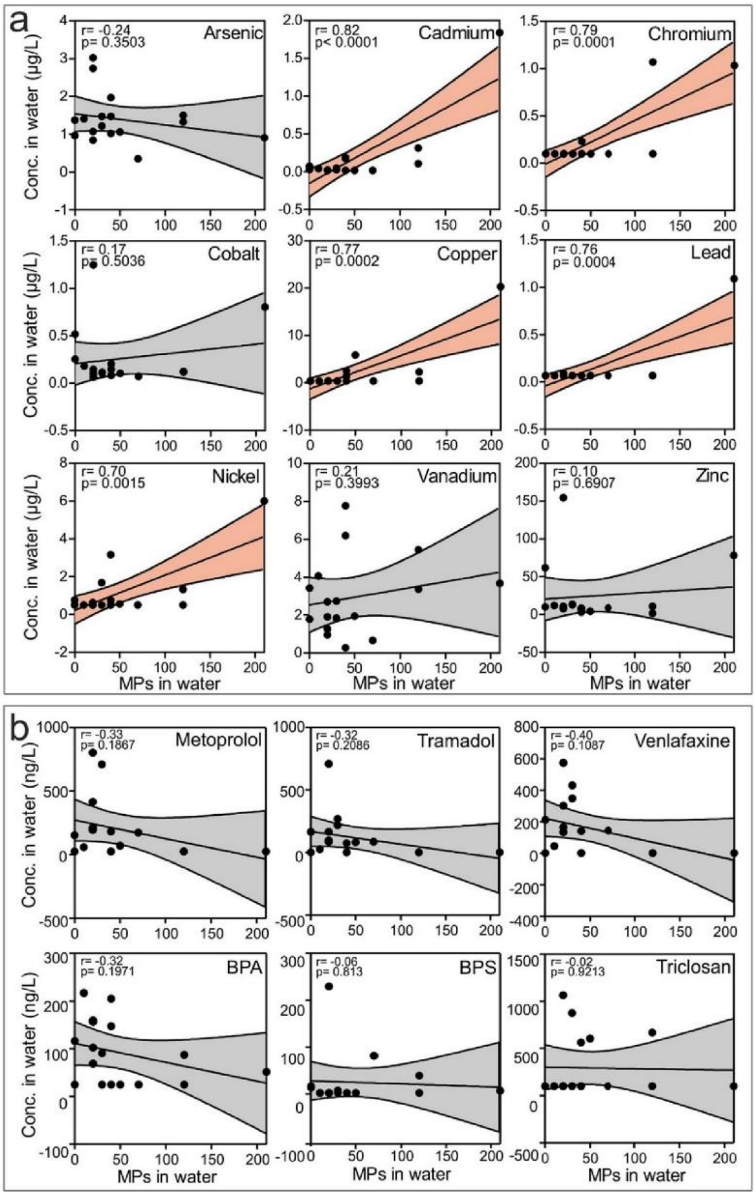

Fig. 6. Relationships between (a) MP abundance in water and HMs and (b) MP abundance in water and detected micropollutants. Note: Orange graphs show a significantly positive correlation.

zones be determined. This would aid in decision making regarding the upgrade of sewage treatment facilities as well as policy formulation addressing the use of tramadol-based drugs.

3.3.3. Venlafaxine

Venlafaxine, a serotonin-norepinephrine selective reuptake inhibitor, is one of the most often prescribed psychiatric drugs for the treatment major depressive disorders, social disorders and panic attacks in humans (Maulvault et al., 2018). The highest measured concentration of venlafaxine in the current investigation was 576 ng L^{-1}, with a mean concentration across three zones of Maharashtra coast was ranged from 24.6 to 234 ng L^{-1} (Supplement 11). These concentrations were high in comparison to Brisbane Estuary (86.2 ng L^{-1}; Anim et al., 2020), Yarra River Estuary (10.0 ng L^{-1}; Anim et al., 2020) and Sydney Estuary (5.2 ng L^{-1}; Birch et al., 2015; Anim et al., 2020). Both human health and the aquatic biota are likely to be in risk from higher detected concentrations of venlafaxine. For example, venlafaxine impacts fish exploration, decreased fish activity, and shoal cohesiveness, increasing the threat posed to them by potential predators (Maulvault et al., 2018). Additionally, it has been demonstrated that ocean acidification and warming accelerate the bioaccumulation of venlafaxine in aquatic organisms (Maulvault et al., 2018), which is expected to have a significant negative impact on human health especially fish consumers. According to Mutiyar and Mittal (2013), 64 % of Indians, only rely on recommendations from peers and their personal experience when they make drug purchases without a prescription. More than ten folds higher venlafaxine concentrations in the south and central zones likely to shade light on inadequate wastewater treatment facilities in these areas and the drug use patterns in these areas. Despite having lower population densities than the north zone (urban zone with metropolitan city like Mumbai), the central and south zones have a considerably high concentration of venlafaxine in their coastal waters, which is certainly a cause for concern. A thorough source-specific investigation on venlafaxine in these areas is thus strongly advised.

3.3.4. Triclosan

Triclosan, which has antimicrobial properties, is an important ingredient (0.1 to 0.3 % by weight) in a variety of frequently used consumer goods, including toothpaste, soap, deodorant, cosmetics and skin care lotions, textiles, and healthcare items (Chakraborty et al., 2018). The highest measured concentration of triclosan in the current investigation was 1064 ng L^{-1}, with a mean concentration across three zones of Maharashtra coast was ranged from 211 to 433 ng L^{-1} (Supplement 11). Triclosan has been detected in a variety of freshwater and marine environments across the world, including the Bhavani River, a tributary of the Kaveri (139 ng L^{-1}; Ramaswamy et al., 2011), the Hudson River in the United States (20 ng L^{-1}; Wilson et al., 2009), and the German Bight, North Sea (0.8–6870 pg L^{-1}; Xie et al., 2008). However, it is evident that the observed concentration of triclosan in Maharashtra's coastal waters is significantly greater than that reported before in several studies, with the exception of the estuary of the Guadalete River in SW Spain (310 ng L^{-1}; Pintado-Herrera et al., 2014), where it is comparable. We found that the mean concentration of triclosan in coastal waters tested from the northern zone was approximately two times higher (433 ng L^{-1}) than that in the central zone (211 ng L^{-1}) and southern zone (227 ng L^{-1}). This could be attributed to two factors: (1) the zone's extremely high population, which leads to increased use of products containing triclosan; and (2) the presence of an industrial cluster that includes a variety of large- to small-scale industries, particularly those in the textile and pharmaceutical sectors (Kumkar et al., 2021), where TCS is frequently used (Ramaswamy et al., 2011; Chakraborty et al., 2018). However, detailed source-specific investigations on triclosan in these areas is warranted.

3.3.5. Bisphenols

One of the well-known and widely produced chemicals, bisphenol A (BPA), is used as a raw material for manufacturing dental composites and sealants, coatings for beverage and food cans, and epoxy resins/polycarbonate plastics (Huang et al., 2012; Chakraborty et al., 2018; Ullah et al., 2018). Similarly, Bisphenols S (BPS) is an alternative to BPA that has been put to the market as a result of BPA being banned in some countries due to toxic effects of bisphenol A (Ullah et al., 2018). Average BPA levels in the current study across three zones of Maharashtra coast ranged from 75.3 to 101 ng L^{-1}, with the maximum recorded value being 218 ng L^{-1} (Supplement 11). The maximum recorded BPS concentration in the current study was 230 ng L^{-1}, while the mean concentration across three zones of Maharashtra coast ranged from 75.3 to 101 ng L^{-1} (Supplement 11). Compared to BPA levels in the Mediterranean Sea (13.80–15.34 g L^{-1}; Ozhan and Kocaman, 2019), Tokyo Bay, Japan (325 ng L^{-1}; Yamazaki et al., 2015), and the Black Sea, Bosphorus, and Sea of Marmara (8.85–14.76 g/L; Ozhan and Kocaman, 2019), the mean current level BPA measured along the Maharashtra coast is relatively lower. The level of BPS was similarly lower along the Maharashtra coast than Tokyo Bay, Japan (373 ng L^{-1}; Yamazaki et al., 2015). We found comparatively higher concentrations of BPA than BPS in all three zones across Maharashtra, despite BPS being reported to be an effective substitute for BPA and higher toxic effect of BPA on humans and aquatic biota (Ullah et al., 2018). This necessitates strict enforcement of policies on the use of chemical additives as well as their regular monitoring. MPs are one of the sources of Bisphenol A in the marine environment (Liu et al., 2019). Our findings are congruent with those of Liu et al. (2019), as we discovered higher quantities of BPA in the northern and central zones of the Maharashtra coast, where we detected more MPs than in the southern zone.

3.4. MPs as vector for contaminants

It is well-known that MPs can serve as a vector for HMs, PPCPs and bisphenols (Atugoda et al., 2021; Liu et al., 2021b; Wagstaff et al., 2022). Although MPs do not exhibit direct porosity, they can acquire contaminants like HMs and PPCPs from their surroundings through weathering, abrasion, and photo-oxidation processes, resulting in the formation of pits, cracks, and cavities on their surfaces (Atugoda et al., 2021; Liu et al., 2021b; Kumkar et al., 2021). The physical interactions between MPs and HMs can be determined using SEM-EDX analysis. We observed comparable morphological features, such as flakes, eroded surfaces, trenches, and cavities on the surfaces of the extracted MPs (Fig. 7a–c), indicating polymer ageing and mechanical and oxidative weathering. An earlier study by Tiwari et al. (2019) reported similar observations for MPs collected from Girgaon Beach sediment in Mumbai, Maharashtra (northern zone). The surface distribution of the elements is shown in Fig. 7d–f. The uneven distribution of elements on the MPs surface might be attributed to their rough surfaces, providing more evidence of the oxidative weathering of MPs, which promotes HMs adsorption (Shruti et al., 2019). Although we detected several HMs in coastal waters, only two (Al and Ti) were found on the surfaces of the MPs (Fig. 7d–f). This could be related to the analysis of a small subset of MP particles or the low detection limit of the method. In addition, several factors are known to influence HM adsorption onto MPs, including HM concentrations in the surrounding environment, organic matter, MP ageing, water salinity, and MP size (Khalid et al., 2021). Currently, we do not know which of these factors influence the interaction between MPs and HMs in the study region, which required additional research. Although we could not discover any PPCPs and bisphenol on the surface of the MPs, results of LC-MS/MS in present study confirm their presence in the coastal waters of Maharashtra. Non-covalent interactions between contaminants (PPCPs and bisphenols) and MPs caused these contaminants to rapidly desorb in the digestive tract of aquatic organisms (Bakir et al., 2014; Atugoda et al., 2021). Several aquatic organisms are now polluted with these contaminants. For example, three North East Atlantic Ocean marine fish species (*Dicentrarchus labrax*, *Trachurus trachurus*, and *Scomber colias*) have considerably higher BPA contents, and MP abundance and bisphenol contamination (in fish) have a strong positive relationship (Barboza et al., 2020). Likewise, Ali et al. (2018) reported the presence of seven PPCPs in macro algae (Methylparaben max. = 43 ng/g), 11 PPCPs in barnacles (Amitriptyline max. 18 ng/g), and 17 PPCPs in fish (Metronidazole max. = 82 ng/g) collected from polluted coastal waters of the Saudi Red Sea. Although we did not perform a direct analysis of either HMs, PPCPs and bisphenols in fishes, the high levels of these contaminants reported in coastal waters of Maharashtra is likely to contaminate aquatic biota, including fishes. Therefore, we highly recommend a screening of HMs, PPCPs and bisphenols in commonly consumed aquatic species (fish, prawns, lobster, crabs etc.) from major fish landing centres across the Maharashtra coast.

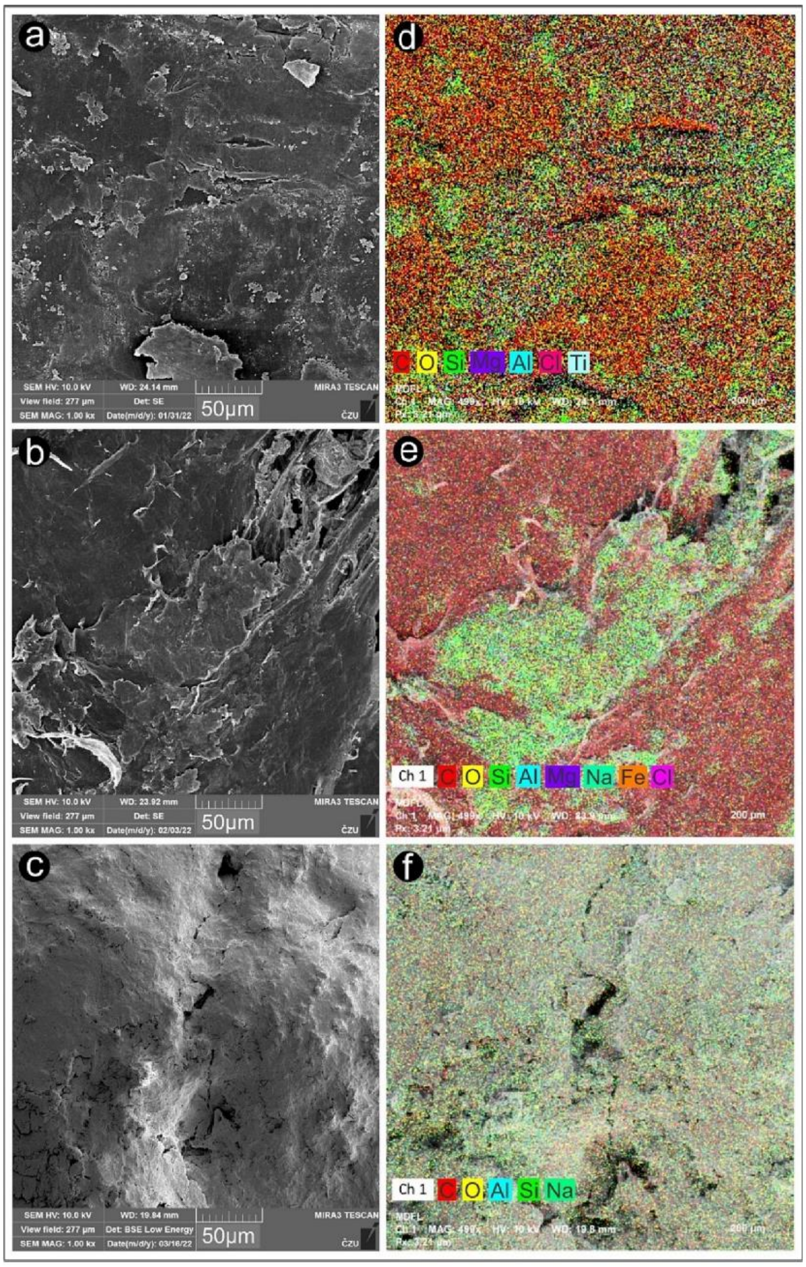

Fig. 7. SEM-EDX analysis of MPs: (a, b, and c) disintegrated or aged or weathered MP surface with flakes, trenches, and cavities; and (d, e and f) patchy distribution of elements on the MPs surfaces.

Nonetheless, the current study provides important information on the presence of HMs, PPCPs and bisphenols in Maharashtra's coastal waters and the ability of MPs to serve as vector/carrier for HMs, which could form the basis for future studies.

3.5. Phthalate esters as additives in plastic

In addition to acting as carriers for pollutants like HMs, PPCPs and bisphenols, MPs are also source of certain chemical additives (phthalates/plasticisers/PAEs) used in the production of plastic and plastic-based products. The MP samples collected from seven sampling stations (N1, N2, N3, N4, N5, C5, and S4) were positive for PAEs (Supplement 12). We detected di-n-butyl phthalate (DBP) and bis(2-ethylhexyl) phthalate (B2EHP or DEHP) in the analysed samples. MPs collected from the northern zone were found to contain both B2EHP and DBP, in contrast to the MPs collected from the central and southern zones, which contained only B2EHP. The lack of PAEs in other samples may be due to comparatively low levels of PAEs in the sample that are below the detection limit. However, the likelihood of PAEs in more samples cannot be ignored. The presence of PAEs in the environment (air, surface water, sediments, and soil) has been reported in numerous studies (as reviewed Net et al., 2015). However, our study is the first to detect their presence in association with MPs obtained from the Maharashtra coast. The disintegration of plastics in marine environments is known to release PAEs (Zhang et al., 2018a). MPs along the Maharashtra coast are subject to weathering, abrasion, and photo-oxidation processes (SEM-EDX data presented above), which are likely to result in the release of PAEs into the marine environment. PAEs are known to have a range of harmful effects, such as endocrine disruption, developmental defects in embryos, behavioural alterations, tissue damage in the liver, oxidative stress and alteration of gene expression (heat shock protein 70) in a variety of organisms including both vertebrates and invertebrates (Harris et al., 1997; Chen et al., 2014; Ye et al., 2014; Net et al., 2015; Kumkar et al., 2022). For improved policy formation and management of coastal ecosystems, we recommend detailed investigations pertaining to the spatiotemporal distribution of PAEs across the Maharashtra coast as well as experiments to examine the impact of PAEs on marine organisms both individually and in combination with MPs.

3.6. Ecotoxicological risk assessment

The ecotoxicological risk to three distinct groups of organisms (fish, algae, and crustaceans) representing three different levels (vertebrates, invertebrates, and plants) was analysed for eight HMs, four PPCPs, and two plasticisers identified in Maharashtra's coastal waters. The details of the contaminants, pollutant type, collection sites, and zone-wise ecotoxicological risk posed to fish, algae, and crustaceans are provided in Fig. 8a. Based on the estimated HQ scores, >50 % of the study sites in all three coastal zones pose a high ecological risk (>1) for all three types of organisms. Based on the HQ scores, >70 % of study sites from all three coastal zones pose a high to medium ($1 > HQ > 0.1$) ecological risk to all three types of species (Fig. 8a), indicating serious concern. Over 60 % of the organisms evaluated are at high or medium risk due to present pollution levels (Fig. 8a). Fish and crustaceans (35.3 % each) show a higher level of risk than algae (29.5 %), which is mostly represented by a medium risk level (46.8 %). This implies that animals like vertebrates and invertebrates are more susceptible to pollutants like HMs, PPCPs, and plasticisers than algae.

The details of site- and organism-specific HQ scores and risk levels (low, medium, and high) are provided in Fig. 8b. The presence of HMs in the environment poses a hazard to both the health and existence of organisms (Ścibior et al., 2021). According to our ecotoxicological risk assessment, V, Cu, Zn, and As pose a high risk to all three types of organisms, while Co, Cd, and Pb all pose medium to low levels of risks throughout the Maharashtra coastal region (Fig. 8b). Surprisingly, fish along the Maharashtra coast were not at high risk from Cr and Pb; however, Cr posed a high risk to algae and crustaceans. Owing to their ability to bioaccumulate and become bio-magnified at multiple trophic levels, HMs are categorised as potentially harmful substances (Liu et al., 2021b). Vanadium is known to accumulate in various tissues (skin, muscles, gills, intestine, liver, gonads, digestive glands, branchial heart, kidney, and pericardial renal appendages) of fish and invertebrates including *Parupeneus multifasciatus* (Common goatfish), *Mugil cephalus* (striped mullet), *Psettodes erumei* (Indian halibut), *Perca fluviatilis* (European perch), *Acanthopagrus latus* (Yellowfin seabream), *Gnathanodon speciosus* (Golden trevally), *Gerres oyena* (Common silver-biddy), *Eledone cirrhosaare* (Horned octopus), *Sepia officinalis* (Common cuttlefish), *Octopus vulgaris* (Common octopus), *Nautilus macromphalus* (Bellybutton nautilus), *Nautilus pompilius* (Emperor nautilus), *Architeuthis dux* (Atlantic giant squid), *Penaeus semisulcatus* (Green tiger prawn), *Metapenaeous affinis* (Jinga shrimp), and *Tripneustes gratilla* (Collector urchin) (as reviewed in Ścibior et al., 2021). Similar findings are reported in a previous study by Velusamy et al. (2014), who found Cu, Cd, Cr, Pb, and Zn in the muscle of 17 marine fish species caught in Mumbai Harbor, India. Several studies have highlighted the bioaccumulation of HMs in marine fishes and invertebrates and their possible adverse effects on consumer health (Velusamy et al., 2014; Mziray and Kimirei, 2016; Alizada et al., 2020; Venkateswarlu and Venkatrayulu, 2020). Although our estimated ecological risk offers valuable information on the potential impacts of HMs on plants, invertebrates, and vertebrates, further in-depth research is required to fully comprehend the direct and synergistic effects of these pollutants at various trophic levels.

Tramadol and BPS posed the least risk to all three types of organisms along the Maharashtra coast; metoprolol posed a high risk to fish in all three zones; whereas venlafaxine posed a high to medium risk to fish in the central and southern zones (Fig. 8b). Metoprolol and venlafaxine are commonly detected in municipal wastewater effluents, exposing non-target organisms including fish (Best et al., 2014; Sun et al., 2015; Thompson et al., 2017; Thompson and Vijayan, 2021; Salahinejad et al., 2022; Thompson et al., 2022). Metoprolol can inhibit growth, change gonadotropin and vitellogenin gene expression, stimulate detoxification, and cause oxidative stress in fish (Sun et al., 2015; Gröner et al., 2017). Similarly, embryonic venlafaxine exposure in zebrafish (*Danio rerio*) has been shown to impair larval behaviour, diminish juvenile growth, disrupt developmental programming, influence brain function, lower whole-body insulin levels, and disrupt glucose uptake in the brain (Thompson et al., 2017; Thompson and Vijayan, 2021). Best et al. (2014) showed that venlafaxine can also impair tissue metabolic capabilities and impede adaptive responses to acute stressors in rainbow trout (*Oncorhynchus mykiss*). The high risk that metoprolol poses to fish along the Maharashtra coast is a cause for concern. Furthermore, the high risk posed by venlafaxine, particularly in the central and southern zones, highlights the need for wastewater treatment plant upgrades to guarantee reduced release into the marine environment. In all three zones, triclosan posed a high risk to crustaceans and medium risk to fish and algae (Fig. 8b). Although BPA has a similar risk trend (medium to high), it affects all three types of organisms along the Maharashtra coast. The bioaccumulation potential of triclosan, along with its slower breakdown rate, has been observed to have adverse effects on a range of aquatic organisms. For example, according to Dann and Hontela (2011), aquatic organisms, including algae, invertebrates, and some types of fish, are much more sensitive to triclosan than mammals. Furthermore, because of its structural resemblance to existing oestrogenic and androgenic endocrine-disruptive chemicals, triclosan operates as a putative endocrine disruptor that affects thyroid hormone homeostasis and possibly the reproductive axis (Dann and Hontela, 2011). Numerous studies have demonstrated that BPA has antagonistic or hormonal agonistic effects on aquatic organisms, including invertebrates. For example, BPA has a severely deleterious influence on the embryonic growth of sea urchins (*Echinometra lucunter*) (Da Silva and de Souza Abessa, 2019). BPA is also a potential endocrine disruptor similar to triclosan (Vandenberg et al., 2012; Morales et al., 2020). Kim et al. (2019) reported that BPA interferes with moulting and reproduction in *Daphnia magna*. Furthermore, BPA bioaccumulates in aquatic organisms including fish (Careghini et al., 2015). BPA is also implicated in the aetiology of several endocrine disorders, including female and male infertility, early puberty, and hormone-dependent malignancies, such

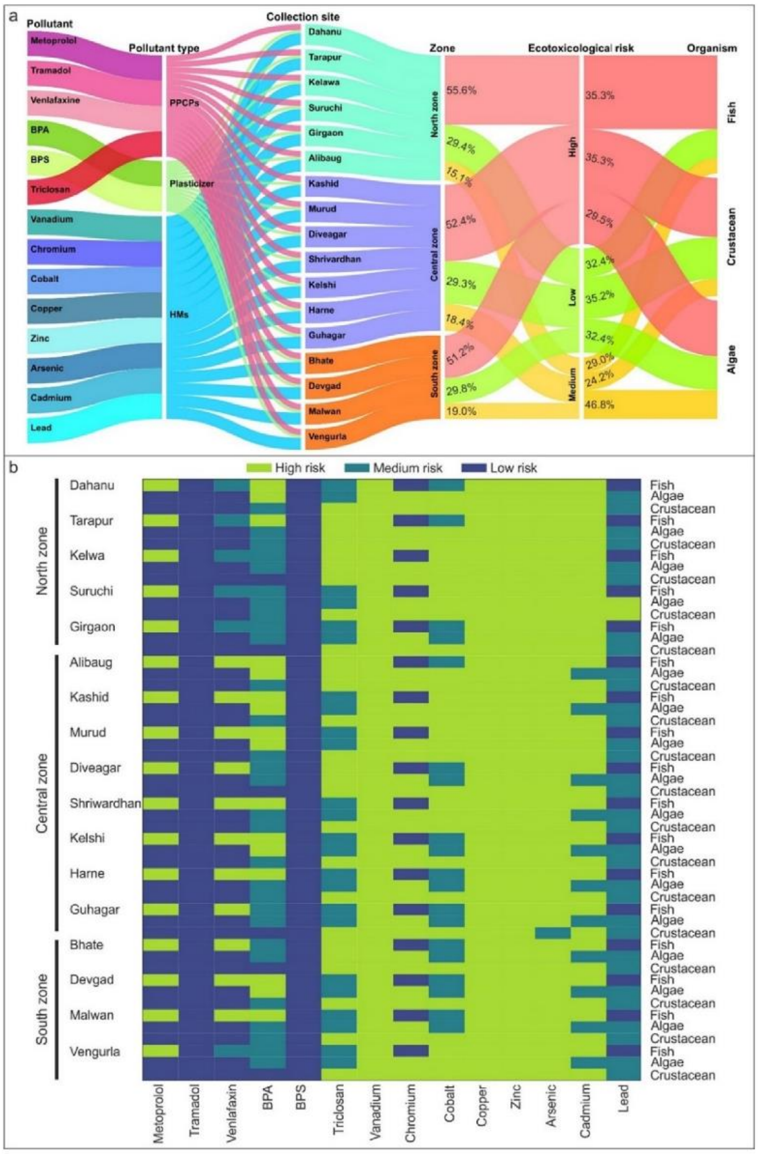

Fig. 8. (a) Alluvial plot illustrating the pollutants at different sites and percent ecotoxicological risk posed to various group of organisms in the three zones; (b) Matrix plot depicting the pollutant-specific ecotoxicological risk posed to different organisms at specific sites in the coastal waters along the Maharashtra.

as breast and prostate cancer (Diamanti-Kandarakis et al., 2009). As a result, the bioaccumulation potential (especially in fish that are regularly consumed by people) as well as the aetiology of many endocrine diseases are clearly a matter for worry for the whole population living along the coast of Maharashtra.

4. Conclusions

The current study offers the first data on the occurrence of several emerging contaminants along the Maharashtra coast of India, including MPs, PPCPs, bisphenols, PAEs, and HMs. Moreover, using the estimated values of eight HMs, four PPCPs, and two plasticisers (BPA and BPS), the current study also assessed ecotoxicological risk to three different groups of organisms (fish, algae, and crustaceans) representing three different levels (vertebrates, invertebrates, and plants). The northern Maharashtra coast is regarded as a zone with significant pollution impact due to the high number of MPs in sediment and coastal water as well as estimated PLI values. The potential of PAEs to contaminate the coastal waters of Maharashtra by leaching is demonstrated by the presence of PAEs in extracted MPs, such as DBP and B2EHP or DEHP. Maharashtra's coastal waters have high levels of HMs, particularly copper and zinc. The coastal waters of Maharashtra clearly contain a variety of contaminants, and it is clear that these contaminants are interacting with one another to act as carriers for one another and intensify the risks they offer. Metoprolol, tramadol, venlafaxine, and triclosan are a few of the pharmaceuticals that are prevalent in the coastal waters of Maharashtra and have much higher quantities than in most other regions in the globe. Based on the estimated levels of pollutants in the current study, 70 % of the sampling sites across Maharashtra coast demonstrates a high to medium ecological risk to fish, crustaceans, and algae, indicating an alarming scenario. However, it should be noted that the majority of the pollutants identified in this study had substantially higher concentrations and are known to have bioaccumulation potential (especially in marine fishes), posing a serious risk to human health. This information is crucial for better policy formulation and coastal conservation management in India in general, and Maharashtra in particular. Overall, future research in this region should focus on source-specific effective mitigation of emerging pollutants such as MPs, HMs, PPCPs, and PAEs from Maharashtra coastal water, as well as risk assessment using appropriate model organisms at environmentally relevant concentrations of contaminants provided in the current study.

CRediT authorship contribution statement

SMG, PBK, LK: conceptualization. SMG, PBK, MP: fieldwork and figure preparation. SMG, PBK, CRV, FM, MB, LP, ŠH, SŹ, FM, KH: methodology and data analysis. SMG, PBK, CRV: MS preparation. RR, PK, MP, LK and all other authors: reviewing the final draft.

Data availability

Data will be made available on request.

Declaration of competing interest

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

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Appendix A. Supplementary data

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5.3. Big eyes can't see microplastics: Feeding selectivity and eco-morphological adaptations in oral cavity affect microplastic uptake in mud-dwelling amphibious mudskipper fish

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Big eyes can't see microplastics: Feeding selectivity and eco-morphological adaptations in oral cavity affect microplastic uptake in mud-dwelling amphibious mudskipper fish

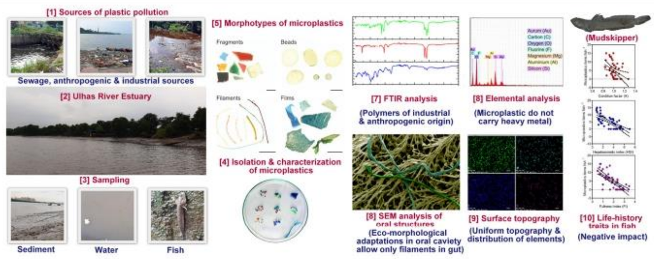
Pradeep Kumkar [a,1], Sachin M. Gosavi [b,*,1], Chandani R. Verma [a], Manoj Pise [a], Lukáš Kalous [a,**]

[a] Department of Zoology and Fisheries, Faculty of Agrobiology, Food, and Natural Resources, Czech University of Life Sciences Prague, Czech Republic
[b] Department of Zoology, Maharashtra College of Arts, Science and Commerce, 246-A, Belasis Road, Opp. Alexandra Cinema, Mumbai 400 008, Maharashtra, India

HIGHLIGHTS

- Microplastics were studied in an anthropogenically impacted urban estuary.
- Fragment morphotypes and white-coloured microplastics were the most common.
- Adaptation in mudskippers oral morphology allows only filament into gut.
- Microplastic abundance in gut affects mudskippers' life-history traits.
- Microplastic surface topography and weathering affects heavy metal binding and transit into fish gut.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastic contamination is a widespread global problem. Plastic pollution in the oceans has received a lot of news coverage, but there is a significant gap in our knowledge about its effect in estuarine areas and a profound regional bias in available information. Here, we estimated the degree of microplastic pollution, its impact on a selected fish, and its function as a vector for heavy metals in the Ulhas River estuary, which is one of the most fragile, polluted, and anthropogenically impacted estuaries in India. Using mudskipper fish, we have also assessed how the feeding guild and ecomorphological adaptations in the feeding apparatus affected the microplastic intake and life history traits of the fish. Sediment, water and fish samples were collected from three sampling localities (S1, S2 and S3) in the Ulhas River estuary and analysed. Findings showed an increase in microplastic abundance from S1 (suburban) to S3 (urban industrial belt) in sediment (96.67–130.0 particles kg^{-1}), water (0.28–0.41 particles L^{-1}) and fish (3.75–6.11 particles per fish). Fragments, followed by pellets and filaments largely contribute to the plastic morphotypes in sediment and water. FTIR analysis revealed polymers of anthropogenic and industrial origin such as polypropylene, Surlyn ionomer, low-density polyethylene, and polyethylene or polybutylene terephthalate. Only filaments were found in the guts of 74% of the mudskippers examined, which may be due to their filter-feeding habit and unique anatomical arrangement of oral structures that effectively filter large microplastic particles. Microplastic abundance showed a strong negative correlation with condition factor, fullness index and hepatosomatic index of fish. SEM-EDS analysis revealed that the microplastic surface

* Correspondence to: S. M. Gosavi, Department of Zoology, Maharashtra College of Arts, Science and Commerce, Mumbai, India.
** Correspondence to: L. Kalous, Department of Zoology and Fisheries, Faculty of Agrobiology, Food, and Natural Resources, Czech University of Life Sciences, Prague, Czech Republic.
E-mail addresses: schn.gosavi@gmail.com (S.M. Gosavi), kalous@af.czu.cz (L. Kalous).
[1] Equally contributing authors.

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topography played an important part in adsorbing heavy metals from a water body containing these contaminants. Results highlight the contamination of vulnerable estuarine habitats, harmful effects on resident biota, and health threats to dependent populations.

1. Introduction

In the mid-1900s, the distinctive properties and versatile applications of plastics made them an indispensable part of human life and an essential commodity in commerce (Thompson et al., 2009; McNeish et al., 2018; Rochman et al., 2019; de Vries et al., 2020). Plastic waste is ubiquitous, being found in even the most remote, uninhabited areas of the world, such as the Barents Sea and Arctic Ocean, Henderson Island, deep ocean trenches, and at the summit of Mount Everest (Browne et al., 2011; Van Cauwenberghe et al., 2013; Woodall et al., 2014; Lusher et al., 2015; Lavers and Bond, 2017; McNeish et al., 2018; Napper et al., 2020). Negative effects from plastics are inevitable as co-existing organisms interact continuously with their surroundings and each other (Laist, 1997; Barnes et al., 2009; Gall and Thompson, 2015; De Souza e Silva Pegado et al., 2018; Parker et al., 2020). The concerns about harm to living organisms from plastic contamination increased many-fold when it was revealed that many common types of plastic are further fragmented into micro-scale particles generally referred to as 'microplastics' (McNeish et al., 2018; Rochman et al., 2019). Their small sizes (<5 mm) enable them to enter organisms at a lower trophic level, from which they can reach higher trophic levels in the food web (Blight and Burger, 1997; Fossi et al., 2012; Cole et al., 2013; Lusher et al., 2013; Zettler et al., 2013; McCormick et al., 2014; Peters et al., 2017; Payton et al., 2020). Higher trophic-level species, such as fish also ingest microplastics directly because of their resemblance to natural prey. This can cause deleterious effects such as blockage and damage to the digestive tract (Ory et al., 2018), decreased food intake (Besseling et al., 2013; Wright et al., 2013), endocrine disruption (Rochman et al., 2014), reproductive failure (Sussarelle et al., 2016), growth suppression and reduced juvenile survival (Naidoo and Glassom, 2019), alteration of blood enzymes, decreased protein synthesis, immunosuppression, electrolyte changes in the blood (Banaee et al., 2020), oxidative damage to the brain and liver, adverse histo-morphological changes to the intestine and liver, and even alteration of gene expression (Rochman et al., 2013; Romano et al., 2020), which eventually leads to decreased overall species fitness and survival (Fonte et al., 2016; Naidoo and Glassom, 2019). In addition, the plastic microparticles can also transport organic pollutants and heavy metals (Haghi and Banaee, 2017; Banaee et al., 2019), which subsequently pose serious health risks to fish and fish consumers (Rios et al., 2007; Brennecke et al., 2016; Karami et al., 2017; Godoy et al., 2019; Naqash et al., 2020).

The current literature on worldwide distribution of microplastic pollution reveals mounting contamination of the oceans, bodies of freshwater and terrestrial ecosystems (Barnes et al., 2009; Horton et al., 2017; De Souza e Silva Pegado et al., 2018; Parker et al., 2020). However, compared to other aquatic bodies, there is a dearth of information on microplastic pollution in estuarine regions, and only a few regions have been studied (Shim and Thompson, 2015; Auta et al., 2017; Sruthy and Ramasamy, 2017). Estuaries are entry points from which plastic reaches the oceans, and several earlier studies have documented the highest level of microplastics in estuarine water and sediment (Galgani et al., 2000; Browne et al., 2010; Zhao et al., 2014; McEachern et al., 2019; Leads and Weinstein, 2019). Therefore, it is a little surprising that there have been so few studies on plastic pollution of estuarine ecosystems in under-developed countries such as India, which has the second highest human population in the world. Furthermore, the effect of microplastics on resident estuarine fauna has received little attention (Maghsodian et al., 2020) and warrants urgent investigation.

Estuarine mudflats are often inhabited by amphibious, euryhaline fish called mudskippers. Their life cycle encompassing both mudflats and water as well as their indiscriminate feeding habits make mudskippers highly susceptible to microplastics in sediments and estuarine water. As there is a strong correlation between the form and quantity of microplastics found in fish and their feeding habits (Ory et al., 2017), mudskippers are an excellent choice for investigating the relationship between microplastic pollution in estuarine mudflats and the resident biota. According to Markic and Nicol (2014) and Naidoo and Glassom (2019), microplastic exposure inhibits fish growth and fitness and thus can have a damaging effect on fisheries. Mudskippers, which are highly nutritious, are widely sold in international markets and are considered a delicacy in China, Vietnam, Thailand, Japan and India (Mahadevan et al., 2020). Thus, microplastic-induced growth impairment is likely to cause economic loss to fisheries (Lamberth and Turpie, 2003). As far as we know, there have been no studies from India estimating the level of microplastics in estuarine sediment and water and its effects on mudskippers. There is also almost no data on how microplastics in the estuarine ecosystem enter the food chain through ingestion by mudskippers and their effects on the fishes' growth (length and weight), reproduction, energy reserves, and survival.

Therefore, the present study was conducted in the Ulhas River estuary (Thane District, Mumbai, Maharashtra, India), which has been declared one of the one hundred most polluted rivers in the country by government pollution monitoring authorities. It receives an immense amount of domestic and industrial waste because of its proximity to densely populated areas and industrial belts (Menon and Mahajan, 2011; Fernandes and Nayak, 2012; Raut et al., 2019). The areas along the Ulhas estuary support a number of small villages (Vehele, Surai, Alimghar, Kasheli, Kalher, Dive-Kevani) inhabited by thousands of artisanal fishers. The extensive mudflats and mangrove habitats of the Ulhas estuary provides an excellent opportunity for artisanal fishers to catch estuarine fish and sell them in urban fish markets such as the Thane fish market (Menon and Mahajan, 2011; Fernandes and Nayak, 2012). We chose *Boleophthalmus dussumieri* Valenciennes, 1837, commonly known as the mudskipper, for the current study because of its abundance in the estuarine mudflats of the study sites, importance to estuarine food webs, and fisheries. It is one of the primary food items in the diet of artisanal fishers and a delicacy in urban population. Earlier investigations in the Ulhas River estuary showed the presence of heavy metals in the water and its detrimental effects on consumers of mudskippers (Menon and Mahajan, 2011; Fernandes and Nayak, 2012; Raut et al., 2019). Since microplastic particles are well-known for transporting organic contaminants and heavy metals, which pose significant health risks to fish and fish consumers (Rios et al., 2007; Brennecke et al., 2016; Haghi and Banaee, 2017; Karami et al., 2017; Banaee et al., 2019; Godoy et al., 2019; Naqash et al., 2020), the likelihood of heavy metals being ingested by mudskipper through microplastics must be addressed, as this has implications for human health.

The objectives of the present study were: (a) to collect, identify and characterize (size, type, colour and polymer chemical) microplastics from sediment, water and mudskippers of the Ulhas River estuary; (b) to determine the association between microplastic abundance in sediments and water and the amount of microplastics in mudskippers; (c) to assess the role of feeding habits and feeding apparatus in the abundance of microplastics in the mudskipper gut; (d) to examine whether the traits of length, weight, physical condition and energy reserves of mudskippers are affected by the microplastic load in its gut; and (e) to determine whether microplastics serve as a vehicle for transferring heavy metal contaminants in the estuarine mudflat ecosystem to the mudskipper gut.

2. Materials and methods

2.1. Study locations

For the present study, we chose the Ulhas River estuary (Fig. 1), which originates in the Rajmachi region of the Western Ghats Mountain Range and passes through densely populated suburban and urban areas such as Karjat, Ambarnath, Kalyan, Dombivali and Thane until its final confluence with the Arabian Sea in Vasai. Samples of sediment, water and fish were collected from three sampling stations: Sarang (S1: 19°23′ N and 73°06′ E), Alimghar (S2: 19°20′ N and 73°03′ E) and Kalher (S3: 19°24′ N and 73°00′ E) in September 2020. The rationale behind the selection of these three study sites is that they cover major areas and sources of microplastic contamination in the Ulhas River estuary and therefore could reflect a true microplastic pollution scenario. Sarang lies in the immediate vicinity of a densely populated industrial area and therefore receives large quantities of sewage and industrial effluent from surrounding locations. Alimghar, although situated in close proximity to an urban area, has a large population of artisanal fishers and tribal people mainly dependent on fishing. Kalher and its nearby areas is truly an industrial hub with several hundred chemical, plastic, paint and textile factories.

2.2. Sediment sampling and extraction of microplastics

A sediment sample (approximately 2 kg) from the top 5 cm layer was obtained from each sampling station using a shovel and a stainless-steel container. Three samples were collected from each

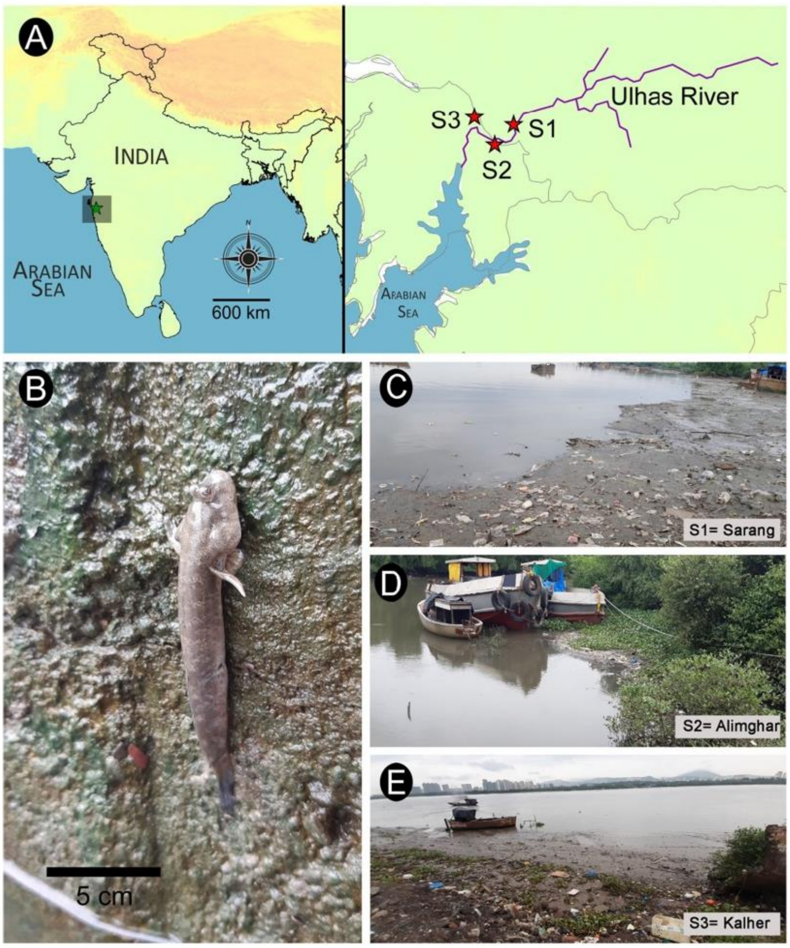

Fig. 1. (A) Map of Ulhas River estuary showing three sampling stations (S1 = Sarang, S2 = Alimghar and S3 = Kalher); (B) study species, *Boleophthalmus dussumieri* at the study site (S2); (C, D and E): sampling stations showing heavy plastic pollution.

locality, maintaining a distance of 100 m between any two sampling points to ensure precision of the sampling, which produced nine sediment samples. Sediment samples were processed according to the protocol of Galgani et al. (2013), Sruthy and Ramasamy (2017) and Patchaiyappan et al. (2020) with minor modifications. Briefly, wet sediment samples were dried at 50 °C in a hot-air oven for 72 h. Dry samples (1 kg; three replicates from each sampling location) were disaggregated, carefully checked visually for large plastic particles (meso-plastics, 5 mm to 2.5 cm, and macro-plastics, >2.5 cm). Meso- and macro-plastic particles were isolated, sorted and stored in glass Petri dishes. The same dried sample (1 kg) was again filtered through a 5 mm sieve to ensure that no particles greater than 5 mm remained in the sample. The dried sediment (100 g) of each replicate was further processed in compliance with the National Oceanic and Atmospheric Administration (NOAA) protocol guidelines (Masura et al., 2015). The samples were subjected to a process of wet peroxide oxidation with 30% H_2O_2 that ensures full digestion of the organic matter present in the samples, followed by density separation using a flotation method with NaCl ($d = 1.3$ g ml^{-1}) and vacuum filtration using Whatman GF/A filter paper, 25 mm. The filters were then carefully removed, dried in a desiccator and stored in glass Petri dishes.

2.3. Water sampling and microplastics extraction

Water samples were collected from the three sampling stations in triplicate using the bottle sampling method (Vermaire et al., 2017; Crew et al., 2020; Zhang et al., 2020) with minor modifications. Before sampling, the container was washed thrice with filtered distilled water at the sampling station. During sampling, 100 L of water was collected using a 2 L stainless steel jug at a depth of 0 to 25 cm below the surface, and the water was filtered through a stainless steel sieve (30 cm diameter, mesh size 50 μm). A separate strainer was used for replicative sampling ($n = 3$ for each sampling site). Each strainer was then wrapped in aluminium foil and transported to the laboratory where it was processed for microplastic isolation. Using a stereo-zoom microscope, the strainers were carefully checked for microplastic particles, which were segregated from the non-plastic material. In the event of an ambiguity in separating plastic from non-plastic, the hot needle test was used (De Witte et al., 2014), as plastic particles would melt or curl in the presence of a very hot needle. Microplastic particles were then retrieved using a metal forceps and a natural fibre brush. In order to ensure complete recovery of the particles, each strainer was rinsed three times using 500 mL of distilled water and vacuum filtered as mentioned earlier in Section 2.2. A procedural blank test was also run to ensure that the filtered distilled water used was free from contamination with microplastic particles.

2.4. Fish sampling and microplastics extraction

Fifty fish were collected from all three sampling localities ($S1 = 16$; $S2 = 16$; $S3 = 18$) with the help of local fishers. Fish were immediately euthanized by placing them in a 5 L steel jar containing MS-222 solution (tricaine methane sulphonate; 0.25 g L^{-1}). Each fish was then individually wrapped in aluminium foil and transported to the laboratory, where it was immediately processed for the isolation of microplastics from the gut. Upon reaching the laboratory, each fish was dried with tissue paper and the length in mm and the mass in g were recorded using digital callipers (precision, 0.1 mm; Mitutoyo, Japan) and balance (precision, 0.001 g; Contech, India), respectively. A ventral incision was made using a sterilised scalpel and the fish was cut open to remove the gut. The entire gut from the throat to the anus was dissected with the aid of sterilised scalpel, forceps and scissors, placed in a glass tray and the length and weight of the gut were measured. At the same time, the oral cavity (upper jaw, lower jaw and pharyngeal region) was also dissected and observed under a stereo-zoom microscope for any adherent microplastic particles. For microplastic isolation, each gut was transferred to a 100 ml conical flask containing 10% KOH (w/v), approximately four times the volume of tissue, covered with aluminium foil, and incubated overnight at 60 °C to isolate microplastic from the gut as described by Dehaut et al. (2016) and Daniel et al. (2020) with some modifications. The next day, the flask was carefully checked for complete tissue digestion, cooled to room temperature, and the liquid from the dissolved tissues vacuum filtered. In the event of incomplete tissue digestion, the flask was incubated for an additional 24 h and the contents filtered. The filters were carefully removed, dried in a desiccator and stored in glass Petri dishes.

2.5. Microplastics segregation and enumeration

The filters from sediment, water and fish containing microplastic particles were carefully examined under a stereo-zoom microscope (Olympus, Germany). The microplastics were then enumerated, measured, and sorted by colour and type. The microplastics abundance in sediment is reported as the number of items/particles kg^{-1} dry weight (d.w.) of sediment. The microplastic abundance in water is reported as the number of items/particles L^{-1} of water, and in fish as the number of items/particles per fish. Isolated particles were sorted into five major morphotypes as suggested by Karthik et al. (2018): fragments, foam, pellets/beads, film and line/fibres/filaments. Each morphotype from each collection site was further classified based on its colour as follows: black, blue, white/opaque, transparent, red, green and other (purple, pink, violet, grey, yellow or brown).

2.6. Polymer characterization using FTIR

Because of its accuracy and need for minimal sample processing, Fourier transform infrared (FTIR) spectroscopy was used to determine the chemical composition of the isolated plastic particle morphotypes (Galgani et al., 2013; Daniel et al., 2020). Ninety-six microplastic particles representative of the different morphotypes from sediment, water and fish were selected for chemical analysis. The microplastic particles were subjected to FTIR analysis (Shimadzu IRSpirit spectrum range 4000 cm^{-1} to 500 cm^{-1}) to obtain clean spectra. The resulting spectra were then analysed using the built-in software (IR Solutions) and the polymer type was identified by comparing the spectra with the reference library.

2.7. Surface topography, elemental composition and distribution analysis

Earlier studies provided concrete evidence that analysis of the microplastic surface structure can provide important information on polymer ageing, weathering and mechanical damage (Zbyszewski et al., 2014; Sathish et al., 2020), which increases the surface area of the particle. This can make it a carrier for heavy metal elements that pose a threat to the health of fish and their consumers (Zbyszewski et al., 2014; Sathish et al., 2020). Scanning electron microscopy (SEM) coupled with energy-dispersive spectroscopy (EDS) is a widely accepted technique to examine surface morphology of microplastic particles and to determine whether heavy metals are adsorbed to them (Zbyszewski et al., 2014; Sathish et al., 2020). Therefore, each representative microplastic morphotype was examined using FESEM-EDS (field emission scanning electron microscopy-energy dispersive spectroscopy) to determine its surface morphology (presence of cracks, crevices, pits, and adherent particles), the elemental analysis, and elemental mapping. Each morphotype in its native form without washing was selected using stainless steel forceps and placed on double-sided adhesive carbon tape, mounted on aluminium stubs, and sputtered with gold. These stubs were imaged by FESEM (FEI Nova NanoSEM 450 Bruker XFlash 6130) operating at an accelerating voltage of 3.00 kV in order to obtain the information about surface morphology of microplastic particles. The identification and distribution of elements and adsorbed heavy metals on the microplastic surface (mapping) was determined using the EDS technique (FESEM: xT microscope control EDS: Espirit 1.9).

2.8. SEM analysis of oral structures

Earlier field studies and laboratory experiments using a range of examples from marine and freshwater habitats showed that the amount of microplastics in fish was directly linked to the feeding mode and habitat (McNeish et al., 2018; Sathish et al., 2020; Zhang et al., 2021). Because of the distinctive mode of mudskipper feeding (filter-feeding on diatoms) and the habitat (mudflats), an SEM analysis of the oral structure was performed to determine if feeding traits, which primarily depend on the oral structure, and eco-morphological adaptations in the oral structure (Maghsodian et al., 2020) affect the contents of microplastics in the mudskipper's gut. Oral structures (upper jaw, lower jaw and pharyngeal apparatus) were dissected out and examined using the FESEM (FEI Nova NanoSEM 450).

2.9. Fish condition, stomach fullness index (SFI) and hepatosomatic index (HSI)

Fish condition, stomach fullness index (SFI), and hepatosomatic index (HSI) are some of the commonly used parameters to assess the effect of microplastic ingestion on fish life history traits (Alomar and Deudero, 2017; Mizraji et al., 2017; Critchell and Hoogenboom, 2018; Wieczorek et al., 2018; Yin et al., 2018; Arias et al., 2019; Filgueiras et al., 2020; Müller et al., 2020; Sbrana et al., 2020). The condition was assessed using measurements of length (TL) and weight (Wt) according to the following equation (Froese, 2006): Condition factor $(K) = Wt / TL^b \times 100$, where '*b*' is the length-weight relationship parameter. The stomach and liver were removed from each fish specimen, transferred to a clean petri dish and weighed. The stomach and liver weights were then used to determine the stomach fullness index (SFI) and hepato-somatic index (HSI) by the following formula (Gosavi et al., 2020): SFI = (stomach weight / total weight of fish) $\times$ 100 and HSI = (liver weight / total weight of fish) $\times$ 100. The estimated values of K, SFI and HSI were further used to determine if the abundance of microplastic particles affected the condition, food intake (stomach fullness) and the overall energy reserve (hepato-somatic index) of the fish.

2.10. Contamination controls

In order to avoid microplastic contamination from external sources, appropriate quality control measures were employed. Samples were collected and stored only in steel containers with tightly fitting lids. To reduce background contamination, only cotton aprons and nitrile gloves were used. All the glassware used in the procedures was sterilised and thoroughly washed thrice using filtered distilled water and kept wrapped in aluminium foil until use. The extraction processes were performed in a laminar-flow hood with surface cleaning and disinfection using double distilled water and 70% alcohol after processing each batch of samples. The fish were rinsed thoroughly with filtered distilled water before being processed to reduce the risk of contamination from particles present on the exterior of the sample. Laboratory blanks and field blanks were used during extraction and identification steps to test for contamination from air-borne plastic particles.

2.11. Data treatment and statistical analysis

Before data processing, the normality (Shapiro-Wilk test) and the homoscedasticity (Levene's test) of the data were tested using PAST (Hammer et al., 2001; Freeware, http:/folk.uio.no/ohammer/past/). Because the data followed a normal distribution ($p > 0.05$), site-specific variations in microplastics abundance in sediment, water and fish were analysed using one-way ANOVA ($\alpha = 0.05$) followed by Tukey's post-hoc test for multiple pairwise comparisons. The percent abundance of each type of microplastic morphotype, colour and polymer type in sediment, water and fish was calculated using Microsoft Excel (Ver. 2010). Pearson's correlation analysis was performed using GraphPad Prism software (trial version) to analyse possible relationships between the microplastics abundance (%): (a) in fish and sediment, (b) in fish and water, (c) of different filament colour in the sediment and fish, (d) of different filament colour in the surface water and fish, and (e) the microplastics particles in fish and their condition factor, (f) the microplastics particles in fish and their SFI, and (g) the microplastics particles in fish and their HSI. Since fish gut had only one kind of morphotype (filament/fibre), we were unable to conduct a correlation analysis between the percent abundance of the various microplastic morphotypes in surface water or sediment and fish.

3. Results and discussion

3.1. Abundance of microplastics in sediment, water and fish

Microplastic particles were detected in sediment, water and fish from all three sampling stations (S1 to S3) on the Ulhas River estuary (for details refer Supplement 1). Numbers ranged from 96.67 $\pm$ 12.02 particles kg^{-1} of dry weight (S1) to 130 $\pm$ 5.77 particles kg^{-1} of dry weight (S3) of sediment, whereas the abundance of microplastics in water ranged from 0.23 $\pm$ 0.02 particles L^{-1} (S2) to 0.41 $\pm$ 0.05 particles L^{-1} (S3) (Fig. 2A, B). Site-specific differences in microplastics abundance were not significant in sediment ($F = 3.950$; d.f. = 2, 6; $p = 0.0804$; Fig. 2A) and water ($F = 5.051$; d.f. = 2, 6; $p = 0.0517$; Fig. 2B). However, a marked trend of increasing mean concentrations of microplastics from S1 to S3 was noticeable. The higher abundance of microplastics at site S3 in both sediment and water is likely associated with its dense population and nearby industrial hubs along the coastline and upstream of the Ulhas River estuary which discharges large volumes of industrial effluent and poorly treated wastewater (Fernandes and Nayak, 2012; Raut et al., 2019). Although the areas upstream of the study sites have operational sewage treatment plants, they are in the process of being upgraded in order to increase their efficiency in processing sewage and industrial effluents (MPCB, 2020). Additionally, the non-point sources of microplastics in the form of sewage derived from adjacent areas of the collection sites are discharged directly without treatment resulting in severe estuarine plastic pollution (Su et al., 2016; Robin et al., 2020).

From a sampling of 50 mudskippers, 37 (74%) fish had microplastics in their digestive tract, which is higher than previous investigations across the globe (Table 1). The average number of plastic microparticles per fish ranged from 3.75 $\pm$ 1.26 to 6.11 $\pm$ 1.17 (Fig. 2C). The particle content per fish was higher (50% of the fish had more than four particles each; eight fish had more than ten particles each) than in the previous study by Robin et al. (2020) along the southwest coast of India, which found four particles in only one fish. Site-specific differences in microplastic contamination in fish were not significant ($F = 1.158$; d.f. = 2, 47; $p = 0.3228$; Fig. 2C). The highest microplastic abundance was observed in mudskippers sampled from S3 (83.33%), followed by S2 (81.25%) and S1 (56.25%), indicating that microplastic abundance in fish was a function of the amount of microplastics in sediment and water. The comparatively higher microplastic abundance in the Ulhas River mudskippers overall, and per fish, can be attributed to their occupation of epipelagic and mudflat habitats (dual habitat occupation) and their feeding behaviour. Owing to the proximity of shallow water habitats to anthropogenic sources (Sathish et al., 2020), epipelagic fish are significantly more vulnerable to direct exposure to microplastics than mesopelagic fish. In earlier research, Sathish et al. (2020) demonstrated that epipelagic fish such as *Harpodon nehereus*, *Chirocentrus dorab*, *Sardinella albella*, and *Rastrelliger kanagurta* accumulated higher levels of microplastics than mesopelagic fishes like *Katsuwonus pelamis* and *Istiophorus platypterus*. In addition, mudskippers tend to live in

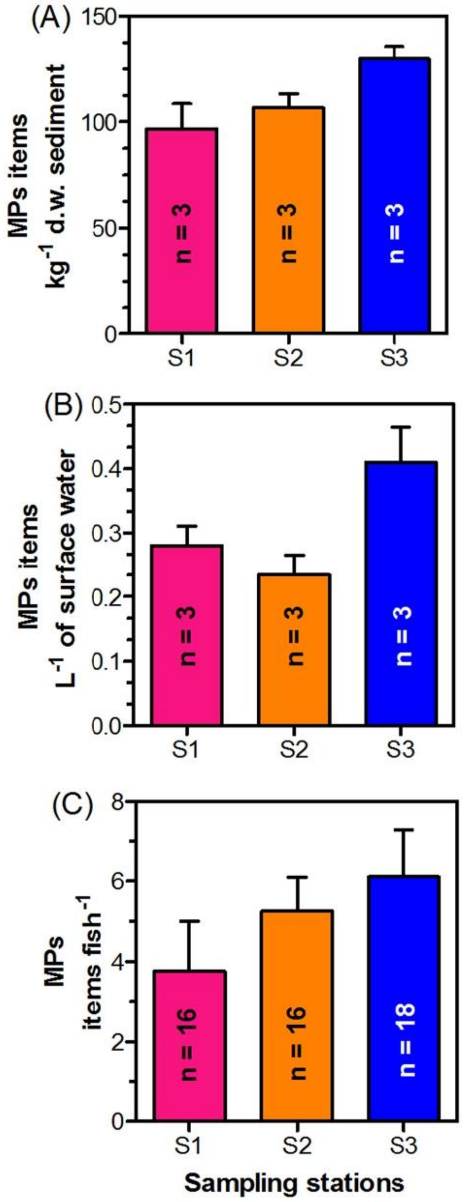

Fig. 2. Microplastics (MPs) abundance (mean ± SE) in (A) sediment, (B) water and (C) fish collected from the Ulhas River estuary at three sampling stations (S1 = Sarang, S2 = Alimghar and S3 = Kalher). Value within each bar indicates number of replicates.

estuarine environments that have a higher plastic retention potential than coastal areas (Martin et al., 2019; Maghsodian et al., 2020; Naidoo et al., 2020). Plastic microparticles entering this mangrove ecosystem may remain there, and could readily be passed to mudskippers through their food from water and mud either directly or indirectly (Farrell and Nelson, 2013; Naidoo et al., 2016).

3.2. Microplastic morphotypes in sediments, water and fish

Microplastics collected from all three sampling locations were categorized into the following types: fragments, pellets/beads, film, foam, and line/fibres/filaments (Fig. 3A–D). Fragments were the most common in sediment and water (Fig. 3E, F). The percent contribution of each morphotype (maximum to minimum) in sediment was: fragments (70.80 ± 2.16%), pellets (15.04 ± 1.13%), and filaments (14.16 ± 3.15%) (Fig. 3E). No foam or film particles were found in any sediment samples (Fig. 3E). The percent contribution of each morphotype (maximum to minimum) in water was: fragments (67.14 ± 4.19%), pellets (19.19 ± 3.62%), filaments (7.87 ± 0.30%), films (3.58 ± 0.49%), and foam (2.23 ± 0.75%) (Fig. 3F). Details of the site-specific contribution of each morphotype are provided in Supplement 2. The results of the present study showed that the numbers of plastic fragments in sediment and water from all study sites was highest, followed by pellets and filaments, which agrees with the study carried out by Maes et al. (2017), Karthik et al. (2018), Dowarah and Devipriya (2019) and Robin et al. (2020). Earlier investigations by Karthik et al. (2018), Goswami et al. (2020) and Robin et al. (2020) presented evidence for the sources of fragments in sediment and water including breakdown, disintegration and weathering of larger plastic pieces, transport of fragments along with water, discharge of effluent from sewage treatment plants and industrial processes and waste from fishing, cargo and sand mining activities. Since our study sites are situated in urban areas in close proximity to industrial hubs discharging large volumes of effluents from STPs and manufacturing activities, the abundance of fragments is entirely reasonable. In addition, the study sites targeted the downstream portion of the Ulhas River which flows through the suburban area and carries an immense amount of plastic and plastic items that are likely to be trapped in the mangrove area (Martin et al., 2019; Maghsodian et al., 2020; Naidoo et al., 2020) where they are subject to weathering, breakdown and disintegration, resulting in large numbers of fragments in sediment and water.

In the present study, the category of pellets/beads and filaments were next in abundance after fragments in sediment and water samples. A large number of plastic beads of different sizes, shapes and appearances (transparent to translucent; Fig. 3B) were observed at all sampling stations, especially at the S3 site. Pellets and beads are considered the primary plastic raw materials for the preparation of plastic items commonly used in industry, ingredient of personal care items such as shower gels, face scrubbers, and face packs (Fendall and Sewell, 2009; Eerkes-Medrano et al., 2015; Karthik et al., 2018; Goswami et al., 2020). In addition, certain everyday personal care items such as shower gels, face scrubbers, and face packs contain pellets or microbeads that contribute to their occurrence in coastal waters (Fendall and Sewell, 2009; Eerkes-Medrano et al., 2015; Goswami et al., 2020). The study sites are close to Thane City, which has a large population [clothes washing has been established as a major source of plastic fibre; (Browne et al., 2011)] and a number of factories producing plastic items; therefore, a higher concentration of pellets and filaments in water and sediment is not unexpected.

Surprisingly, only filaments (100%) were detected in the fish gut (Fig. 3G). The shortest filament was 0.7 mm long, while the longest was 11.3 mm [average length of the plastic filaments (n = 103) ingested = 3.3 ± 0.22 mm]. Several fish guts contained 15 or more microplastic filaments. Although similar findings on the ingestion of only filaments by marine and estuarine fish species have been reported in earlier investigations (Ramos et al., 2012; Ferreira et al., 2016; Mizraji et al., 2017; Sun et al., 2017; Vendel et al., 2017; Dai et al., 2018; Ferreira et al., 2018; Halstead et al., 2018; Jabeen et al., 2018; Zhao et al., 2018; Wang et al., 2019; Naidoo et al., 2020; Sathish et al., 2020), this study provides the first empirical evidence (for details refer Section 3.5) on

Table 1
Comparative analysis of the number of fish species studied, the number of fish examined, and the percentage of microplastics (MPs) found in the guts of fish around the world.

Study location	No. of fish species studied	No. of fish analysed	% fish with MPs	Reference
North Pacific Gyre, Northern Pacific Ocean	6	670	35%	Boerger et al., 2010
Goiana Estuary, Brazil	3	182	23%	Possatto et al., 2011
English Channel, UK	10	504	36.5%	Lusher et al., 2013
North Sea, Atlantic Ocean	7	1203	2.6%	Foekema et al., 2013
Coast of Portugal, Portugal	26	263	19.8%	Neves et al., 2015
Paotere Fish Market, Indonesia	11	76	27.63%	Rochman et al., 2015
Fish market in Half Moon Bay and Princeton, California, USA	12	64	25%	Rochman et al., 2015
Brazos River Basin, Central Texas, USA	2	436	45%	Peters and Bratton, 2016
Urban port in South Africa	1	70	73%	Naidoo et al., 2016
Texas Gulf Coast, Texas	6	1381	42.4%	Peters et al., 2017
Tamil Nadu coast, India	5	79	10.1%	Karthik et al., 2018
Amazon estuary, Brazil	46	189	13.7%	Pegado et al., 2018
Kerala coast, India	23	70	21.40%	Robin et al., 2020
Mangrove of KwaZulu-Natal, South Africa	4	174	52%	Naidoo et al., 2020
Ulhas River Estuary, Mumbai, India	1	50	74%	Present study

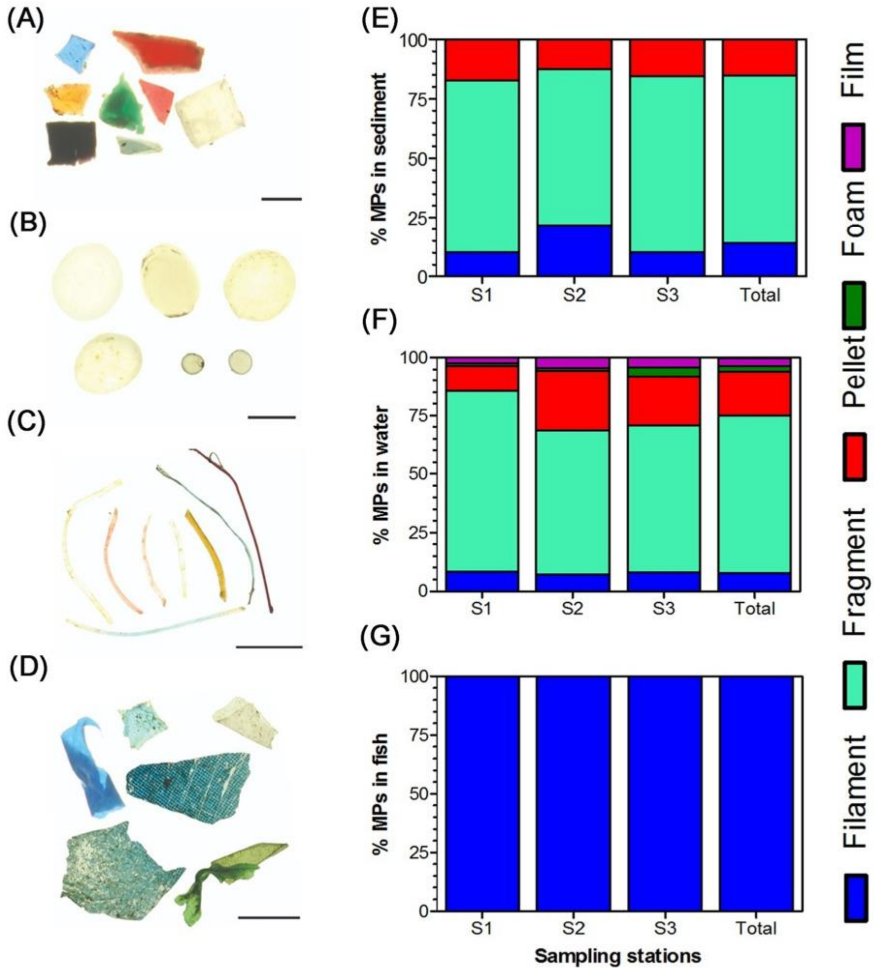

Fig. 3. Microplastic morphotypes: fragments (A), beads (B), filaments (C) and film (D) and their distribution (%) in the sediment (E), water (F) and fish (G) collected from the Ulhas River estuary at three sampling stations (S1 = Sarang, S2 = Alimghar and S3 = Kalher), and in total. (Scale bar = 1 mm).

how selective feeding habits and eco-morphological adaptations in the oral structures (Yang et al., 2003; Wu et al., 2009; Ravi, 2013; Mizraji et al., 2017; Sathish et al., 2020; Tran et al., 2020) play a critical role in microplastic ingestion in species of the genus, *Boleopthalmus*. The species of this genus have a dual lifestyle and are thus vulnerable to microplastic filament pollution from both terrestrial and surface water sources.

3.3. Colour-based distribution of microplastics in sediments, water and fish

The percent contribution of particles of each colour to the total (maximum to minimum) in sediment was: white (24.41 ± 4.39%), red (22.39 ± 3.71%), blue (21.03 ± 0.35%), green (16.83 ± 1.25%), and other (10.17 ± 1.13%) (Fig. 4A). The percent contribution of particles of each colour to the total in water was: white (39.84 ± 9.48%), blue (20.51 ± 0.96%), red (16.80 ± 4.38%), green (14.55 ± 2.38%), and other (6.70 ± 2.46%) (Fig. 4B). The percent contribution of each coloured microplastics other than blue in fish was black (23.23 ± 2.80), red (1.97 ± 1.68), green (0.79 ± 0.65) and white (0.39 ± 0.32) (Fig. 4C). Data on the numbers of site-specific plastic particles according to colour are provided in Supplement 3. Coloured microplastics have a

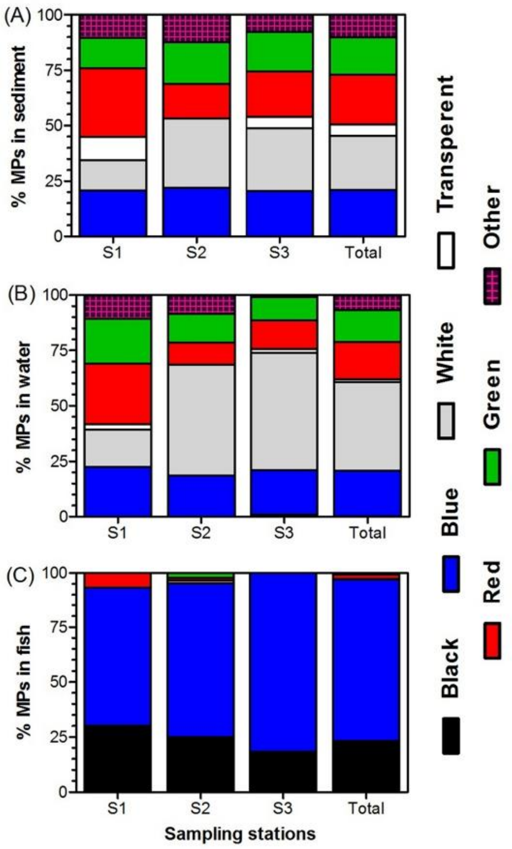

Fig. 4. Colour-based distribution (%) of microplastics (MPs) in (A) sediment, (B) water and (C) fish collected from the Ulhas River estuary at three sampling stations (S1 = Sarang, S2 = Alimghar and S3 = Kalher), and in total.

wide range of uses, including primary materials for household packaging, medical goods, dairy products, toys, garments, *etc.* (Robin et al., 2020) and thus their occurrence in sediment and water is not surprising. Despite the greater numbers of white microplastic particles in sediments and water, blue microplastics (73.62 ± 4.40%) were most common in fish (Fig. 4C) at all three collection sites. The colour of microplastic particles is known to play a significant role in the dietary preferences of fish that use visual cues in feeding (Ory et al., 2017; Naidoo et al., 2020). For instance, Ory et al. (2017) found that amberstripe scad, *Decapterus muroadsi* (Temminck and Schlegel, 1844), primarily ingest blue microplastic particles because they look similar to their copepod prey. Likewise, estuarine fish species such as the Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852), the Jarbua terapon, *Terapon jarbua* (Forsskål, 1775), the Malabar glassy perchlet, *Ambassis dussumieri* Cuvier, 1828 and the flathead grey mullet, *Mugil cephalus* Linnaeus, 1758, are also known to feed primarily on blue filaments that resemble copepods and other prey animals (Carpenter et al., 1972; Wright et al., 2013; Karthik et al., 2018; Naidoo et al., 2020). The ingestion of only filaments by mudskippers, predominantly blue followed by black, red and green, may be related to their dietary preference for diatoms and algae, but further feeding trials and laboratory experiments with consideration of various colour preferences for prey are needed before a conclusion can be made.

3.4. Characterization of microplastic polymers using FTIR

Determination of the microplastic chemical composition is of vital importance as it can provide valuable information on the probable source of the plastic pollution (Galgani et al., 2015; Ballent et al., 2016; Robin et al., 2020). Ninety-six morphologically distinct microplastic particles (based on shape and colour), 31 from sediment, 39 from water and 26 from fish, were analysed to identify common polymers in a representative subset of particles recovered from the three sites. Details of the microplastic polymer composition for each site are provided in Supplement 4. FTIR analysis of microplastics from sediment revealed the following numbers of polymer types as a percent of the total: polypropylene (29.03%), Surlyn ionomer (22.6%), low-density polyethylene (LDPE; 12.9%), polyethylene terephthalate (9.68%), cellulose (9.68%) and polybutene (6.44%) (Fig. 5A, D). FTIR analysis of microplastics in water revealed the following polymers: Surlyn ionomer (28.2%), polypropylene and polyethylene terephthalate (18.0% each), low-density polyethylene (12.8%) and cellulose (7.69%) (Fig. 5B, D). Minor polymer forms identified in sediment and water included polybutylene terephthalate, polybutylene and gum arabic. FTIR analysis of microplastics from fish gut showed only two polymers: low-density polyethylene (65.38%) and cellulose (34.62%) (Fig. 5C, D).

Most of the polymers identified in this study are commonly used in the manufacturing of plastic products (Sruthy and Ramasamy, 2017; Karthik et al., 2018; Goswami et al., 2020; Maghsodian et al., 2020; Robin et al., 2020), which confirms the anthropogenic link between plastic pollution and industrial activities such as textile and plastic manufacturing in the vicinity of the study sites. Polypropylene and Surlyn ionomer, for instance, are used for food packaging, manufacture of medical appliances and equipment, fishing tackle, plastic pipes, automotive and electrical parts, housewares, furniture, toys, luggage, clothing and sports equipment (Sruthy and Ramasamy, 2017; Karthik et al., 2018; Goswami et al., 2020; Maghsodian et al., 2020; Robin et al., 2020). Low-density polyethylene (LDPE) is used to make fishing tackle (Lusher et al., 2013, 2015; Sruthy and Ramasamy, 2017; Karlsson et al., 2017; Syakti et al., 2018; Dowarah and Devipriya, 2019). Polyethylene terephthalate (PET) is the best choice for manufacturing of flexible materials such as films, fabrics and plastic beverage bottles (Krishnan and Kulkarni, 2008; McKeen, 2013; Lim, 2017; Koshti et al., 2018). Polybutylene terephthalate (PBT) is commonly used for insulating electrical wires, in making keyboards, and for showerheads, toothbrushes, false eyelashes (Krishnan and Kulkarni, 2008; Lim, 2017). Polybutene

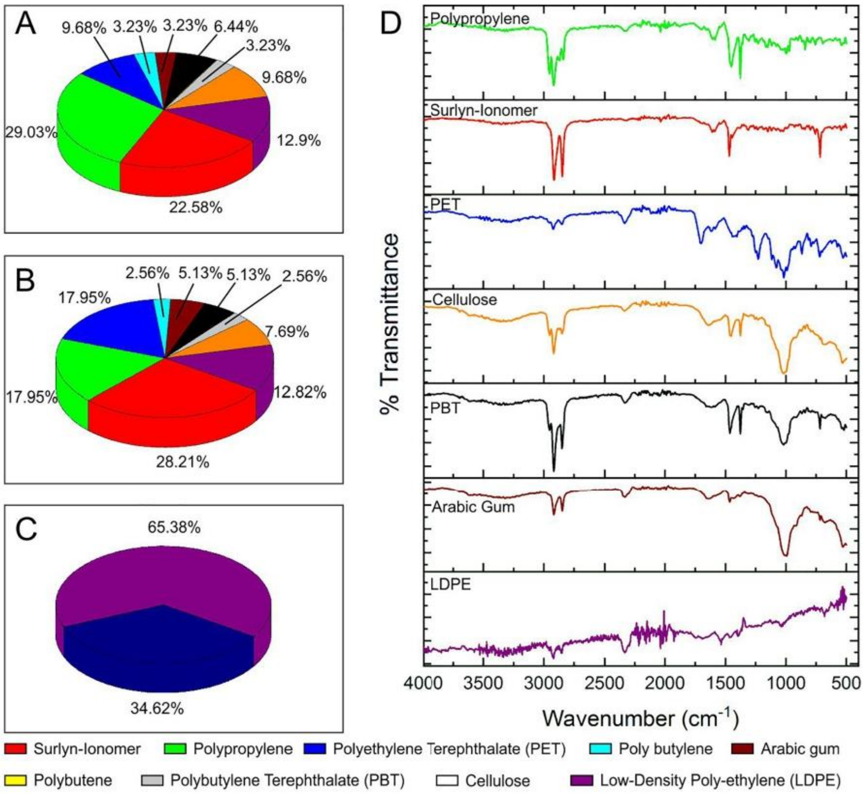

Fig. 5. Pie chart showing distribution of different types of polymers in (A) sediment, (B) water and (C) fish collected from the Ulhas River estuary. (D) Representative spectra of the different types of polymers described. Each colour represents one type of spectrum. The colour codes are at the bottom left of the diagram.

is used in the cosmetics and beauty products sector (Lochhead, 2007; Niederer et al., 2016; Chuberre et al., 2019) while polybutylene is mostly used for the manufacture of water supply lines and plumbing pipes and parts (Walsh, 2011). Apart from LDPE (65.4%), the significant level of non-plastic cellulose fibres (34.6%) in the fish intestine appears reasonable, as the study sites are near textile and dyeing plants. Earlier investigations have demonstrated that large quantities of microfibres [1900 microfibres in wastewater from washing a single garment in a domestic washing machine; Browne et al., 2011] are discharged by the apparel and home textile industries that can have a detrimental effect on the environment (Henry et al., 2019; Savoca et al., 2019). Cellulose *per se* is not an environmental hazard, but cellulose-based synthetic fibres such as rayon and cellulose-associated dyes or additives (Direct Blue 20 and Direct Red 28) have been identified as potent carcinogens and mutagens that adversely affect the reproduction of mammals and fish (Remy et al., 2015). Research has clearly shown that animals with the ability to digest cellulose are more prone to adverse effects from cellulose-associated dyes and additives (Remy et al., 2015; Hermsen et al., 2017). In the present study, we did not quantify the types and concentration of dyes associated with cellulose, but the fibres ingested by mudskippers showed a negative correlation with life-cycle traits (lower stomach fullness index and hepatosomatic index), which may support earlier studies showing a negative impact of microfibres on fish (Henry et al., 2019; Savoca et al., 2019).

3.5. Effects of mudskipper oral structures on microplastic ingestion

Out of all the morphotypes of microplastics identified in mud and water, only fibres were detected in the gut of the mudskipper indicating that the morpho-functional characteristics of its mouth and jaw (Fig. 6A–C) effectively filtered out the other types of microplastics. In a large number of specimens of mudskippers we observed large filaments trapped in the pharyngeal region (Fig. 6B, C) as this region allows only particles of a certain size to pass. Members of the Gobiidae: Oxudercinae sub-family in the *Boleopthalmus* genus are well known for their distinctive oral morphology, specialized dentition, skeletal elements, pharyngeal apparatus and well-developed musculature that support their unique filter-feeding habit (Murdy, 1989; Clayton and Snowden, 2000; Polgar et al., 2017; Tran et al., 2020). Mudskippers exhibit unique feeding behaviour with side-to-side head movements as they graze on diatoms on the surface of mudflats (Clayton and Snowden, 2000; Polgar et al., 2017; Tran et al., 2020). An earlier study by Tran et al. (2020) showed that the feeding apparatus of the mudskipper was adapted to collecting and transporting only small food particles like diatoms from the mudflats. They stated that the specialized anatomical configuration of the teeth on the pharyngeal plate promoted the efficient passage of the diatoms caught on the gill-rakers into the oesophagus. The inability of microplastic fragments, pellets, foam and film to enter the digestive tract may be due to their relatively large size, which causes them to be filtered out before they reach the digestive

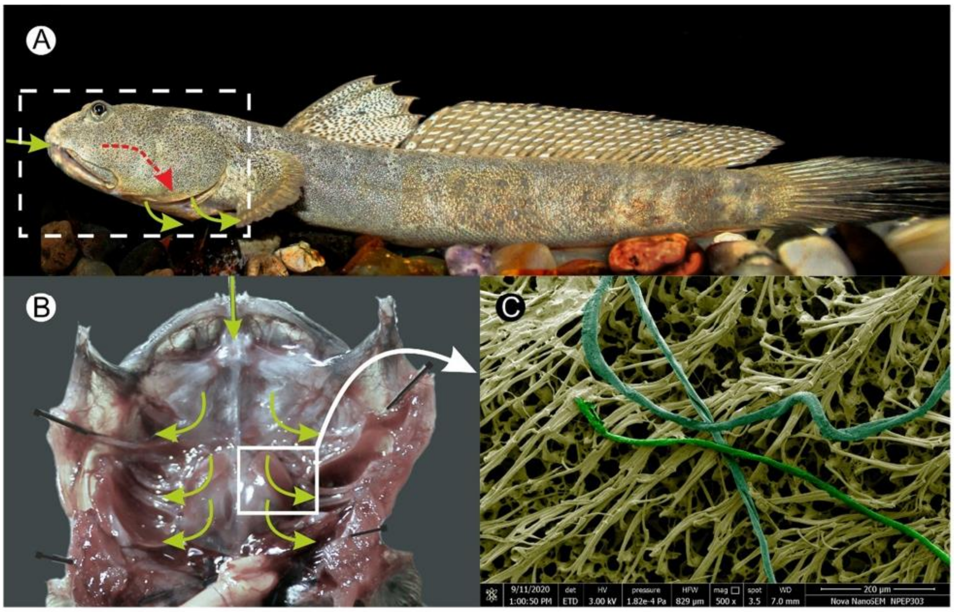

Fig. 6. Morphology and anatomy of oral structures in mudskipper. (A) Lateral view showing large mouth and operculum. (B) Floor of the oropharyngeal cavity with gill arches and pharyngeal apparatus which together act as a sieve for filter feeding. (C). Enlarged view of the pharyngeal plate with microplastic filaments Arrow indicates pathway for straining mud from water.

tract. The elongated shape, flexibility and small diameter of the plastic fibres allow them to be retained and transported into the digestive tract. Our observation that only microplastic fibres are found in the gut of mudskippers is in agreement with earlier investigations on Walton's mudskipper, *Periophthalmus waltoni* Koumans, 1941 (Maghsodian et al., 2020). However, the present study offers the first empirical evidence in the form of SEM (Fig. 6C) that most microplastic morphotypes are trapped in the pharyngeal apparatus so that only the filamentous forms pass into the fish intestine. This also proves that feeding behaviour plays a substantial role in determining the types of microplastic ingested by fish. Still, the occurrence of microplastics in the gut of mudskippers is of concern, because they could enter the food chain and be consumed by humans, because *B. dussumieri* is one of the most common estuarine fish caught by artisanal fishers and consumed by the urban population in study area (Mahadevan et al., 2020).

3.6. Interaction of microplastics with mudskipper life-history traits

No significant correlation was seen between microplastics in fish and sediment (Pearson $r = 0.927$, $r^2 = 0.861$, $p = 0.243$) and water (Pearson $r = 0.592$, $r^2 = 0.351$, $p = 0.596$). Furthermore, there was no clear relationship between the microplastic fibres ingested by fish and the colour of microplastics in sediment and water (for all colours $p > 0.05$). However, there was a significant negative correlation between the abundance of microplastics in fish and the condition factor (Pearson $r = -0.373$, 95% CI $= -0.5903$ to -0.1058, $r^2 = 0.139$, $p = 0.007$; Fig. 7A) suggesting that the microplastics in the gut likely

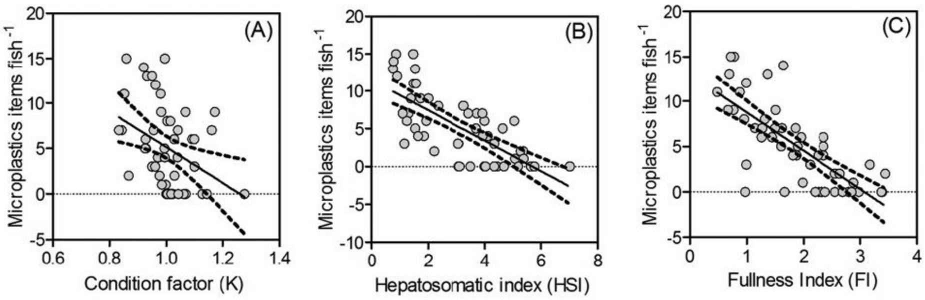

Fig. 7. Negative linear relationship between microplastics in *Boleophthalmus dussumieri* and the condition factor (A), hepatosomatic index (B) and fullness index (C). The 95% confidence interval is indicated by the black dashed lines.

to pose a negative impact on the health of fish. We also found a significant negative association between the abundance of microplastics in mudskippers and the hepato-somatic index, HSI, (Pearson $r = -0.728$, 95% CI $= -0.8372$ to -0.5648, $r^2 = 0.531$, $p < 0.0001$; Fig. 7B) and FI (Pearson $r = -0.745$, 95% CI $= -0.8478$ to -0.5892, $r^2 = 0.555$, $p < 0.0001$; Fig. 7C). The negative effects of microplastic ingestion on body condition have already been reported in the omnivorous fish, *Girella laevifrons* (Tschudi, 1846) and the planktivorous fish, *Acanthochromis polyacanthus* (Bleeker, 1855) (Mizraji et al., 2017; Critchell and Hoogenboom, 2018). On the contrary, a few earlier investigations found no correlation between microplastic ingestion by fish and body condition (Foekema et al., 2013; Morgana et al., 2018; de Vries et al., 2020). This may be due to the low microplastic load (one particle per fish or less), as Müller et al. (2020) demonstrated in laboratory

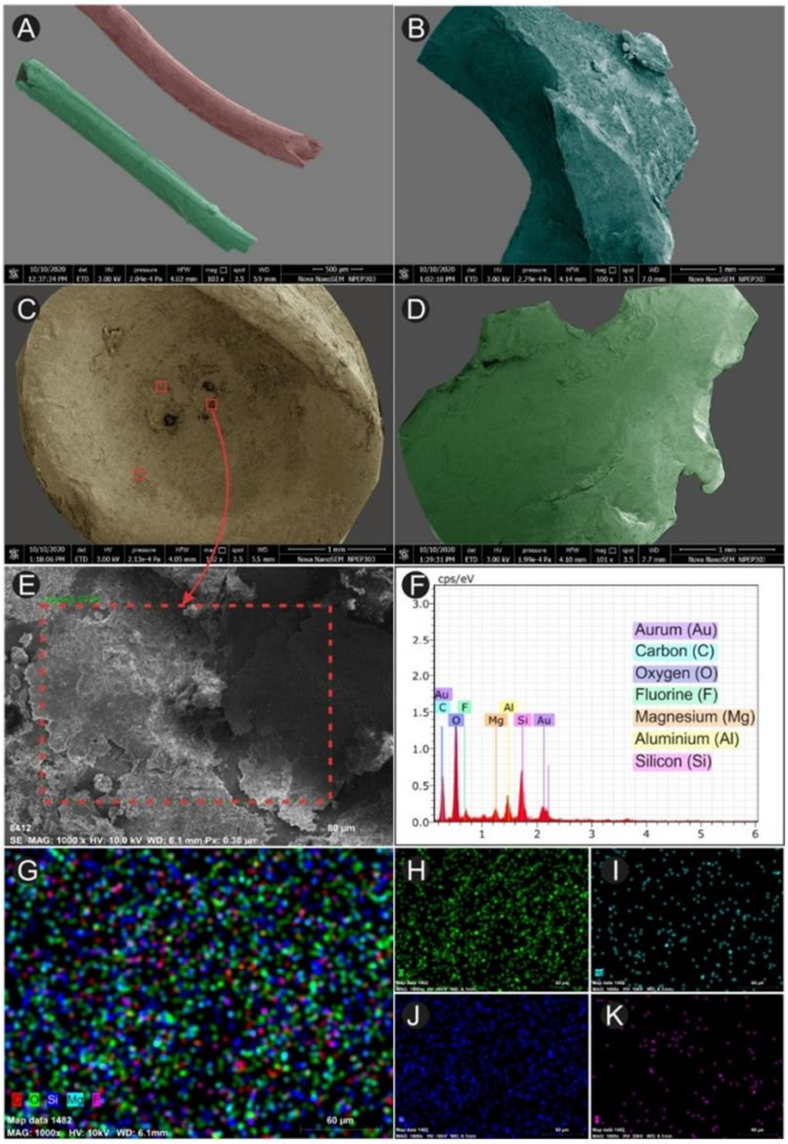

Fig. 8. Surface topography and elemental analysis of microplastics using FESEM-EDS. (A) Filaments showing relatively smooth, homogeneous topography compared to fragments (B), beads (C) and foam (D) with a slightly rougher surface topography but with very few pits, cracks, flakes and particles (E). (F) Representative image showing elemental analysis. (G) Representative image showing the distribution of elements on the microplastics pellet: (H) oxygen, (I) silicon, (J) magnesium, and (K) fluorine.

experiments on white seabream, *Diplodus sargus* (Linnaeus, 1758), that only high levels of microplastic ingestion can affect growth and condition. In the present study, 25 fish (50% of total specimens collected) had more than four microplastic particles each and eight fish had more than ten particles each, which is ten times greater than what was previously reported and may well have an adverse effect on condition, HSI and FI. Ingested microplastics are known to have a range of lethal or sub-lethal effects on the health of marine organisms, such as reduced growth, intestinal blockage, false sense of satiation, hepatic stress, physical injury and reproductive impairment (Browne et al., 2008; Rochman et al., 2013; Wright et al., 2013; Clark et al., 2016; Sussarelle et al., 2016; Banaee et al., 2020; Romano et al., 2020). In the digestive tract of several mudskippers, we found 15 fibres with a length of up to 11.4 mm, which could obstruct their intestines, produce a feeling of satiety and prevent feeding. However, more research and laboratory experiments, similar to those conducted by Müller et al. (2020), are needed to determine the extent of microplastics in fish guts and their effects on life-history traits.

3.7. Surface topography, elemental composition and mapping

FESEM analysis of representative microplastic morphotypes showed that filaments had a relatively smooth and homogeneous topography (Fig. 8A) compared to fragments (Fig. 8B), beads (Fig. 8C) and foam (Fig. 8D), which have a slightly rougher surface topography but with few pits, cracks, flakes and particles attached to their surface (Fig. 8E). Among the small number of microplastic morphotypes studied in this report, the surface elemental analysis showed presence of carbon (C), oxygen (O), fluorine (F), magnesium (Mg), aluminium (Al) and silicon (Si) on their surface (Fig. 8F). Details of the percent occurrence (weight and atomic %) of each element on a specific plastic morphotype are given in Supplement 5. Microplastics can act as carriers for heavy metals in the marine environment (Brennecke et al., 2016) and surface topography and weathering have been reported to affect adsorption of heavy metals onto the plastic surface (Kowalski et al., 2016; Wang et al., 2017; Aghilinasrollahabadi et al., 2020). Earlier investigations by Fernandes and Nayak (2012) and Raut et al. (2019) confirmed the presence of heavy metals such as lead (Pb), nickel (Ni), chromium (Cr), copper (Cu), zinc (Zn), mercury (Hg) and cadmium (Cd) in the Ulhas River estuary. In the present analysis, however, no heavy metals were found on any of the microplastic morphotypes examined. Elemental mapping clearly demonstrated an even distribution of the elements detected on the surface of microplastics (Fig. 8G–K), which can only result from microplastics' homogeneous surface topography, reducing heavy metal adsorption. If the examined particles were weathered and had heterogeneous surface topography, the detected elements would have an irregular, patchy distribution, increasing the chances of heavy metal adsorption. However, it does not fully rule out the possibility of heavy metal adsorption on microplastics in the Ulhas River estuary, therefore further research involving more microplastic particles at different weathering stages, as well as a thorough elemental determination in water using sensitive analytical techniques such as ICP-MS (inductively coupled plasma mass spectrometry), is needed.

4. Conclusions and implications for human health, the ecosystem and fish conservation

The abundance of microplastics in the estuarine ecosystem and microplastic contamination of fish is an indicator of an unhealthy ecosystem that may have a detrimental effect on human health. The negative impact of microplastics on the condition factor and direct communication with fishers living in the study region indicates that mudskipper populations are adversely affected by anthropogenic activities such as release of industrial effluents and untreated wastewater containing plastic microparticles. Mudskippers are a high-priced and high-demand fish in local markets in Mumbai and are a part of the diet of thousands of tribal people (Mahadevan et al., 2019, 2020). The occurrence of microplastics in the mudskippers' intestines and their ability to translocate to various organs (Karami et al., 2017; Abbasi et al., 2018; Elizalde-Velázquez et al., 2020; Prata et al., 2020) are likely to pose a health threat to dependent tribal and urban populations. It is therefore important to focus on implementing effective management strategies to protect fragile mangrove ecosystems and their fauna, so that the indigenous peoples as well as the urban population can continue to enjoy eating fish from unpolluted habitats that are clean and safe.

CRediT authorship contribution statement

Pradeep Kumkar: Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing – original draft. **Sachin M. Gosavi:** Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing – original draft. **Chandani R. Verma:** Methodology, Investigation, Formal analysis, Validation, Writing – review & editing. **Manoj Pise:** Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing – original draft. **Lukáš Kalous:** Conceptualization, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147445.

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5.4. Effect of diethyl phthalate on predator–prey chemo-ecology in *Lepidocephalichthys thermalis*

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Effect of diethyl phthalate on predator–prey chemo-ecology in *Lepidocephalichthys thermalis*

Chandani R. Verma · Sachin M. Gosavi · Manoj Pise · Lukáš Kalous · Pradeep Kumkar

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Abstract Phthalates, an additive used in the plastics manufacturing industries, are also common pollutants in the aquatic environment. However, little is known about how phthalates affect chemo-recognition in fish controlling vital ecological decisions such as predator–prey cognition. Therefore, we investigated the effect of diethyl phthalate (DEP) on predator recognition mechanism using the common spiny loach, *Lepidocephalichthys thermalis*, as a model prey fish and dwarf snakehead, *Channa gachua,* as sympatric native predator. We also performed paired choice predation assay to evaluate if DEP exposure impacts prey survival in an actual predation event. Short-term exposure (STE) or Long-term exposure (LTE) to DEP followed by assessment of anti-predator responses using ethological assay clearly shows that DEP impairs innate predator recognition in common spiny loach. DEP-exposed prey fish did not recognize sympatric predator kairomones, alarm signals from conspecifics, and dietary cues from fed predators. The inability of DEP-exposed *L. thermalis* to distinguish conspecific alarm signals will likely to have an additional impact on associative learning, putting it at risk of predation by non-native predators. Anti-predatory responses of prey fish in LTE treatment groups were found to be more impacted than the STE treatment group. Furthermore, actual predation studies demonstrated that DEP-exposed *L. thermalis* had a lower survival rate than unexposed fishes, confirming that DEP exposure decrease survival in prey fish. Given the critical importance of chemically-mediated anti-predator strategies and associative learning ability to counter both native and non-native predators, ability of plasticizers to impose anti-predator behavioral suppression and reduce survival is likely to have long-term detrimental effects on fish fitness.

Keywords Cobitidae · Plasticizers · Fisheries · Info-disruption · Cognition · Chemo-ecology

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C. R. Verma · M. Pise · L. Kalous · P. Kumkar
Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

S. M. Gosavi (✉)
Department of Zoology, Science and Commerce, Maharashtra College of Arts, 246-A, Jehangir Boman Behram Marg, Mumbai, Maharashtra 400008, India
e-mail: schn.gosavi@gmail.com

Introduction

Aquatic animals, such as fishes, larval amphibians, and invertebrates communicate primarily by visual and/or chemical cues (Ferrari et al. 2010; Mogali et al. 2011; Batabyal et al. 2014; Polo-Cavia and Gomez-Mestre 2014). Due to various limitations such as turbidity, lack of sunlight, and the short detection range associated with the use of visual signals by

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aquatic animals, prey animals evolved to rely predominantly on chemical signals commonly referred to as "chemical cues" (Ferrari et al. 2010; Mogali et al. 2012; Chivers et al. 2013). Chemical signals allow aquatic animals to differentiate between conspecifics, recognize predators, and are vital in associative learning to cope with invading predators (Wisenden 2000; Ferrari et al. 2010; Tapkir et al. 2017). Furthermore, they also help to detect potential mates, nearby food supplies, establish dominance, and defend their territory (Lima and Dill 1990; Wisenden 2000; Ferrari et al. 2010; Seuront 2018; Maximino et al. 2019). As a result, species that fail to perceive and respond to such chemo-signals may jeopardize their survival and fitness (Ferrari and Chivers 2011; Lahman et al. 2015; Tapkir et al. 2019). Hence, the ecological significance of correctly identifying the predators based on chemical cues is critical.

Chemical contaminants are ubiquitous in aquatic bodies and are well-known to impair chemosensory abilities and disruption of the chemical cues in aquatic animals (Tierney et al. 2010; Moore et al. 2015; Tapkir et al. 2019; Gosavi et al. 2020). For instance, water body contaminated with herbicide glyphosate is capable of inactivating conspecific alarm signals in larval amphibians and fish (Moore et al. 2015; Tapkir et al. 2019). Similarly, the negative effect of copper on the chemically mediated behavioral responses of several species of fishes such as *Ptychocheilus lucius*, pike minnow; *Oncorhynchus kisutch,* coho salmon; *Oncorhynchus* keta, chum salmon; *Carassius auratus*, goldfish; *Oncorhynchus tshawytscha*, Chinook salmon; *Danio rerio*, zebra fish and *Lepidocephalichthys thermalis*, common spiny loach is well-documented (Tierney et al. 2010; McIntyre et al. 2012; Sovová et al. 2014; Gosavi et al. 2020). Furthermore, several earlier investigations have conclusively proven that aquatic prey animals such as fishes and anuran tadpoles exposed to chemical pollutants have impaired their anti-predator responses and are thus more vulnerable to predation (Tierney et al. 2010; McIntyre et al. 2012; Tapkir et al. 2019; Gosavi et al. 2020). As a result, any chemical contaminant that compromises predator detection in prey species is potentially hazardous, and thus research into the harmful impact of chemical contamination on ecological processes is vital.

Plastic contamination is one of the most severe environmental issues. Approximately 8300 million metric tons of plastic are produced yearly (Geyer et al. 2017; Prieto Amador et al. 2021). Apart from raw plastic material, catalysts and other compounds, known as plasticizers, are often used in plastic production processes. Phthalates are a kind of plasticizer that is widely utilized in the plastic manufacturing process. According to Mackintosh et al. (2006) and Gao et al. (2018), global phthalate production has surpassed 6 million tons per year in the past decade. However, because phthalates are not covalently linked to the plastic polymer, they can readily leach into the environment, making them ubiquitous environmental pollutants (Shaha and Pandit 2020; Chakraborty et al. 2021; Prieto Amador et al. 2021). Among the phthalate group of chemicals, diethyl phthalate (DEP) is the one which has many industrial applications and is known to be a common contaminant of freshwater and marine ecosystems (Ghorpade et al. 2002; Chakraborty et al. 2021; Prieto-Amador et al. 2021; Kumkar et al. 2022). The detrimental effects of phthalates, including DEP, on aquatic ecosystems and resident biota, have been well-documented (Ghorpade et al. 2002; Kaplan et al. 2013; Shaha and Pandit 2020; PrietoAmador et al. 2021; Kumkar et al. 2022). Phthalates are known to act as endocrine disruptors, induce oxidative stress, alter development and growth patterns, shorten the lifespan of exposed aquatic animals, and even alter gene expressions (Ghorpade et al. 2002; Pradhan et al. 2018; Zhang et al. 2018; Shaha and Pandit 2020; Weaver et al. 2020; Prieto-Amador et al. 2021; Zhang et al. 2021; Kumkar et al. 2022). In addition to physiological, metabolic and molecular changes in exposed animals, phthalates have been linked to changes in fish behavior such as shoaling (Kaplan et al. 2013), which is a crucial phenomenon in predator avoidance (Wibe et al. 2002). Although phthalates are one of the most prevalent pollutants in numerous aquatic bodies, there is a complete lack of knowledge regarding how phthalates alter chemically mediated communication in fish and influence predator–prey interactions in aquatic species. Furthermore, phthalates are naturally lipophilic and have a tendency to bind to sedimentary particles, therefore, they are more likely to be detected at the bottom and make benthic organisms more vulnerable than pelagic ones (Woin and Larsson 1987; Verma et al. 2021; Kumkar et al. 2022). Therefore, a study on the effects of DEP on bottom dwelling aquatic organisms such as loaches is required. Studies examining the effects of anthropogenic stressors like DEP on loaches is important since bottom-dwelling loaches occupy lower trophic level in ecosystems, and anything that impacts their population would indeed negatively impact higher trophic level organisms. Furthermore, phthalates like DEP are known to induce stress in fish (Kumkar et al. 2022); it has not yet been established how this stress would affect species survival under actual predation circumstances.

Due to the wealth of information on predator–prey interaction and the ease of measuring ethological responses (activity measurement), fish could be considered an ideal choice for eco-toxicological studies, particularly DEP or phthalate-based investigations (Kumkar et al. 2022). The common spiny loach, *Lepidocephalichthys thermalis* (Valenciennes 1846), is well suited for eco-toxicological investigations due to the availability of basic information on the predator–prey interaction, such as innate predator recognition, the ability to learn about invasive predators, the types of chemical cues used for predator detection (Tapkir et al. 2017, 2019), high sensitivity of species towards pollutants (Gosavi et al. 2020), environmental sources and sub-lethal concentrations of DEP (Kumkar et al. 2022). Therefore, in the present study, we focused on how DEP influences predator–prey interactions (prey anti-predator tactics and survival), primarily based on detecting chemical signals by prey species. We used *L. thermalis*, a freshwater fish, in a series of ethological studies to investigate the following questions: (1) Which different chemical cues found in water does the common spiny loach utilize to recognize its predator; (2) Does short-term exposure (STE) or long-term exposure (LTE) to DEP impact *L. thermalis* innate ability to recognize their natural predators; and (3) Does DEP exposure impair *L. thermalis* ability to survive against their natural predators?

Materials and methods

Chemicals

Acetone, diethyl phthalate (DEP), Tricane methane sulfonate (MS-222) were procured from commercial suppliers (Merck, India). Chemicals were 99% pure (analytical grade) as per the manufacturers.

Prey and predator fish

Live individuals of *L. thermalis* (standard length: 55–65 mm) were purchased commercially at Lonawala (18.746°N; 73.449°E), Maharashtra, India, and transported to the laboratory in large plastic bags (25 individuals per bag) filled with 5 L of dechlorinated oxygenated water. Fish were kept in a large aquarium ($120 \times 60 \times 60$ cm) supplied with an aerator and filled with 20 L of dechlorinated water at 25 °C under a natural photoperiod (12L:12D). Twenty individuals of the sympatric native predator dwarf snakehead *Channa gachua* (standard length: 250–280 mm) were also collected from the same place and kept separately in the aquarium ($120 \times 60 \times 60$ cm) with 20 L of dechlorinated water. The identification of the gender of prey fish is limited by the absence of externally visible sexually dimorphic characteristics. However, to validate their sexual maturity, three of the smallest individuals used in the study were dissected, and their gonads were examined using a stereo microscope (Olympus SZ61). No instances of cannibalism were observed during the experiments. All aquaria had their water changed twice a week, and the fish were fed artificial fish food *ad-libitum*.

Chemical cues

Chemical cues (kairomones, alarm cues, and dietary cues) were prepared using the standard methodology described by Woody and Mathis (1998), with modifications following Tapkir et al. (2019). For kairomone preparation, the predatory snakehead (standard length: 255 mm) was starved for two days to clear its gut before being transported to a separate tank filled with 10 L of dechlorinated water. Kairomone was collected and frozen in 100 ml aliquots after 48 h of transfer. Dechlorinated water was used as a control, prepared using the same process but without placing the predatory fish in the aquarium (Woody and Mathis 1998; Tapkir et al. 2017). To prepare alarm cues, three *L. thermalis* were euthanized with MS-222 solution. The fish were washed thoroughly and quickly snap-frozen in liquid nitrogen to remove any traces of anesthesia. Small skin pieces were cut from the lateral side of the fish body and immediately immersed in 10 ml chilled distilled water. Tissue pieces were then homogenized in 50 mL of distilled water and filtered. The skin extract was then diluted to 10 L and stored at -20 °C in 100 mL aliquots until used in the experiment. To prepare the dietary cues, starved predatory fish was allowed to feed on the *L. thermalis* before being transported to a separate tank filled with 10 L of dechlorinated water. Dietary cues were collected and frozen in 100 ml aliquots after 48 h of transfer (Tapkir et al. 2017).

DEP- formulation and dosing

The lethal concentration (LC_{50}) value of DEP for the *L. thermalis* is 44.53 mg/L (Kumkar et al. 2022). Therefore, DEP concentration of 22.26 mg L^{-1}, which is less than half of the estimated LC_{50} was chosen for the present investigation. At this concentration, we observed no mortality of the *L. thermalis* during and after experimentation. Furthermore, according to Zheng et al. (2019), the highest concentration of DEP recorded in freshwater is 21 mg L^{-1}, which is very close to concentration used in present study. Acetone (0.004% v/v) was used as a solvent for DEP formulation. DEP stock solution (100 mg/L) was prepared in acetone and diluted to a final concentration of 22.26 mg L^{-1}.

Treatment and experiments

The identical dosage protocol was used to dose all of the fish used in the experiments. The details of the experimental design are provided in Fig. 1. The individuals of *L. thermalis* were divided into five treatment groups: D/W-control (Group-1), acetone-STE (Group-2; solvent control), DEP- STE (Group-3), acetone- LTE (Group-4; solvent control), and DEP- LTE (Group-5). The fish were then placed in a separate aquarium (120 × 60 × 60 cm) with 25 L of clean water and fitted with a stone aerator at a density of 4 individuals per liter. Following a 2 h acclimation period, 10 mL of chemical (DEP or acetone or distilled water) was introduced. The *L. thermalis* were given STE for 24 h and eight days for LTE treatment before being removed and used in behavioral trials.

Five treatment groups (Fig. 1) of *L. thermalis* allowed us to assess three different aspects. (1) Confirmation of innate predator recognition in *L. thermalis*: Group 1 (D/W-control) received only 10 mL of distilled water (D/W) and will act as a positive control, reconfirming the innate predator recognition in *L. thermalis* and the types of cues (kairomones, conspecific alarm cues and dietary cues) used by the species to identify their native-predator; (2) Response to acetone treatment: Groups 2 and 4 received only 10 mL of acetone (STE or LTE), serving as solvent controls and allowed us to evaluate the suitability of acetone as a vector for DEP delivery and whether acetone itself has any effect on predator recognition and the type of cues used by the *L. thermalis*; and (3) Response to DEP treatment: Groups 3 and 5 received

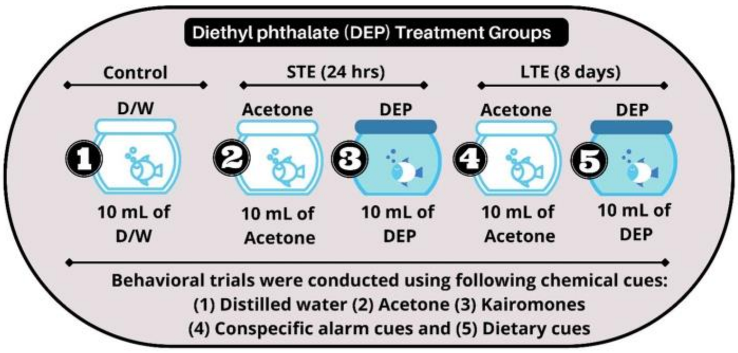

Fig. 1 Schematic illustration of experimental design depicting five different treatment groups, details of treatments and chemical cues utilized for post-treatment behavioral trials. (STE: Short-term exposure, LTE: Long-term exposure)

10 mL of DEP, which allowed us to determine whether DEP exposure (STE or LTE) has any effect on predator recognition ability and types of chemical cues used by the *L. thermalis*. Each treatment group was tested separately for ethological assay.

Ethological assay

Following treatment, we employed a well-established method of measuring activity to assess the effect of DEP exposure on prey fish anti-predator responses (Batabyal et al. 2014). Behavioral trials were carried out in a custom-designed glass testing chamber (100 L × 10 W × 12 H cm; see Fig. 2). We drew 9 lines on the bottom of the glass chamber using a black glass marker pen and divided it into 10 equal portions, each 10 cm long and 10 cm wide. The test chamber was cleaned with plenty of water before each trial to ensure no traces of cues from the previous trial remained and then filled with 2 L of clean water. One individual of *L. thermalis* was then introduced into the test chamber for 20 min to acclimate. The pre-stimulus activity was then quantified by counting the number of lines crossed during a 4-min interval. The test solution (stimulus: clean water or acetone or chemical cues; 5 ml) was carefully administered to each end of the glass tank using glass syringe without disturbing the prey fish. The diffusion time of the test solution is 5 min (estimated using the $KMnO_4$ dye diffusion method). The post-stimulus activity was then assessed for 4 min. The following formula was used to determine the change in activity: Change in activity = Post-stimulus activity—Pre-stimulus activity. The observer was about a meter away from the test chamber. The vertical one-way mirror glass was hung to provide a barrier between the observer and the test chamber to avoid the influence of the observer's presence on fish behavior. The observer was blind to the treatments, and the order of the treatments was randomized in all cases.

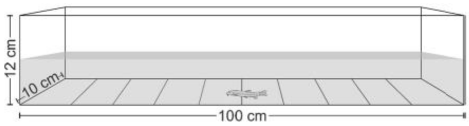

Fig. 2 Testing chamber used for the behavioral trials. Gray color indicates water level

Paired choice predation assay

This experiment was undertaken to see if the DEP-induced behavioral suppression to identify predators affects prey survival in actual predation scenarios. Paired choice predation assay was performed following the methodology given by Gosavi et al. (2020). A paired predation assay was performed between (1) the control group and the DEP-STE group and (2) the control group and the DEP-LTE treatment group. One individual of *L. thermalis* from each control, DEP-STE or DEP-LTE treatment groups was randomly paired and introduced to a separate aquarium containing 20 L of clean water and one 48 h starved dwarf snakehead (predatory fish). The time required the predator to kill or eat each *L. thermalis* individual was recorded. A total of 20 trials were performed in each case. Two observers simultaneously recorded the predation event to ensure the correct identification of the first individual killed/eaten. Each trial had a time limit of 30 min.

Statistical analysis

The data from each treatment group was tested for normality (Shapiro–Wilk test, $P > 0.05$) and homoscedasticity (Levene's tests) prior to analyses. The findings are reported as mean standard error (M ± SE), unless otherwise specified. Due to the non-normal distribution of data, each treatment group's activity change in response to various types of cues (clean water, acetone, kairomone, conspecific alarm cues, and dietary cues) was analyzed separately using the Kruskal–Wallis H-test followed by post-hoc Dunn's test. We were able to quantify the effect of D/W (control), acetone (solvent control), and DEP by analyzing each group independently (STE and LTE). An unpaired t-test was used to compare prey fish survival in real predation trials between control and short or long-term exposure to DEP. The results were only considered significant if the $P < 0.05$. Statistical analysis was performed using PAST freeware (version 3.14) (Hammer et al. 2001). In the figures, the colors light green and light orange represent STE and LTE treatment groups, respectively. Please refer to the online version of the article for color figures.

Results

Response to distilled water exposure

When *L. thermalis* were exposed to distilled water and tested using different chemical cues, a significant reduction in activity was observed (Kruskal–Wallis $H = 40.52$; $P < 0.0001$; Fig. 3A). The presence of kairomones (K), conspecific alarm cues (CC) and dietary cues (DC) of the native predator as a stimulus caused a significant decrease in the activity level of *L. thermalis* (Dunn's test, $P < 0.0001$; Fig. 3A). However, *L. thermalis* exposed to distilled water (control) did not show significant change in activity (Dunn's test, $P > 0.05$; Fig. 3A) when tested against clean water (W) and acetone (AT).

Response to acetone (solvent control) exposure

When *L. thermalis* were exposed to acetone and tested using different chemical cues, a significant change in activity was observed in both STE (Kruskal–Wallis $H = 42.15$; $P < 0.0001$; Fig. 3B)

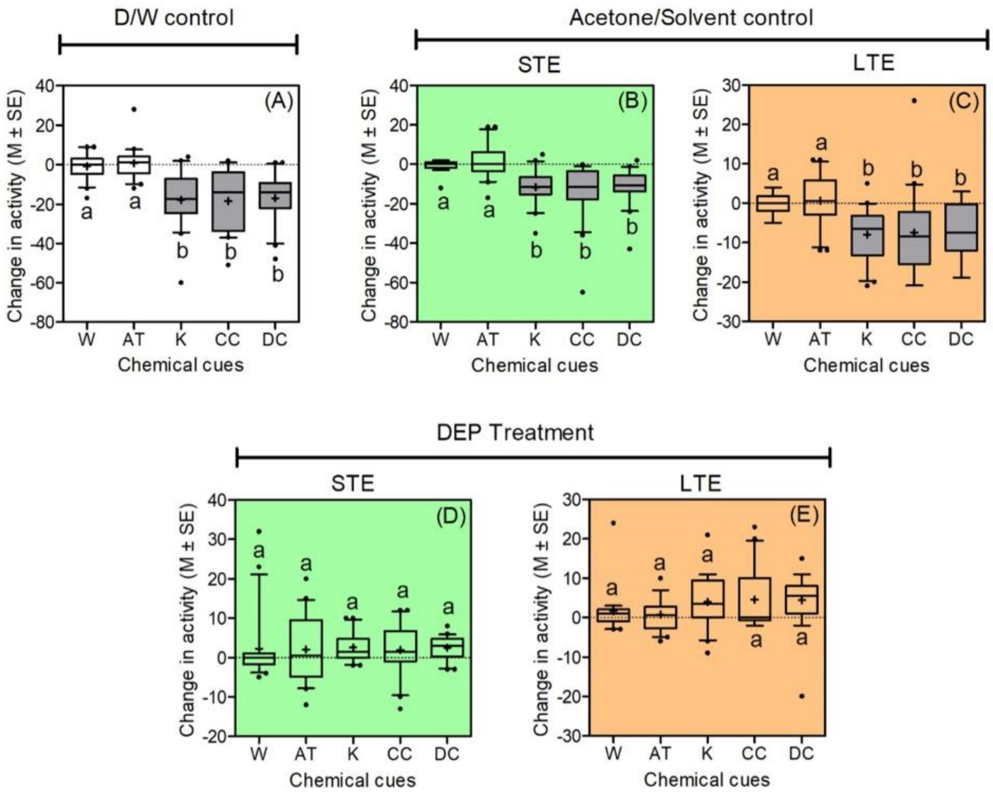

Fig. 3 Mean (± SE) change in activity level of the prey fish exposed to **A** distilled water (D/W) control; **B** acetone-STE; **C** acetone-LTE; **D** DEP-STE and, **E** DEP-LTE. Each treatment group was tested using five different test cues: Clean water (W), Acetone (AT), Kairomones from dwarf snakehead (K), Conspecific alarm cues (CC), and dietary cues (DC). Grey color box (panel A, B and C) indicates significant ($P < 0.05$) reduction in the activity level of prey fish. "+" symbol and horizontal line in each box indicate mean and median values respectively. Dissimilar letters above boxes indicate significant difference in the activity levels between tested cues. Outliers are indicated by black dots above or below the box in each panel. STE stands for Short-term exposure whereas, LTE stands for Long-term exposure

as well as LTE treatment groups (Kruskal–Wallis $H=28.79$; $P<0.0001$; Fig. 3C). The results of STE-acetone and LTE-acetone treatment group were comparable to those of distilled water exposure. Individuals of *L. thermalis* from both STE as well as LTE-acetone treatment groups did not show a significant change in activity when tested against clean water (W) and acetone (Dunn's test, $P>0.05$; Figs. 3B and C respectively). However, a significant reduction in activity was observed in *L. thermalis* individuals of the STE-acetone or LTE-acetone (AT) treatment groups which were tested using kairomones (K), conspecific alarm cues (CC) and dietary cues (DC) of the native predator (Dunn's test, $P<0.0001$; Figs. 3B and 3C respectively).

Response to DEP exposure

When *L. thermalis* were exposed to DEP and tested using different chemical cues, no significant difference in activity was observed in both STE (Kruskal–Wallis $H=4.768$; $P<0.3119$; Fig. 3D) as well as LTE (Kruskal–Wallis $H=8.606$; $P<0.0717$; Fig. 3E) treatments. Likewise, after DEP- STE or DEP-LTE, the individuals of *L. thermalis* did not show significant change in activity for any tested cues (Dunn's test, $P>0.05$; Figs. 3D and 3E). When we compared the response of *L. thermalis* to chemical cues (K, CC, and DC) between DEP-STE and DEP-LTE treatment groups, the DEP-LTE treatment group showed considerably greater impairment (Fig. 4).

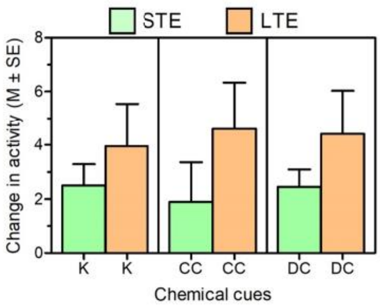

Fig. 4 Comparison of activity levels (Mean ± SE) of DEP treated prey fish between short-term exposure (STE) or Long-term exposure (LTE). Trials were performed using kairomones (K), Conspecific alarm cues (CC), and dietary cues (DC)

Prey survival between control and DEP treatment

The individuals of *L. thermalis* in the control group survived more against the predatory fish than in the DEP-STE treatment group ($t=2.378$; *d.f.* $=36$; $P=0.022$; Fig. 5A) or DEP-LTE treatment group ($t=2.690$; *d.f.* $=38$; $P=0.011$; Fig. 5B). The *L. thermalis* individuals either from STE or LTE treatment group showed reduced survival rates because they were often consumed early than the control ones (Figs. 5C and D). When compared to the STE-DEP treatment group (7.18 ± 0.43 min; Fig. 5E), the control *L. thermalis* individuals were able to tackle the predatory fish for a significantly ($t=3.473$; *d.f.* $=36$; $P=0.0014$) longer duration of time (14.32 ± 0.62 min; Fig. 5E). Likewise, the unexposed *L. thermalis* individuals were able to tackle the predatory fish for a significantly ($t=4.025$; *d.f.* $=38$; $P=0.0003$) longer time (15.31 ± 0.64 min) than the individuals from LTE-DEP treatment group (6.16 ± 0.47 min; Fig. 5F), confirming that DEP exposure has a detrimental effect on *L. thermalis* survival.

Discussion

The reduction in the activity level of the *L. thermalis* following exposure to distilled water clearly suggests that it recognizes and responds to the chemical cues emitted by the dwarf snakehead (predatory fish), validating the innate predation recognition toward native predators described in this species (Tapkir et al. 2017, 2019). The current study clearly shows that to assess the predatory threat and tackle native predator, the *L. thermalis* uses a variety of chemical cues, including kairomones from a predator, conspecific alarm cues, and conspecific dietary cues. The ecological significance of precisely recognizing predators based on a range of chemical cues has been proven advantageous for prey (Ferrari et al. 2010; Mcintyre et al. 2012; Polo-Cavia and Gomez-Mestre 2014; Tapkir et al. 2019; Gosavi et al. 2020). Similarly, when the individuals of *L. thermalis* were treated with solvent acetone, either for short time or long time (STE or LTE), their behavior did not alter, and exposed individuals still responded in the same way to various chemical stimuli. This clearly shows that acetone did not appear to influence the anti-predator responses of

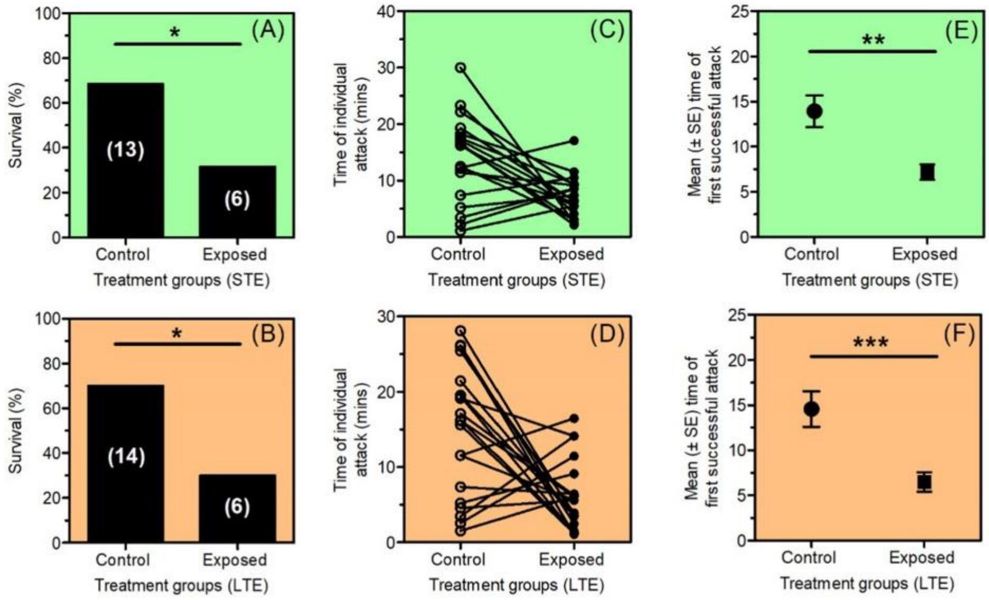

Fig. 5 (**A and B**) Survival (%) of control and short-term and long-term DEP exposed prey fishes in real predation trials with the dwarf snakehead, respectively; (**C and D**) Comparison of the time required for a successful attack between the control and exposed prey fish (from STE and LTE treatment groups) during the real predation trials; (**E and F**) Comparison between the times (Mean ± SE) required for the first successful attack between the control and exposed (STE and LTE treatment groups) prey individuals. Results were considered significant when * ($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$)

the *L. thermalis*. The suitability of the acetone as a vector for phthalates delivery has been already tested for common spiny loach (Kumkar et al. 2022) as well as other fish species, *Fundulus heteroclitus* (Kaplan et al. 2013), where stress physiology and shoaling behavior have been tested respectively.

Several previous studies have provided a plethora of evidence regarding the loss of chemosensory abilities in contaminant-exposed fishes, including phthalates, which has a negative impact on ecological decisions such as predator detection (Tierney et al. 2010; McIntyre et al. 2012; Kaplan et al. 2013; Sovová et al. 2014; Tapkir et al. 2019; Gosavi et al. 2020; Pilehvar et al. 2020). For example, the mummichog, *F. heteroclitus*, was exposed to benzyl butyl phthalate (BBP) at a concentration of 0.1 mg/L, which caused agitation and shoal choice disruption (Kaplan et al. 2013), putting the mummichog at danger of predation. The current study shows that *L. thermalis* treated with sub-acute DEP concentrations, either from STE or LTE group, exhibit anti-predator behavioral suppression and fail to recognize their native predator based on chemical signals (kairomone, conspecific alarm cues and conspecific dietary cues). Individuals of *L. thermalis* exposed to DEP (either STE or LTE) did not reduce their activity in response to any of the chemical signals tested in this investigation.

Earlier studies have demonstrated that prey animals with compromised anti-predator skills are more vulnerable to predation (McIntyre et al. 2012; Polo-Cavia and Gomez-Mestre 2014; Gosavi et al. 2020). Active prey fish are more accessible for predators to detect, as activity reduction is a well-known survival strategy against aquatic predators (Wisenden 2000; Hazlett 2003; Gonzalo et al. 2007; 2009; Ferrari et al. 2010; Batabyal et al. 2014). For example, juvenile coho salmon, *Oncorhynchus kisutch*, exposed to low-levels of copper (5–20 μg/L), fail to perceive predatory chemical cues and remain active, rendering them more vulnerable to cutthroat trout predators (McIntyre et al. 2012). In the present study, direct predation trials using DEP exposed and unexposed prey fish provide direct evidence that DEP treated *L. thermalis* individuals either from STE or LTE treatment groups face higher predation from the dwarf snakehead predator. In addition, DEP-exposed *L. thermalis* individuals have been shown to be consistently eaten before unexposed *L. thermalis* individuals and could only handle predators for significantly shorter periods of time, leading to higher mortality in predatory attacks. Such maladaptive anti-predatory responses and increased predation risk are anticipated to have long-term implications for prey species fitness (Ferrari et al. 2010; Ferrari and Chivers 2011; Lahman et al. 2015; Tapkir et al. 2019). Contaminants in aquatic ecosystems have a negative impact on predator–prey interactions via a variety of mechanisms. For instance, increased level of copper in aquatic ecosystems is known to damage the olfactory system of prey fish (Tierney et al. 2010), rendering prey species unresponsive to chemical signals and therefore increasing their vulnerability to predators (McIntyre et al. 2012). Glyphosate inactivates chemical signals from injured conspecifics in aquatic ecosystems, and drastically altering anuran tadpoles' ability to perceive chemical cues in their proximity (Moore et al. 2015). Similarly, using the common spiny loach, Gosavi et al. (2020) demonstrated that even copper in the aquatic ecosystem disrupt alarm cues, making prey species more vulnerable to predation. At the moment, determining the precise mode of action by which DEP acts on prey animals to diminish their anti-predator response is challenging, thus more research into the mechanism of action of phthalates on predator–prey chemical cues is required. However, Kumkar et al. (2022) found evidence that DEP exposure induces stress and liver damage in *L. thermalis*, which is likely the reason of the decrease in *L. thermalis* activity and their increased mortality in real predation experiments.

To counter alien predators, aquatic prey has developed a unique strategy in the form of associative learning, which they need to acquire during their lifetime when they receive mixtures of chemical cues (invasive predator kairomones + visual or conspecific alarm cues) (Brown and Chivers 2005; Ferrari et al. 2005; Gonzalo et al. 2007; Brown et al. 2011). Associative learning broadens the range of predator detection, giving prey individuals better opportunity to recognize novel predators with whom they have limited evolutionary co-existence (Brown and Chivers 2005; Gonzalo et al. 2007; 2009; Ferrari et al. 2010). As a result of this added benefit, associative learning is a quite widespread phenomenon in animal world (Ferrari et al. 2010). When kairomones from invasive predators and conspecific alarm cues are combined, the *L. thermalis* learns about them associatively (Tapkir et al. 2017). In the current study, DEP-exposed (either STE or LTE treatment group) *L. thermalis* completely fail to recognize conspecific alarm cues, which is critical for learning about invasive predators. This indicates that DEP-exposed *L. thermalis* with diminished ability to distinguish conspecific alarm cues will likely have no opportunity for associative learning. Because prey animals only innately recognize certain predators, the survival benefits of prey species that display associative learning are always greater (Ferrari et al. 2005, 2008; Schoeppner and Relyea 2005; Gonzalo et al. 2007; Pueta and Perotti 2016). Phthalates, such as Di(2-ethylhexyl) phthalate (DEHP), have been shown to impair learning and memory in rats (Ran et al. 2019) through alteration of neuronal function. The observed impact of failing to detect the alarm cues could be related with similar neurotoxicity, but further detailed investigation is required to make conclusive statements. The habitat acquired by the *L. thermalis* is frequently reported to be inhabited by invasive predators such as Mozambique tilapia, *Oreochromis mossambicus*, and walking catfish, *Clarias batrachus*. As a result, inability to learn about invading predators would further increase predation pressure on *L. thermalis*, thus negatively impacting the population of the study species in the wild.

In conclusion, DEP exposure has a major impact on the *L. thermalis* chemosensory abilities and survival that are critical for predator deterrence. Individuals of *L. thermalis* exposed to DEP not only fail to recognize native predators but also have a detrimental influence on associative learning and survival. Further research is needed to determine if such DEP-induced malfunction in anti-predatory responses is reversible, because few pollutants, such as copper at greater concentrations, are known to permanently alter olfactory-mediated behavior in fishes (Tierney et al. 2010). Nonetheless, the global increase in phthalate usage, increased dependence on plastic items, and mounting plastic pollution in the aquatic ecosystem will likely put aquatic animals at risk of extinction. As a result of the global decline in freshwater ichthyofauna caused by anthropogenic stresses and the wide spread of invasive species across global rivers, the findings of this study are relevant. Plastic management measures are anticipated to promote species survival while minimizing the disturbance of various chemosensory activities. This includes, for example, the government's rigorous enforcement of rules controlling the manufacturing and use of commercial items made of plastic.

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Data availability No data is associated with this manuscript.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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5.5. Impact of diethyl phthalate on freshwater planarian behaviour, regeneration, and antioxidant defence

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Impact of diethyl phthalate on freshwater planarian behaviour, regeneration, and antioxidant defence

Chandani R. Verma [a,1], Tushar Khare [a,b,1], Paromita Chakraborty [c,d], Sachin M. Gosavi [e], Miloslav Petrtýl [a], Lukáš Kalous [a], Pradeep Kumkar [a,*]

[a] Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Czech Republic
[b] Department of Biotechnology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, India
[c] Environmental Science and Technology Laboratory, Centre for Research in Environment, Sustainability Advocacy and Climate Change (REACH), Directorate of Research, SRM Institute of Science and Technology, Chengalpattu District, Tamil Nadu, 603203, India
[d] University of Lodz, Faculty of Biology and Environmental Protection, UNESCO Chair on Ecohydrology and Applied Ecology, Banacha 12/16, 90-237 Lodz, Poland
[e] Department of Zoology, Maharashtra College of Arts, Science and Commerce, Mumbai, Maharashtra, India

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ABSTRACT

Diethyl phthalate (DEP) has been widely used as a plasticiser in various consumer products, including cosmetics, personal care items, and pharmaceuticals, and recent studies reported a higher abundance of this priority phthalate in the aquatic environment. DEP is a potential endocrine disruptor, affecting immune systems in humans and wildlife even at low-level chronic exposure. As concern over phthalates increases globally, regulatory bodies focus more on their environmental impact. However, limited research is available, particularly using model organisms like planarians. Planarians are ideal for toxicological studies and may provide insightful information on pollutants' neurotoxic, developmental, and ecological effects, especially in freshwater environments where planarians play a vital role in ecosystem balance. Therefore, the objective of the current study was to examine the toxicity of DEP using the freshwater *Dugesia* sp., as an experimental animal. The LC_{50} for the test organism was calculated using DEP concentrations of 800, 400, 200, 100, and 50 μM, with an estimated LC_{50} of 357.24 μM. Furthermore, planarians were exposed to sub-lethal DEP concentration (178.62 μM) for one day as well as eight days to evaluate the impact of DEP on planarian locomotion, feeding behaviour, and regeneration ability. At sub-lethal concentration, locomotion and feeding ability were decreased, and regeneration was delayed. Furthermore, neuro-transmittance in planaria was altered by sub-lethal DEP concentration, as indicated by a reduced acetylcholinesterase (AChE) activity. DEP exposure induced oxidative damage in the tested planarians as shown by a marked increase in stress biomarkers, including lipid peroxidation levels and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and glutathione S-transferase (GST). Our study revealed that DEP exposure may prove fatal to freshwater organisms, such as planarians. The observed alterations in behaviour and regeneration ability demonstrate the severity of the effects exerted by DEP as a toxicant in aquatic ecosystems, thereby indicating the need to restrict its usage to protect aquatic environments.

1. Introduction

Phthalic acid esters (PAEs) are synthetic additives used to enhance the flexibility of plastics (Geyer et al., 2017; Zhang et al., 2021). Amongst them, diethyl phthalate (DEP) is one of the most common and ubiquitously used plasticisers (Zhang et al., 2021; Prieto-Amador et al., 2021). DEP is prevalent in consumer goods, including personal care products, pharmaceuticals, and packaging materials, and thus one of the most widely detected phthalates in the environment (Mansouri et al., 2019; Seyoum and Pradhan, 2019). Chemically, DEP is a diester of phthalic acid and ethanol with a molecular weight of 222.24 g mol^{-1}, a boiling point of 298 °C, slightly water solubility (1.08 mg/L at 25 °C), highly soluble in organic solvents, and a vapour pressure of 1.97×10^4 mm Hg at 25 °C (EPA, 2012). These properties enable DEP to persist in

* Corresponding author.
E-mail address: kumkar@af.czu.cz (P. Kumkar).
[1] Equal contribution.

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various environmental matrices, including air, water, and soil (Mackintosh et al., 2006; Verma et al., 2023). Globally, there is a lack of sufficient regulatory oversight on the use of phthalates, including DEP (Seyoum and Pradhan, 2019), along with a scarcity of research on its effects. Predictions indicate that rapid economic growth will lead to a significant expansion of the global phthalate market, projected to reach USD 698,414.17 thousand by 2030, with an annual growth rate of 4.1 % from 2023 to 2030 (Shaha and Pandit, 2020; Verma et al., 2023). This trend is particularly notable in developing countries, where there is increasing phthalate demand (Data Bridge Market, 2023), coupled with limited regulatory measures. DEP enters the environment through industrial discharges, product leaching, and atmospheric deposition, exhibiting considerable persistence due to its moderate water solubility and biodegradation resistance (Mansouri et al., 2019; Verma et al., 2023). Consequently, DEP is a priority phthalate and is one of the most widely distributed contaminants in aquatic ecosystems. Earlier research has demonstrated that DEP is linked to numerous adverse effects on both organisms and humans (Zhang, 2015; Yin et al., 2018). Prior research has indicated that phthalate concentrations in riverine water range from 0.1 to 370 μg/L (Sha et al., 2007; Bono-Blay et al., 2012). A previous study conducted in the Kaveri River in India found that DEP accounts for 22 % of the phthalates detected in the river water (Selvaraj et al., 2015). This highlights the extensive pollution of aquatic ecosystems with phthalates, including DEP (Kumkar et al., 2022; Verma et al., 2023).

The primary concern of DEP lies in its ability to disrupt endocrine function. Phthalates, including DEP and its metabolites, mimic natural hormones, binding to hormone receptors and disrupting normal hormonal signalling pathways (Kavlock et al., 2002). Additionally, DEP interferes with enzymes critical for steroidogenesis, thereby affecting the synthesis and metabolism of hormones (Diamanti-Kandarakis et al., 2009). Furthermore, DEP induces epigenetic modifications such as DNA methylation and histone modification, altering gene expression related to endocrine function (Singh and Li, 2012). These mechanisms collectively contribute to DEP's ability to disrupt endocrine processes. For instance, in common carp, *Cyprinus carpio* and Zebrafish, *Danio rerio*, DEP contributes to reproductive dysfunction by causing impaired reproductive output, altering sex ratios, and inducing developmental anomalies (Barse et al., 2007; Oehlmann et al., 2009). DEP can disrupt normal behavioural patterns in fishes, affecting their survival and reproductive behaviours (Barse et al., 2007; Cheng et al., 2021). Moreover, the long-term consequences of DEP exposure lead to weakened immune systems in fish (*D. rerio*) and heightened mortality rates, ultimately leading to population declines across affected species (Yin et al., 2018). Additionally, DEP can accumulate in the tissues of aquatic organisms, leading to biomagnification and increasing concentrations in organisms higher up the food chain (Zhang, 2015; Baloyi et al., 2021). This can ultimately disrupt entire aquatic food webs and eventually impact human health. This suggests that DEP's chemical properties, widespread use, environmental persistence, and endocrine-disrupting capabilities make it a significant environmental contaminant with adverse effects on human health and wildlife. Robust regulations and continued research with appropriate model organisms are crucial to address DEP-related challenges and protect ecosystems and public health.

Planarians are well-known animal models in ecotoxicology due to their robust regeneration capacity and high sensitivity to water contaminants (Barbosa et al., 2022; Zhang et al., 2023; Wu et al., 2024). However, there is limited information on comprehensive assessments focusing on the impact of DEP on planarians. Thus, this study aimed to evaluate the lethal and sub-lethal effects of DEP on *Dugesia* sp.. This widespread aquatic planarian has been a globally validated test species in ecotoxicology (López et al., 2019). Using laboratory experiments, we evaluated the impact of DEP on planarians by measuring mortality, locomotor velocity (*p*LMV), feeding rate, and regenerative ability. Additionally, we assessed the oxidative stress induced by DEP by measuring the activity of key antioxidant enzymes. Changes in neurotransmitter function were also examined by measuring acetylcholinesterase (AChE) activity to explain DEP-induced behavioural effects like *p*LMV and feeding. This research will deepen our understanding of DEP's effects on aquatic life and aid in designing strategies to reduce its environmental impact.

2. Materials and methods

2.1. Organisms and maintenance

Live planarians were collected from Pashan Lake (18.533 N, 73.785 E), Pune, Maharashtra, India, and transported to the laboratory in borosilicate glass containers filled with 5 L of dechlorinated oxygenated water. The planarians were maintained in borosilicate glass containers filled with 3 L of American Standard Test and Materials (ASTM) medium (ASTM, 1980) with a stocking density of 50 individuals L^{-1} at 22 ± 1 °C under near-dark conditions (except during feeding and media renewal). Planarians were fed twice a week with chicken liver (ad libitum), and the media was renewed after three hours of feeding. In addition, media was renewed two to three days after feeding. The organisms were maintained in the laboratory for two weeks. Prior to conducting the bioassay, planarians were starved for a week to obtain homogeneity in the physiological state of the population. Active planarians with approximately 7–10 mm length, without any lesions/abnormalities, were used for further experiments. The random planarian representatives were selected and subjected to molecular identification (supplementary material).

2.2. Diethyl phthalate

Diethyl phthalate (DEP; CAS 84–66–2, 99.5 % purity) was procured from Sigma-Aldrich, India. Analytical-grade acetone (CAS 67–64–1, >99 % purity, Merck Millipore, India) was used as a solvent for the preparation of DEP stock solutions (10 mM). The stock solutions were then diluted with ASTM medium to achieve the concentrations required for subsequent experiments.

2.3. Determination of lethal concentration (LC_{50})

The LC_{50} of DEP concentration for planarians was estimated via the guidelines of the Organization for Economic Cooperation and Development (OECD) using a static renewal system. Planarians were exposed to a series of DEP concentrations (800, 400, 200, 100, 50 μM) along with a control (no DEP) and solvent control (acetone, 0.004 % v/v). The exposure experiment was conducted in borosilicate glass beakers containing the ASTM medium with different DEP concentrations (ten planarians per beaker), with five replicates for every concentration. Experimental solutions were replaced every day. Exposure was conducted under dark conditions at 22 ± 1 °C, and the planarians were not fed during the experimental period. Planarian mortality was recorded every 24 h, and the experiment was terminated after 96 h. The online Probit analysis tool (http://14.139.232.166/Probit/probitanalysis.html) was used to calculate the LC_{50} value. The sub-lethal (½ LC_{50}) DEP concentration was calculated as half of the obtained LC_{50} value and used for further experimentation.

2.4. Experimental design for assessing the behavioural and regeneration endpoints

Planarians were exposed to ½ LC_{50} DEP concentration for one day (short-term exposure: STE) and eight days (long-term exposure: LTE). For both STE and LTE, the treatments were formulated in ASTM as (1) control (no DEP); (2) acetone (0.004 %, solvent control); and (3) DEP (½ LC_{50}). Each exposure treatment was conducted in five replicates in borosilicate glass beakers holding experimental solutions (50 planarians per beaker). Following short or long-term exposure, planarians from all replicates were transferred to clean water and distributed randomly for the assessment of post-exposure effects of DEP on planarian locomotor velocity, feeding, and regeneration. The schematic of conducted experiments is presented (Fig. 1).

2.4.1. Planarian locomotor velocity (pLMV)

The *p*LMV was evaluated using the procedure described by López et al. (2019), with minor modifications. Grid lines spaced 1 cm apart were marked on the outer-bottom surface of the borosilicate glass petri dish. Before each trial, the petri dish was thoroughly washed with distilled water and filled with 15 mL of ASTM water. Next, a single planaria individual was placed in the petri dish and left to acclimatise for 1-minute. The number of lines crossed or re-crossed by each planarian during the 2-minute observation period was used to determine post-exposure *p*LMV. Twenty trials were conducted for each treatment group, with the observer being blinded to the treatments and the sequence of the treatments being randomised in all cases. To avoid the impact of the observer's presence on planarian behaviour, a vertical one-way mirror glass was mounted to form a barrier between the viewer and the test chamber.

2.4.2. Feeding activity assay

The post-exposure feeding rate was assessed by placing ten planarians per treatment into Petri plates (90 × 15 mm) with 20 mL of ASTM media. For each replicate, planarians were offered thirty live *Chironomus circumdatus* larvae (total length: 0.58 ± 0.061 cm). After 24 h, the number of ingested larvae per planarian was counted.

2.4.3. Head regeneration

To determine the post-exposure effects of DEP on planarian head regeneration, five planarians (after LTE) were decapitated with a single incision below the auricles (under a stereo zoom microscope, Olympus, Germany). Decapitated planarians were then kept in crystallising plates with 20 mL of ASTM media. The key phases of the planarian regeneration process (blastema development, photoreceptor regeneration, and auricle formation) were then meticulously examined every six hours and photographed using a stereo zoom microscope (Olympus, Germany). Temporal progression of blastema development, regeneration of new photoreceptors, and auricle formation were assessed via a chronometric analysis involving the quantification of post-decapitation time intervals.

2.5. Determination of antioxidant parameters and acetylcholinesterase activity

Following STE and LTE at a ½ LC_{50} DEP concentration, the treatment solution was gently removed, and the planarians were rinsed with milli-Q water to ensure the complete removal of ASTM medium and traces of DEP. This was immediately followed by homogenising of the planarians in 1 mL of phosphate buffer (0.1 M, containing 0.1 % Triton X-100, pH 7.5) on ice. Next, the homogenate was centrifuged at 15,000 rpm for 20 min at 4 °C. The resulting supernatant was used to measure lipid peroxidation (LPO) levels, enzymatic activities, and total protein content. The LPO levels and activities of all enzymes were normalised to the protein content of the extract. LPO levels were determined via a thiobarbituric acid reactive substances (TBARS) assay, which measures the malondialdehyde (MDA) content at 532 nm (Bird and Draper, 1984). Superoxide dismutase (SOD) activity was measured in terms of the inhibition of photochemical reduction in nitro-blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). Catalase (CAT) activity was calculated as the hydrogen peroxide decomposition rate at 240 nm (Aebi 1984). Peroxidase (POX) activity was assessed using the method described by Castillo et al. (1984). The activity of glutathione S-transferase (GST) was determined by assessing the conjugation of glutathione with 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). Acetylcholinesterase activity (AChE) was evaluated via Ellman's assay using acetylthiocholine iodide as the enzyme-substrate (Poirier et al., 2017).

2.6. Statistical analysis

Statistical significance of the differences between the control and DEP-treated test sample sets (STE or LTE) was assessed using the Student's *t*-test at $p < 0.05$. The solvent control set was statistically insignificant in all evaluations compared to the control samples. The data were represented by the mean ± standard error to assure the reproducibility of results. Each biological replicate consisted of three technical replicates for the biochemical parameters. Statistical analyses and graphs were constructed using GraphPad Prism software version 9.

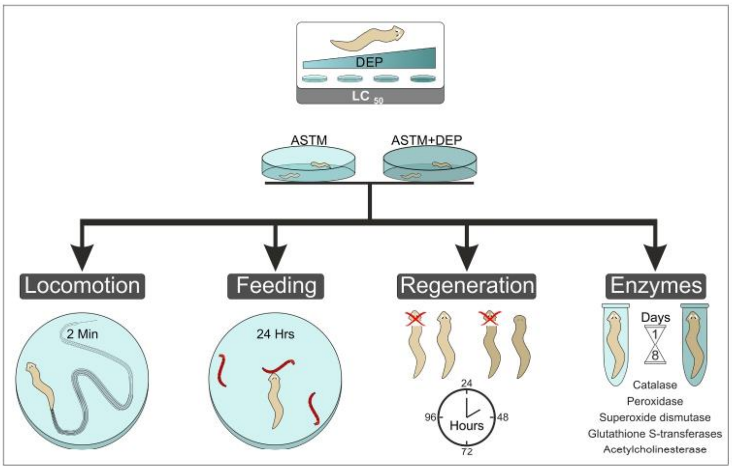

Fig. 1. Schematic of the experimental design, highlighting evaluated endpoints. Planarians were exposed to the variable diethyl phthalate (DEP) concentrations (50–800 μM) for 96 h to calculate the lethal DEP concentration (LC_{50}). The planarians were exposed to a ½ LC_{50} DEP concentration, to evaluate the effect of DEP on locomotion, feeding behaviour, regeneration antioxidative damage, and acetylcholinesterase activity.

3. Results

3.1. Lethal concentration (LC_{50}) of DEP

The LC_{50} of DEP for *Dugesia* sp. was found to be 357.24 µM, based on which the ½ LC_{50} DEP concentration was calculated as 178.62 µM and used for subsequent experimentation.

3.2. Impact of DEP on behavior

A statistically significant difference was observed between planarians' locomotion velocities in the control and treatment groups (t = 11.33, $p < 0.0001$, Fig. 2A). The *p*LMV of the planarian was reduced by 1.8-fold following STE to DEP at ½ LC_{50} DEP concentration (Fig. 2A). On the other hand, LTE to DEP at ½ LC_{50} DEP concentration reduced *p*LMV by 3.4-fold (t = 18.66, $p < 0.0001$, Fig. 2A). The feeding behaviour of planarians exposed to DEP was also significantly reduced compared to that of the control group (t = 11.50, $p < 0.0001$; Fig. 2B). Feeding activity (i.e., larvae consumed per 24 h) of planarians exposed to ½ LC_{50} DEP concentration was markedly reduced by 4.2-fold (Fig. 2B). LTE to DEP at ½ LC_{50} DEP concentration proved even more detrimental for planarian feeding activity, as indicated by a 14-fold reduction in feeding activity following LTE (t = 15.01, $p < 0.0001$, Fig. 2B).

3.3. Impact of DEP on regeneration

Exposure to ½ LC_{50} levels of DEP induced a delay in planarian regeneration (Fig. 3A). First, blastema formation in planaria was significantly delayed following LTE to DEP at ½ LC_{50} DEP concentration, where blastema formation required 1.4-fold more time than that required by the control (t = 9.021, $p < 0.0001$, Fig. 3B). Furthermore, the formation of photoreceptors was delayed following LTE to DEP at ½ LC_{50} DEP concentration, with DEP treated planarians requiring 1.32-fold more time for photoreceptor formation (t = 8.232, $p < 0.0001$, Fig. 3B). Lastly, auricle development also followed the same trend as that of delayed regeneration with planarians treated at ½ LC_{50} DEP concentration requiring 1.23-fold more time for auricle development than that required by the untreated (control) planarians (t = 8.398, $p <$ 0.0001, Fig. 3B).

3.4. Altered stress responses against DEP

STE and LTE to DEP considerably changed all examined stress markers in the planarians. LPO levels were significantly elevated in response to DEP exposure. The LPO levels of planarians subjected to STE were 2.8-fold higher than those in the untreated (control) group (t = 11.07, p = 0.0004). In contrast, LTE elevated LPO levels by 2.3-fold (t = 12.75, p = 0.0002, Fig. 4A). SOD activity in planarians was 1.8-fold higher following STE (t = 4.981, p = 0.0076) and 1.6-fold higher following LTE (t = 6.980, p = 0.0022, Fig. 4B). Similarly, CAT activity was improved 1.9-fold in response to STE (t = 12.76, p = 0.0002) and 2.1-fold in response to LTE (t = 11.95, p = 0.0003, Fig. 4C). Furthermore, POX activities were increased by 3.9-fold (t = 13.48, p = 0.0002) and 4.3-fold (t = 7.245, p = 0.0019, Fig. 4D) due to STE and LTE to DEP at ½ LC_{50} DEP concentration, respectively. Lastly, GST activity in planarians was raised 2.3-fold by STE (t = 10.83, p = 0.0004) and 2.7-fold

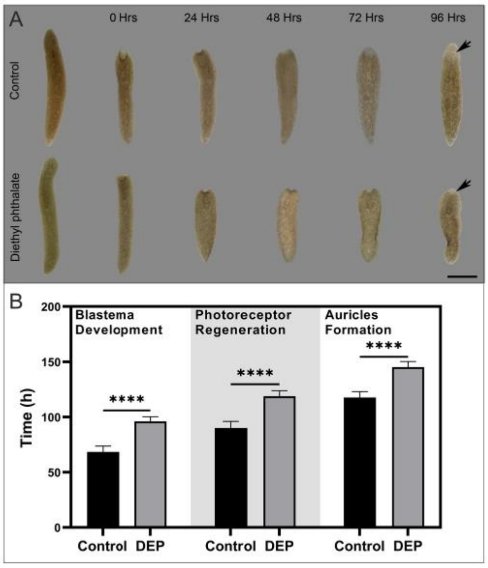

Fig. 3. Effect of diethyl phthalate (DEP) on head regeneration in planarians. (A) Progression of head regeneration in control and DEP-treated planarians as a function of time (0–96 Hrs); (Bar = 2 mm). (B) Delayed blastema development, photoreceptor regeneration, and auricle formation following DEP exposure. Each bar represents the mean ± standard error (S.E.) of five replicates; * = $p <$ 0.05; ** = $p < 0.01$; *** = $p < 0.001$; **** $p < 0.0001$.

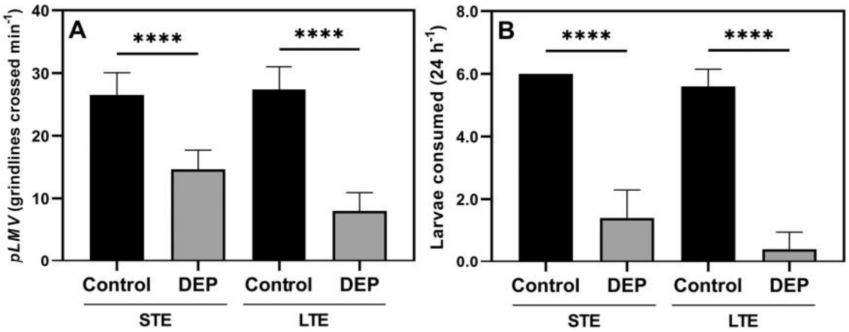

Fig. 2. Effect of short-term exposure (STE) and long-term exposure (LTE) to diethyl phthalate (DEP) on (A) locomotion (gridlines crossed min^{-1}) and (B) feeding behaviour (*Chironomus circumdatus* larvae consumed in 24 h) of planarians. Each bar represents the mean ± standard error (S.E.); * = $p < 0.05$; ** = $p < 0.01$; *** = p < 0.001; and **** = $p < 0.0001$.

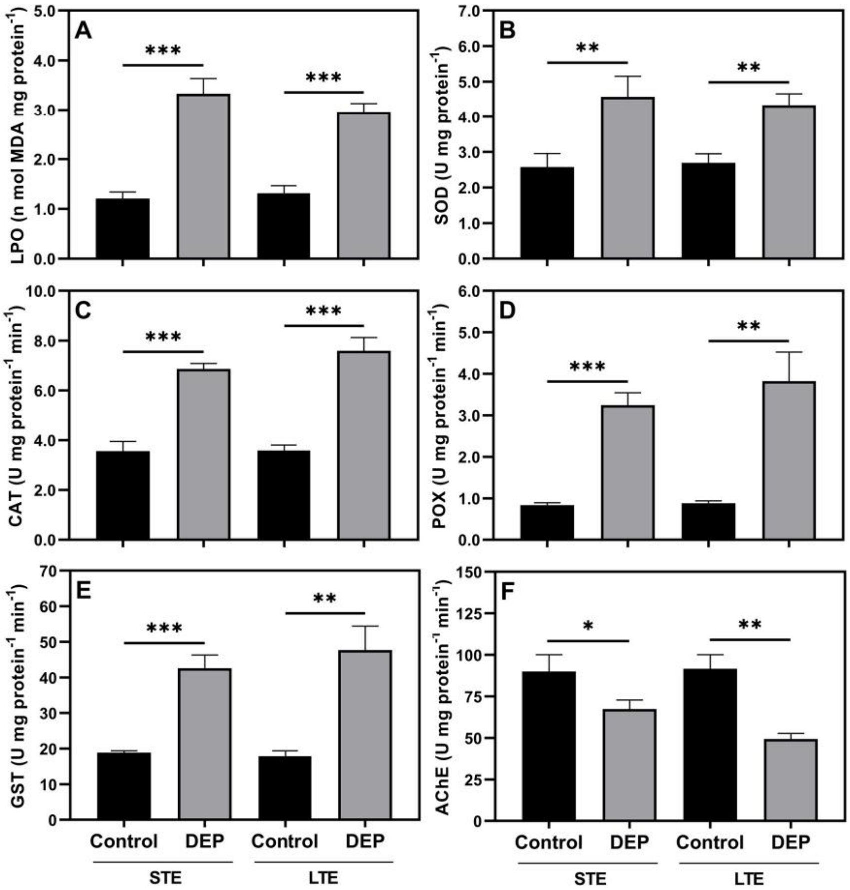

Fig. 4. Effect of short-term exposure (STE) and long-term exposure (LTE) to diethyl phthalate (DEP) on (A) lipid peroxidation levels (in terms of MDA content), (B) superoxide dismutase (SOD), (C) catalase (CAT), (D) peroxidase (POX), (E) glutathione-S-transferase (GST) activity and (F) acetylcholinesterase (AChE) activity in planarians. Each bar represents the mean ± standard error (S.E.) of three biological replicates ($n = 3$); * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. One unit of SOD is equivalent to 50 % inhibition of the NBT photo-reduction rate. One unit of CAT refers to mM H2O2 decomposed. One unit of POX refers to μM of tetraguaiacol formed. One unit of GST refers to μM S-(2, 4-dinitrophenyl) glutathione formed. One unit of AChE refers to the enzyme catalysing thiocholine production.

by LTE ($t = 7.457$, $p = 0.0017$, Fig. 4E).

3.5. Impact of DEP on acetylcholine esterase activity

In present study, AChE activity displayed a decreasing trend in response to DEP treatment. AChE activity in planarians declined 1.3-fold in response to the STE ($t = 3.390$, $p = 0.0275$) and 1.9-fold in response to the LTE ($t = 7.961$, $p = 0.0013$, Fig. 4F).

4. Discussion

4.1. DEP exposure affected planarian survival

Planarians used in the current investigation represent freshwater benthic invertebrates that provide ideal research material due to their extraordinary regenerative abilities and prompt physicochemical responses to a wide range of toxicants (Wu and Li, 2018). Heavy use of phthalates, including DEP, has resulted in their presence in various aquatic ecosystems worldwide (Baloyi et al., 2021). Evaluation of the median lethal concentration (LC_{50}) via standard acute toxicity testing is customarily recommended for preliminary investigations aimed at estimating the toxicity of a substance (Sprague, 1989; Farah et al., 2004). The LC_{50} of DEP for *Dugesia* sp. was found to be 357.24 μM, based on which the ½ LC_{50} DEP concentration was calculated as 178.62 μM and used for subsequent experimentation. Our choice of a higher environmental concentration was driven by the need to simulate and evaluate potential adverse effects in scenarios of significant contamination, a concern heightened by the lack of precise DEP concentration data for many aquatic ecosystems in India. Even after 96 h of exposure, no mortality was seen in the blank or solvent controls, confirming the appropriateness of these media for planarian maintenance and DEP administration. Planarians have been previously tested for LC_{50} values against heavy metals (chromium, mercury and lead; 52.28, 0.48, and 182.02 mg L^{-1}, respectively, 48 h; Liu et al., 2021), a herbicide (glyphosate, 128 mg L^{-1}, 96 h, Zhang et al., 2023), an insecticide (thiamethoxam, 77.6 mg L^{-1}, 96 h, Barbosa et al., 2022; chlorpyriphos, 622.8 μg L^{-1}, de Souza Saraiva et al. 2023), and biocides (Li, 2019), among others. Similarly, the impact of DEP on various aquatic organisms, such as fishes, including Mrigal carp, *Cirrhinus mrigala* (Ghorpade et al., 2002), Common carp, *C. carpio* (Poopal et al., 2017), Zebrafish, *D. rerio* (Pu et al., 2020; Tseng et al., 2021) and Common spiny loach, *Lepidocephalichthys thermalis* (Kumkar et al., 2022; Verma et al., 2023), the freshwater prawn, *Macrobrachium rosenbergii* (Chen and Sung, 2005), the gastropod, *Physella acuta* (Prieto-Amador et al., 2021), as well as the insect *C. circumdatus* (Shaha and Pandit, 2020) and the amphibian, *Xenopus laevis* (Gardner et al., 2016), has also been reported. However, reports that compare planarian lethality induced by DEPs is yet to be published. Therefore, the current study may provide crucial insights into the lethal impact exerted by DEP on planarians. The LC_{50} values of DEP pertaining to various organisms estimated by previous studies included, 470 mg L^{-1} (96 h) for *D. rerio* embryos (de Almeida et al. 2023), 1516.18 mg kg^{-1} (14 d) for *Eisenia fetida* (Feng et al., 2016), 44.53 mg L^{-1} (96 h) for *L. thermalis* (Kumkar et al., 2022), and 34.63 mg L^{-1} (96 h) for *Indoreonectes evezardi* (Kumkar et al., 2022). This indicates that DEP exhibits different toxicity levels among various aquatic organisms, including vertebrates and invertebrates. Additionally, the susceptibility to DEP varies significantly across different species, which can be attributed to a range of factors. For instance, *Daphnia magna*, another widely studied invertebrate, has an EC_{50} of approximately 90 mg/L (Pereira et al., 2023). This indicates that *D. magna* is more susceptible to DEP compared to *Dugesia* sp. The higher sensitivity of *D. magna* could be attributed to its smaller size, higher surface area-to-volume ratio, and possibly different metabolic processes that affect how the organism interacts with and detoxifies toxicants such as DEP (Hutchinson et al., 1994; Barata et al., 2001). Likewise, freshwater fish, *C. mrigala* has an LC_{50} of approximately 50 mg/L (Ghorpade et al., 2002). Factors contributing to this increased sensitivity may include the relatively high permeability of fish gills to waterborne contaminants, the rapid uptake of DEP, and the potentially high bioaccumulation in fish tissues. The LC_{50} values for *Dugesia* sp. provide crucial insights into the species-specific responses to DEP exposure, informing regulatory policies to mitigate the ecological risks of this widespread contaminant. Additionally, the higher sensitivity of invertebrate species like *Dugesia* sp. and *Daphnia* is particularly concerning due to the occupancy of their lowermost trophic level and crucial ecological niche in aquatic food webs. The negative impacts and decline of these aquatic invertebrates could trigger cascading effects on overall ecosystem health. Based on the LC_{50} values observed, the DEP levels reported by the current study are realistic and should not be acutely lethal to freshwater planarians. However, evaluating sub-lethal impacts is vital for predicting overall adversity to natural planarian populations because exposure times and concentrations do not remain static.

4.2. DEP exposure impaired planarian behavior

Aquatic organisms often change their behaviour in response to environmental contaminants, either directly through exposure or indirectly due to the effects of such exposures (Gosavi et al., 2020; Hamilton et al., 2021; Verma et al., 2023). Behavioural responses such as changes in swimming speed, shoaling behaviour, or predator avoidance strategies, represent a critical and observable response of aquatic organisms to toxicants, serving as sensitive indicators of environmental contamination and providing valuable insights into the ecological impacts of chemical pollutants (Tapkir et al., 2019; Gosavi et al., 2020; Hamilton et al., 2021; Verma et al., 2023). In the present study, the *p*LMV was calculated via a simple quantifiable mean, which demonstrated planarian sensitivity to toxicants (López et al., 2019; Silva et al., 2023), where a significant difference indicated variation in the number of grids crossed by planarians (control vs DEP treated; Fig. 2A). This assessment revealed that both STE and LTE had considerable negative impact on the planarian locomotory behaviour. Furthermore, LTE to DEP had a higher degree of impact on planarian locomotion than STE to DEP at ½ LC_{50} concentration. Previous research has highlighted that exposure to plasticisers such as DEP, di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), and their alternatives such as bisphenol A (BPA) and bisphenol F (BPF), can lead to locomotory impairments in aquatic organisms including planarians (Thuren and Woin, 1991; Yang et al., 2018; Poopal et al., 2017; Zhang et al., 2021; Morris et al., 2022; Wu et al., 2024). For instance, a recent study by Wu et al. (2024) showed that exposure to DBP (1.6 mg/L and 8 mg/L) significantly impaired locomotion in intact freshwater planarians (*Dugesia japonica*). Additionally, Morris et al. (2022) found that bisphenol A (BPA) and bisphenol F (BPF), which are used as alternatives to other plasticisers like DEP, caused noticeable behavioural changes such as impaired phototaxis and abnormal locomotion in regenerating planarians (*Schmidtea mediterranea*). DEHP and DEP have also been shown to disrupt swimming behaviours in various species: DEHP exposure affected the swimming ability of medaka fish, *Oryzias latipes*, while DEP exposure impacted Zebrafish, *D. rerio* (Yang et al., 2018; Tseng et al., 2021). Similarly, in amphipod crustaceans such as *Gammarus pulex*, exposure to DEHP and di-n-butyl phthalate (DBP) led to reduced locomotor activity and decreased survival rates (Thuren and Woin, 1991). In the current study, a reduction in *p*LMV reflected delayed movement. Planarian locomotion is mainly achieved via gliding based on ciliary movements and muscle contractions that require optimal nervous system functioning (Nishimura et al., 2007). Previous studies have demonstrated that a decrease in neoblast numbers notably impacts the upkeep of mature tissues such as nervous systems, protonephridia systems, and the ciliary structure of ventral epithelial cells, resulting in impaired locomotion of planarians (Peiris et al., 2016; Wu et al., 2024). Therefore, the impaired movement observed in our study's planarians may be linked to diminished neoblast cell production, as we have also noted compromised neuronal function and regeneration in planarians exposed to DEP. Planarians are active predators; thus, optimal gliding is a fundamental activity that enables them to capture prey more effectively (Vila-Farré and Rink, 2018). On the other hand, limited locomotory functions may also denote higher susceptibility to predatory attacks. Planarians are always in a state of movement for several reasons, including hunting for prey, escaping from predators, and searching for sexual partners (Macedo et al., 2019). Furthermore, optimal locomotory movements are a must for avoiding hostile environments and improving their chances of survival (Inoue et al., 2004, 2015). Therefore, reduced mobility would ultimately exacerbate the adverse effects on the survival and fitness of planarians.

Similar to its effects on locomotion, DEP exposure also affected the feeding behaviour of planarians (Fig. 2B). LTE to DEP caused a remarkable impact on planarian feeding because the extent of reduction following LTE equalled 14 times that of the control group (Fig. 2B). Feeding behaviour also requires harmonised functioning of the nervous system. For instance, effective feeding requires recognition of prey-borne chemical cues, gliding in the direction of prey (using ciliary and muscular movements), subduing prey via mucus secretion, pharynx eversion, production of digestive enzymes, and ingestion of prey tissue via suction (Inoue et al., 2015; Norena et al., 2014). In this context, locomotory behaviour and feeding behaviour must be allied. The current investigation substantiates this requirement as both *p*LMV and feeding behaviour were reduced in response to STE and LTE to DEP. Considering the vital nature of the physiological aspects, mainly mucus production and muscular functionality, which drive feeding behaviour, feeding behaviour tends to be more sensitive to environmental changes than locomotion (Simão et al., 2020). A similar trend was observed in the current study, where fold reduction was more observable in feeding activity than in *p*LMV. These observations depicting behavioural and movement-related hindrances signify the negative impact exerted by

DEP on planarians and that such impacts should be factored in when advocating for the fitness of wild populations of aquatic organisms. Overall, the impact of DEP on locomotory activity underscores their potential to disrupt fundamental behaviours essential for the ecological fitness of aquatic organisms. These behavioural alterations indicate immediate physiological stress and pose long-term ecological risks to aquatic ecosystems.

4.3. DEP exposure impaired planarian regeneration

Regeneration is a form of asexual reproduction commonly utilised by aquatic invertebrates, allowing an animal to regrow specific body parts from a fragment of the original organism.

The ability of planarians to regenerate relies on pluripotent stem cells known as neoblasts, which are the sole cells capable of undergoing division (Lin and Pearson, 2017). When planarians incur an injury, a significant influx of neoblasts migrates towards the wound area, undergoing rapid proliferation and differentiation to regenerate new tissues and organs (Wu et al., 2024; Huang et al., 2024). The functioning of these neoblasts is vulnerable to environmental toxins (Dong et al., 2021; Leynen et al., 2019). Hence, assessing regenerative capabilities is a critical factor that should be considered when evaluating the toxic impact (teratogenic, cytotoxic, or neurotoxic) of any environmental pollutant or toxicant (López et al., 2019). Exposure to DEP significantly disrupted several crucial regeneration processes in planaria, including blastema formation, eyespot regeneration, and auricle development (Fig. 3A). Previous studies have demonstrated that impaired regeneration in planarians is caused by decreased neoblast proliferation and the dysfunctional, delayed recovery of planarians under the influence of contaminants (Wu and Li, 2018; Cesarini et al., 2023; Wu et al., 2024). Impaired regeneration in planarians has previously been observed when they are exposed to various environmental pollutants, including phthalates (DBP, Wu et al., 2024), nano- and microplastics (Cesarini et al., 2023; Gao et al., 2022), nanoparticles (Xie et al., 2023), fungicides (Dornelas et al., 2022), herbicides (López et al., 2019), heavy metals (Majid et al., 2022), polycyclic aromatic hydrocarbons (Simão et al., 2020), and textile azo dye (Ribeiro and Umbuzeiro, 2014). In the current study, we speculate that the impaired regeneration observed in planarians may be linked to decreased neoblast cells. Previous research using DBP has convincingly demonstrated that exposed planarians exhibit reduced expression levels of genes marking stem cells and various lineage markers, including those for epidermis, muscle, protonephridia, and cilia (Wu et al., 2024). This downregulation impedes both cell proliferation and differentiation, thereby affecting the generation of stem cell components crucial for the regeneration process in planarians, resulting in suppressed regeneration. We propose a similar mechanism that may account for the suppressed regeneration observed in our case, given the structural similarity between DEP and DBP and their comparable uses in various consumer products. Moreover, the findings of decreased locomotor activity also offer corroborative evidence of reduced neoblast production essential for maintaining mature tissues like nervous systems, protonephridia systems, and the ciliary structure of ventral epithelial cells, crucial for movement in planarians (Peiris et al., 2016; Wu et al., 2024). The survival and reproductive potential of planaria are reliant on photoreceptor- and auricle-based photo-sensing and chemo-sensing aimed at detecting light and other environmental stimuli, respectively (Inoue et al., 2004; 2015). Hence, the results of the current study indicate the detrimental effects exerted by DEP, in the form of delayed recovery due to late blastema development and delayed generation of photoreceptors and auricles, which hamper the stimuli-sensing abilities of planarians. Results of the current study validate DEP-induced delays in planarian regeneration, as well as the potential long-term effects of the presence of DEP in freshwater ecosystems on planarian populations and other freshwater communities. Research providing detailed insights into the extent and mechanisms of phthalate toxicity is still in its infancy, and the next vital step in phthalate research should be a detailed molecular characterisation of phthalate-mediated effects on freshwater organisms.

4.4. DEP exposure induced oxidative stress in planarians

In aerobic organisms, reactive oxygen species (ROS) are produced as a result of metabolic redox reactions primarily through the mitochondrial electron transport chain (Birnie-Gauvin et al., 2017). This production of ROS is particularly prominent during instances of chemical-induced oxidative stress, such as during detoxification processes (Valavanidis et al., 2006). The antioxidant defence system comprises a network of enzymes such as such as SOD, CAT, POX, and GST, known as ROS scavengers. SOD catalyses the dismutation of O_2^{*-} into molecular O_2 and H_2O_2, while POX and CAT quench H_2O_2 (Zhang et al., 2019; Kumkar et al., 2022). GST modulates the conjugation of glutathione, an antioxidant which neutralises the harmful products of LPO, thereby reducing the levels of these free radicals in the cell (Rocha-Santos et al., 2018; Kumkar et al., 2022). MDA is a by-product of lipid peroxidation and is a significant biomarker indicating oxidative stress (Wang et al., 2023; Kumkar et al., 2022). These antioxidant enzymes and defence mechanisms do not operate independently but collaborate to efficiently eliminate intracellular ROS and maintain the equilibrium of the oxidant/antioxidant system within the organism (Ye et al., 2022; Kumkar et al., 2022). High levels of ROS can trigger apoptosis via oxidative stress pathways (Dai et al., 2022; Kumkar et al., 2022; Wu et al., 2024). In this study, the significant increase in levels of all stress markers examined—LPO, SOD, CAT, POX, and GST—indicates that exposure to DEP, whether STE or LTE, induced oxidative stress in planarians. Exposure to pollutants, including phthalates DBP (Wu et al., 2024), microplastics (Han et al., 2022), polycyclic aromatic hydrocarbons (Simão et al., 2020), graphene oxide (Xie et al., 2023), perfluorooctanoic acid (Zhang et al., 2019), and heavy metals (Liu et al., 2021) is known to cause oxidative damage in planarians. In the current study, the rise in antioxidant enzyme levels in planarians following DEP exposure indicates that their antioxidant defense system was activated early on, initially neutralizing some ROS to protect against DEP-induced damage. However, even after eight days of exposure, the levels of antioxidant enzymes remained high, suggesting the continuous production and persistence of ROS due to the presence of DEP. The synthesis of antioxidant enzymes in planarians proved inadequate to completely clear the excess ROS over time, resulting in their gradual build-up during later stages. As a consequence, ROS swiftly targets significant molecular components such as phospholipids in biological membranes, membrane receptors, and fatty acids (Yang et al., 2018; Wu et al., 2024), thereby amplifying lipid peroxidation (Fig. 4A). LPO levels customarily serve as markers of oxidative damage (Khan and Panda, 2008). Similar observations were also made by Wu et al. (2024) in planarians exposed to the DBP. The current study shows a significant increase in MDA content, indicating high LPO levels, and, thereby, high oxidative damage. Such toxicant-induced oxidative damage generates ROS, including superoxide ions (O_2^{*-}), hydroxyl radicals ($^*OH$), and hydrogen peroxide (H_2O_2). The cellular system maintains a steady state that signifies an equilibrium between producing and scavenging ROS. Nonetheless, any overproduction of ROS (indicated by high LPO levels) caused by a toxicant may hamper the balance between the production and elimination of ROS, leading to an excess of ROS, which may eventually impact vital biological macromolecules, such as DNA, proteins, and lipids, thereby propelling cells into a state of oxidative damage (Zhang et al., 2019; Samanta et al., 2014). Additionally, MDA could function as an early indicator of toxicity induced by phthalates (Wu et al., 2024). Furthermore, the toxic effects of DEP persisted over time, suggesting potential long-term cumulative effects in planarians. This persistence may be attributed to the suppression of antioxidant enzyme activity and notable elevation in ROS levels and MDA content observed in the experiment's long-term exposure (LTE) phase. Further investigation into the effects of DEP on planarian DNA damage using appropriate methods is needed, as ROS can oxidise DNA directly, leading to disruptions in its helical structure. Moreover, ROS can interact with MDA to produce intermediates that further impact DNA molecules, potentially exacerbating DNA damage (Wang et al., 2023; Wu et al., 2024).

4.5. DEP induce neurotoxicity in planarian

AChE is a crucial enzyme responsible for breaking down the neurotransmitter acetylcholine (ACh) and maintaining its balance within the body. When presynaptic neurons release acetylcholine, AChE is activated to control its concentration (Soreq and Seidman, 2001; Yang et al., 2018). In addition, AChE is an important biomarker of environmental neurotoxicants (Kaviraj and Gupta, 2014; Yang et al., 2018). In the present study, AChE activity displayed a decreasing trend in response to DEP treatment (Fig. 4F). While there is a wealth of literature on changes in AChE expression levels in various organisms in response to contaminants, which ultimately affect their behaviour, such research is scarce for planarians. For instance, exposure to the phthalate DEHP reduces AChE activity in most organs and tissues of bagrid catfish (*Pseudobagrus fulvidraco*) (Jee et al., 2009). Additionally, exposure to heavy metals increases AChE activity in Piava (*Leporinus obtusidens*) and Nile tilapia (*Oreochromis niloticus*) (Gioda et al., 2013; Oliveira et al., 2017). Conversely, agricultural chemicals such as endosulfan suppress AChE activity in Zebrafish (exposed to 2.4 μg/L for 96 h) and *D. magna* (Pereira et al., 2012; Ren et al., 2015; 2016). Various studies have suggested that exposure to toxicants may induce neurotoxicity by altering the activity of AChE, which plays a crucial role in cholinergic neurotransmission (Goncalves et al., 2010; Abdalla et al., 2013; Akinyemi et al., 2017; Yuan et al., 2018). Earlier studies have also highlighted the link between AChE activity and locomotory behaviour (Pereira et al., 2012; Ren et al., 2015; 2016). Reduced AChE activity in both STE and LTE suggests that the DEP induces neurotoxicity in the planarians. Reduction in AChE may cause acetylcholine accumulation in the synapses, resulting in deviated cholinergic neuro-transmittance (Carageorgiou and Katramadou, 2012). Such altered neuro-transmittance at neuromuscular junctions may hinder signal transfers between neurons, leading to a deterioration in the behaviour of toxicant-treated organisms. This may result in a significant reduction in *p*LMV as well as suppression of auricle development during regeneration (Yuan et al., 2014). The current results appear to substantiate these trends. The present study illustrates probable DEP-induced neurotoxicity, which manifests as decelerated locomotion and delayed regeneration due to flawed neuro-transmittance. However, an in-depth analysis that is able to evaluate the neurotransmitter levels precisely as well as the expression patterns of neuronal genes involved in locomotion, is required to elucidate the underlying mechanism of action.

5. Conclusions and recommendations

The study demonstrated that DEP has detrimental effects on freshwater benthic planarians, significantly impacting their locomotion, feeding behaviour, and regeneration. DEP exposure resulted in impaired neuronal function, delayed blastema formation, reduced development of photoreceptors and auricles, and lowered AChE activity, signalling neurotoxicity. Increased LPO levels and sustained activation of antioxidant enzymes indicate oxidative stress in planarians. These findings highlight DEP's harmful effects on freshwater ecosystems, where planarians act as indicators of water quality. We recommend that future research should aim to elucidate the precise mechanisms of toxicity and identify key molecules or genes involved in phthalate-induced damage in aquatic organisms.

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CRediT authorship contribution statement

Chandani R. Verma: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tushar Khare:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paromita Chakraborty:** Writing – review & editing, Supervision, Conceptualization. **Sachin M. Gosavi:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Miloslav Petrtýl:** Writing – review & editing, Visualization, Formal analysis. **Lukáš Kalous:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Pradeep Kumkar:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, 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

No data was used for the research described in the article.

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Supplementary materials

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5.6 Occurrence and distribution of plastispheres in coastal sediments and waters along the maharashtra coast, India

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RESEARCH

Occurrence and Distribution of Plastispheres in Coastal Sediments and Waters along the Maharashtra Coast, India

Chandani R. Verma[1] · Manoj Pise[1] · Štěpán Hýsek[2,3] · Sonia Źółtowska[2] · Pradeep Kumkar[1] · Lukáš Kalous[1] · Sachin M. Gosavi[4]

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Abstract
Microplastics can promote microbial colonisation and biofilm growth, thus being referred to as "plastispheres". The global plastic pollution surge is likely to adversely impact ecology and human health by providing a novel habitat for microbial communities. Even though microplastics in marine environments have been the subject of in-depth research, plastispheres have recently received attention. Thus, the current study investigates the prevalence and distribution of plastispheres along the Maharashtra coast of India, considering their plausible implications for ecology and human health. Microplastics were isolated from sediment and water samples obtained from 10 sampling sites. Subsequently, these microplastic particles were subjected to ATR-FTIR and scanning electron microscopy (SEM) analyses to ascertain their chemical composition, surface topography, and presence of attached biofilms. The predominant polymers composing the microplastic particles were polypropylene (42.8%), polyethylene (28.6%), polystyrene (14.3%), and polyvinyl chloride (14.3%). SEM analysis revealed the presence of topographical structures and degradation effects, facilitating microbial attachment on the microplastic surface. About 50% of the microplastic particles tested positive for biofilms, with over 66% of those collected from Girgaon and Malvan beaches exhibiting biofilm presence. These positively screened particles also displayed comparatively rough surface structures, likely enhancing microbial colonisation. Microplastic ageing and polymer type could positively affect microbial colonisation. Diatoms and fungal hyphae exhibit varied interactions with microplastic polymers. Notably, microplastics host various reproductive stages of fungi, as evidenced by filamentous networks, mycelia, and conidiophores.

Keywords Marine microplastics · Microorganisms · Diatoms · Fungi · Polymers

Introduction

High and low-density polyethylene (HDPE and LDPE), polypropylene (PP), polyvinyl chloride (PVC), and polystyrene (PS) are among the widely manufactured thermoplastics (Sánchez 2020; Zhang et al. 2021). These versatile thermoplastics are used in a wide range of daily items, playing a significant role in the notable rise of worldwide plastic production, which presently amounts to 368 million metric tons annually (Sooriyakumar et al. 2022). However, the surge in plastic production and extensive utilisation has led to a parallel escalation in releasing plastic waste into the environment (Geyer et al. 2017). Microplastics (plastic less than 5 mm) pollution is an emerging environmental concern (Thompson et al. 2004). Microplastics are classified as either primary or secondary. Primary microplastics originate from sources with an initial size of less than 5 mm, while secondary microplastics result from the breakdown

✉ Pradeep Kumkar
kumkar@af.czu.cz

✉ Sachin M. Gosavi
schn.gosavi@gmail.com

1 Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic

2 Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Prague, Czech Republic

3 Institute of Wood Technology and Renewable Materials, Department of Material Sciences and Process Engineering, University of Natural Resources and Life Sciences, Vienna, Austria

4 Department of Zoology, Maharashtra College of Arts, Science and Commerce, Mumbai, India

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of larger plastic fragments (Dong et al. 2021). These tiny plastic particles have become ubiquitous in terrestrial, freshwater, estuarine, marine, polar, and remote regions (Dong et al. 2021). Given their small size, various aquatic organisms, mainly marine fauna, readily ingest microplastics, leading to their potential accumulation and dispersion throughout the food chain (Roman et al. 2021). Numerous studies have extensively documented the adverse impacts of microplastics on a diverse array of organisms, spanning from zooplankton to molluscs, fish, seabirds, turtles, and mammals (Zhang et al. 2021). Moreover, the hydrophobic nature and the high surface area-to-volume ratio of microplastics facilitate the accumulation of various contaminants, including heavy metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, perfluorinated alkyl substances, polybrominated diphenyl ethers, and pharmaceuticals and personal care products (PPCPs) (Bakir et al. 2014a; Caruso 2019; Atugoda et al. 2021; Khalid et al. 2021). The potential of microplastics to act as carriers for a range of contaminants could pose additional threats to ecosystems, wildlife, and potentially human health (Bakir et al. 2014b; Atugoda et al. 2021; Khalid et al. 2021).

Besides serving as vectors for various pollutants, the interaction between microplastics and microorganisms has recently become a topic of great interest (Dong et al. 2021; Stenger et al. 2021; Sooriyakumar et al. 2022). Microorganisms can attach to the surface of microplastics and form biofilms within a relatively short time (Harrison et al. 2014; Khatoon et al. 2018). For instance, studies have shown that bacterial communities quickly colonised LDPE when exposed to coastal sediments, demonstrating the speed of this process within just a week (Harrison et al. 2014). Various factors, including surface texture, hydrophobicity, and the presence of chemical additives, have been identified as influencing the formation of biofilms on the surface of microplastics (Tu et al. 2020; Wang et al. 2021a, b; Sooriyakumar et al. 2022). According to Sooriyakumar et al. (2022), the surface characteristics of microplastics inherently promote microbial colonisation. Microplastics featuring irregular or rough surfaces serve as attachment points for microorganisms, creating microenvironments conducive to microbial colonisation and the development of biofilms. Similarly, the hydrophobic nature and smaller particle size of microplastics, which offer a larger surface area relative to their volume (Atugoda et al. 2021), provide additional sites for microbial attachment.

Biofilms encompass a variety of microorganisms existing in symbiotic collaboration (Sooriyakumar et al. 2022). Among the microorganisms affiliated with microplastics, autotrophs like photosynthetic bacteria and algae can independently produce their own sustenance. Conversely, heterotrophs depend on the surplus food generated by autotrophs during their coexistence (Bolan et al. 2020). Consequently, microplastics offer a distinctive habitat for microorganisms and have been denoted as "plastispheres" (Zettler et al. 2013). Plastispheres signify a novel ecological niche that can have significant environmental implications. As the research on microplastics and their interactions with microorganisms grows, our understanding of the ecological consequences and potential impacts on marine ecosystems will become more comprehensive. Biofilm formation can modify the physical and chemical attributes of microplastics, influencing their degradation and dispersion in the water column (sinking and buoyancy rate), their capacity to adsorb and transport various contaminants, and ultimately the trophic transfer and environmental release of adsorbed chemicals (Rummel et al. 2017; Stabnikova et al. 2021; Stenger et al. 2021; Vaseashta et al. 2021; Zhang et al. 2021). It has been established that a variety of microbial communities, such as toxic, pathogenic, invasive, or plastic-degrading species, can be found in biofilms on microplastics (Zettler et al. 2013; McCormick et al. 2014; Oberbeckmann et al. 2014; Curren and Leong 2019; Li et al. 2019; Zhang et al. 2021). These potentially harmful microorganisms might be extensively disseminated in seawater, shielded by biofilms, endangering human health (Metcalf et al. 2022a). A plethora of investigations have been carried out regarding microplastics in marine environments, addressing diverse topics (Ajith et al. 2020; Gola et al. 2021; Ahmed et al. 2021; Perumal et al. 2022; Biswas and Pal 2023), including their function as transporters for various contaminants (Bakir et al. 2014a; Caruso 2019; Atugoda et al. 2021; Khalid et al. 2021; Kumkar et al. 2023). However, recent focus has shifted towards a more specific examination of biofilms associated with microplastics (Kumar et al. 2022a; Kaur et al. 2022). Furthermore, previous research has indicated that obtaining a more profound understanding of the processes involved in biofilm formation on microplastic surfaces necessitates more detailed insights into the plastisphere compared to naturally occurring substrate-associated aggregates, such as microbial communities on cellulose, wood, and glass (Sooriyakumar et al. 2022).

The projected amount of plastic debris in the Earth's oceans is expected to reach 5.25 trillion particles, with microplastics comprising 92%. Approximately 80% of these plastic particles are linked to terrestrial sources (Coyle et al. 2020; Cincinelli et al. 2021; Gola et al. 2021). Notably, the benthic region of the Indian Ocean is reported to have the highest prevalence of microplastic contamination, quantified at 4 billion fibres per square kilometre (Eriksen et al. 2014; Woodall et al. 2014). Thorough examinations of microplastic occurrence within sedimentary substrates have been extensively documented across various coastal regions of India, including Goa (Veerasingam et al. 2016), Tamil Nadu (Karthik et al. 2018), Karnataka (Tiwari et al. 2019), Kerala (Robin et al. 2020), Odisha (Patchaiyappan et al. 2021), Andhra Pradesh (Sambandam et al. 2022), West Bengal (Kumar et al. 2022b), the Andaman and Nicobar Islands (Goswami et al. 2020; Patchaiyappan et al. 2021), and Maharashtra (Kumkar et al. 2023; Tiwari et al. 2019). However, there is currently a research gap concerning biofilms' presence and prevalence on microplastics along the Maharashtra coast. Moreover, we speculate that significant differences in biofilm density on microplastics obtained from sediment and water samples along the Maharashtra coast are likely due to the region's diverse demographic zones, human activities, and land use patterns. Additionally, the diversity in microplastic origins offers the potential to gain insights into the relationship between microbial diversity residing on the surfaces of different microplastic polymers and the geographical locations from which they are sourced. To address these gaps, our study pursues the following objectives: (1) Collection and examination of microplastics obtained from coastal sediment and water along the Maharashtra coast to discern their surface morphology and disintegration patterns, (2) To identify the microbial communities associated with the microplastics, (3) Characterisation of the microplastics and investigation into the interplay between the diversity of microbial life inhabiting on the surfaces of microplastic polymer types and the specific regions of their origin.

Materials and Methods

Study Locations

Maharashtra, ranked as the second most populous state and covering the third-largest geographical area in India, is bounded by the Arabian Sea on one side and the Western Ghats Mountain ranges on the other. The state encompasses five coastal districts: Thane, Raigad, Mumbai, Ratnagiri, and Sindhudurg. Plastic pollution from anthropogenic activities poses a significant challenge along the Maharashtra coast, with major contributors including densely populated regions, urban development, industrial zones, fishing ports, and coastal tourism (Kumkar et al. 2023). In our investigation, we deliberately chose ten distinct sample sites labelled S1 to S10, strategically positioned across the five coastal districts mentioned above, thus providing a representative overview of the entire Maharashtra coastline (Fig. 1a).

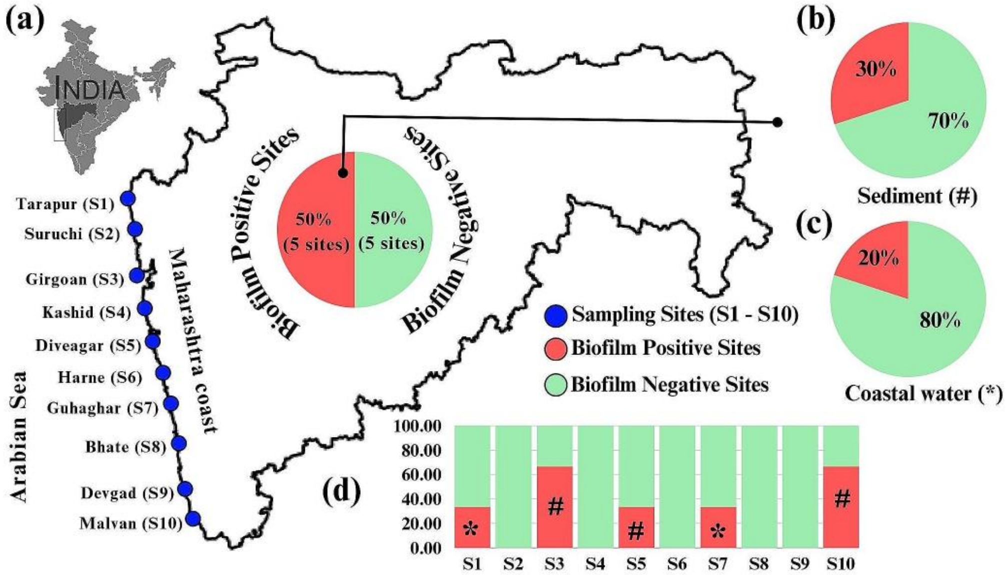

Fig. 1 **(a)** Geographical distribution of the Maharashtra coastline showing collection sites ($n=10$); pie chart within the map of Maharashtra state indicates overall biofilm-positive and negative sites; **(b and c)** Percent distribution of biofilm-positive sites in sediment and coastal water, respectively; **(d)** Site-specific distribution of biofilm-positive microplastics ("#" and "*" indicates biofilm found on microplastics isolated from coastal sediment and water samples, respectively)

Sampling, Microplastic Isolation and Characterisation

In August 2021, we gathered beach sediment and surface water samples from ten locations along the Maharashtra coast, following the methodology outlined by Karthik et al. (2018) and Robin et al. (2020). A slight alteration was made to the procedure, considering our particular emphasis on investigating biofilms on microplastic particles. This modification was implemented to avoid the potential detachment of biofilms from microplastics during sample processing and extraction, which chemical interactions could influence. Stainless steel scoops were employed at each assigned sampling location to collect the uppermost 5 cm of beach sediment. Sediment samples were acquired from three quadrats (30 cm x 30 cm) on each beach, spaced 100 m apart. The sediment was placed in stainless-steel containers and sieved through a 5-mm mesh to remove particles larger than 5 mm. Likewise, 100 L of water were gathered from a depth of 0 to 25 cm below the surface for water samples, utilising a 5 L stainless-steel sampling container. The obtained water was subsequently strained through a stainless-steel sieve with a mesh size of 100 μm.

The sieves underwent thorough inspection, and microplastic particles ranging from 3 to 5 mm in size were manually separated from the sediment and water samples using sterile forceps. These isolated particles were then individually placed into glass vials for subsequent analysis. The focus on choosing larger microplastic particles was intentional in clearly separating them from the samples. This helps to minimise the chance of unintentionally including microplastics from outside sources. In an aseptic laboratory environment, microplastic particles from each sample location were pooled together. Six particles were randomly chosen from this combined microplastic pool for each sampling location—three from water samples and three from sediment samples. To maintain an unbiased selection of microplastic particles, an anonymous individual, unaware of the sample sites or any sampling data, made the particle selections. The glass vials containing the microplastic particles were tightly sealed and covered with aluminium foil to prevent contamination from external sources. The task of impartially selecting microplastic particles was meticulously carried out in a strictly aseptic environment.

The selected microplastic particles were thoroughly examined for their colour (red, green, blue, white, yellow, and black) and morphological characteristics using the Olympus SZ61 stereo zoom microscope. To identify the polymeric composition of the microplastics and determine their potential source, we employed Attenuated Total Reflectance Fourier-transform Infrared Spectroscopy (ATR-FTIR) (Ballent et al. 2016). Following ATR-FTIR analysis using the Thermo Scientific NicoletTM iS20, the spectrum data of the microplastic particles were compared to the OMNiC reference library.

Detection of Biofilms on Microplastics

We employed a high-resolution imaging method to comprehensively understand microplastic surface characteristics, biofilm formation, and changes induced by degradation. The goal was to explore microbial communities and uncover the importance of surface texture and topography in biofilm development. Scanning Electron Microscopy (SEM) was chosen for its effectiveness in revealing surface texture, topographical features, and attached materials on microplastic surfaces, including microorganisms like bacteria, fungi, and algae (Kumkar et al. 2021; Verma et al. 2022). This technique plays a crucial role in unveiling the complexities of microplastic disintegration due to weathering and ageing and understanding its potential role in carrying other contaminant pollutants. In our study, six microplastic particles, including three from sediment and three from water samples at each designated station, underwent preparation for SEM analysis. This preparation involved sputter-coating the particles with a thin layer of gold and imaging using a Scanning Electron Microscope (Tescan Mira3, Tescan Orsay Holding, Brno, Czech Republic).

Data and Image Analysis

All values that are given as %, mean ± standard error (M ± SE), or otherwise specified. PAST freeware Version 4.03 (Hammer et al. 2001) was used for the data analysis.

Results and Discussion

The present research contributes to our comprehension of the prevalence and distribution of biofilm formation on microplastics along the Maharashtra Coast, shedding light on the interactions between microplastics and microbes. These microbial communities, consisting of autotrophs, photosynthetic algae, fungi, and bacteria, form biofilms and exhibit rapid growth on the solid surfaces of microplastics (Bolan et al. 2020; Sooriyakumar et al. 2022). Upon scrutinising microplastic samples from the ten sampling sites, our investigation unveiled the presence of biofilms on microplastic particles collected from half of these sites (50%) (Fig. 1a). Among the samples positive for biofilms, 30% were derived from sediment while 20% were obtained from coastal water samples (Fig. 1b and c). Microplastic particles from sampling sites S1, S3, S5, S7, and S10 were identified as biofilm-positive (Fig. 1d). Among the designated sampling stations, the Girgaon (S3) and Malvan (S10) locations displayed the highest percentage (66.66%) of biofilm positive microplastic (Fig. 1d). In contrast, the Tarapur (S1), Diveagar (S5), and Guhagar (S7) stations had 33.33% of microplastic particles exhibiting biofilm (Fig. 1d). The formation of biofilms and the degree of microbial colonisation can be significantly influenced by various factors, including surface roughness, polymer composition, hydrophilic or hydrophobic characteristics, and other relevant attributes (Sooriyakumar et al. 2022). While microplastics themselves lack direct porosity, their surfaces can develop pits, fissures, and cavities through weathering, abrasion, and photo-oxidation processes (Atugoda et al. 2021; Kumkar et al. 2021, 2023). The presence of such morphological features, including flakes, eroded surfaces, trenches, and cavities, not only facilitates the adsorption of pollutants like heavy metals (Turner and Holmes 2011; Li et al. 2018; Shruti et al. 2019; Atugoda et al. 2021; Khalid et al. 2021; Kumkar et al. 2021, 2023) but also encourages the formation of biofilms (Tu et al. 2020; Wang et al. 2021a, b; Sooriyakumar et al. 2022). Using detailed SEM analysis, Tiwari et al. (2019) demonstrated that microplastics collected from Girgaon exhibit rougher surfaces than those from other places. Our present data align with this past trend, revealing a noticeable pattern of increased biofilm colonisation on microplastic particles with rough surfaces. We observed more microplastic particles with positive biofilm presence in samples collected from Girgaon and Malvan than in other locations. This is likely associated with the rougher surfaces of the microplastics obtained from these specific areas. This holds particular significance as both microplastics and the pollutants they absorb can accumulate in substantial quantities in the environment, potentially exerting adverse effects on ecosystems and food chains. Verdú et al. (2023) showed that biofilm growth considerably affects the vector transport of personal care products such as triclosan-adhered polyethylene microplastics into *Daphna magna*. Furthermore, Guan et al. (2020) conclusively proved that biofilm formation increased microplastics' function in the transport of trace metals, such as nickel (Ni), copper (Cu), zinc (Zn), and cadmium (Cd) in the aquatic environment. In a recent study by Kumkar et al. (2023), various emerging pollutants were identified in the coastal waters of Maharashtra. These pollutants included heavy metals such as vanadium (V), copper (Cu), arsenic (As), nickel (Ni), cobalt (Co), chromium (Cr), cadmium (Cd), and lead (Pb). Additionally, pharmaceuticals and personal care products such as metoprolol, tramadol, venlafaxine, triclosan, bisphenol A, and bisphenol S were detected, along with plasticisers like di-n-butyl phthalate (DBP) and bis(2-ethylhexyl) phthalate (B_2EHP or DEHP). Moreover, earlier investigations have showed weathered microplastic particles' capability to carry these pollutants into fish (Kumkar et al. 2021, 2023; Verma et al. 2022). Considering that fish and fish-related products constitute a primary food source for the population residing in the coastal regions of Maharashtra, the presence of diverse pollutants along the Maharashtra Coast (Kumkar et al. 2023), the transport capacity of microplastics for these pollutants (Li et al. 2018; Kumkar et al. 2021, 2023), and the potential enhancement of microplastics uptake by marine organisms through biofilms (Fabra et al. 2021), pose a substantial threat to aquatic biota and human health.

Among the five distinct morphological categories considered (Supplement 1a), only microplastic fragments and beads exhibited positive biofilm presence (Fig. 2a). In this context, microplastic fragments were notably prevalent, constituting 85.7% of instances with associated biofilms, while beads accounted for 14.3%. Regarding colour, white microplastics showed the highest occurrence of biofilm, making up 28.5% of the total instances, followed by other colour categories, each representing 14.3% (Fig. 2b). Notably, white-coloured microplastics have often been observed in the digestive systems of marine fish (Tanaka and Takada 2016; Pan et al. 2021). Previous research has investigated the impact of microplastic colour and biofilm growth on the ingestion and susceptibility of species to such particles (Peters and Bratton 2016; Ory et al. 2017; Naidoo et al. 2020; Kumkar et al. 2021). This study analysed the polymeric composition of 60 microplastic particles using ATR-FTIR. Spectral analysis (Supplement 1b) revealed the following polymers: polypropylene (PP), polyethylene (PE), polystyrene (PS), and polyvinyl chloride (PVC). The per cent distribution of biofilm-positive microplastic polymers along the Maharashtra coast is as follows: PP (42.8%), PE (28.6%), PS (14.3%), and PVC (14.3%), respectively (Fig. 2c). Over 70% of the polymers identified in microplastic particles consisted of PE and PP. This finding is reasonable, considering that the study sites were either urban (Urban area) or suburban to rural (Tourism) areas, where disposable items like food wrappers, straws, water and beverage bottles, wire insulation, plastic bags, and sachets for personal care products (shampoo, conditioners, and scrub) are commonly utilised. It is crucial to highlight that PP, PE, PS, and PVC have demonstrated a higher affinity for a range of environmental contaminants, including heavy metals and hydrophilic and/or hydrophobic chemicals (Amelia et al. 2021). Specifically, PE exhibits the highest affinity for heavy metals, while PS shows the highest affinity for hydrophilic and hydrophobic chemicals (Amelia et al. 2021). In this context, the presence of biofilm on white microplastics in the study area, coupled with the prevalence of polymer types exhibiting a high affinity for pollutants, presents a dual threat to marine life, raising concerns.

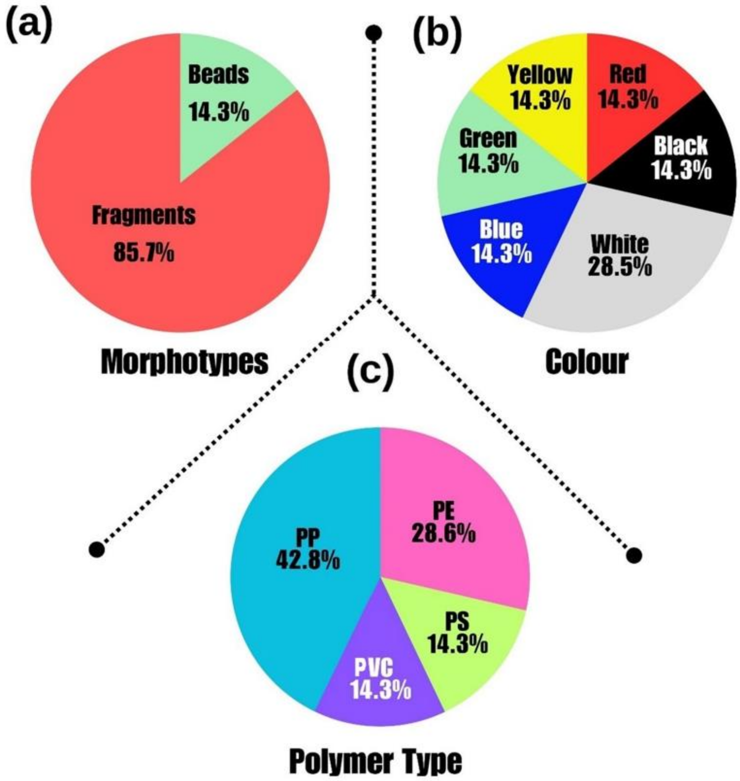

Fig. 2 Categorisation of microplastics isolated from coastal sediment and water sample across Maharashtra state according to **(a)** Morphotype; **(b)** Colour, and **(c)** Polymer type

Plastispheres are recognised for fostering the growth of biofilms, hosting diverse microbial communities (Oberbeckmann et al. 2015; Frère et al. 2018; Debroy et al. 2022; Miao et al. 2021). It is crucial to emphasise the progression from biofilm formation to subsequent biodegradation processes, leading to the deterioration of the polymer's physical structure (Debroy et al. 2022). In our study, all plastispheres exhibited rough surfaces characterised by cracks, fissures, and trenches (Fig. 3a–d). This highlights the complex interplay among microplastics, biofilms, and polymer integrity, contributing to our understanding of their ecological implications. According to Eich et al. (2015), diatoms constitute a significant element of marine biofilms and play a crucial role in their formation and activity. SEM micrographs offer tangible evidence of the substantial colonisation of plastic surfaces by diatoms (Fig. 3a and b) and fungal colonisation (Fig. 3c and d) in the majority of the examined samples. Diatoms were prevalent on microplastic particles obtained from coastal waters of Maharashtra, while fungi (fungal hyphae or spores) were observed to have colonised 60% of the microplastic particles from sediment samples. Similar findings were reported by Eich et al. (2015), where floating plastic exhibited an increased abundance of diatoms. Hence, it is evident that microplastics can act as selective artificial microhabitats, attracting distinct microbial communities.

Though fungal pathogens represent one of the most diverse microbial groups and exhibit a propensity to adhere to plastics, colonising and persisting on plastic pollutants in the environment and posing a potential pathogenic threat, their significance has only recently gained attention from the scientific community (Gkoutselis et al. 2021; Akinbobola et al. 2024). For instance, A recent investigation by Akinbobola et al. (2024) revealed that biofilms linked with plastic could function as a unique reservoir facilitating the environmental persistence of the multi-drug resistant fungal pathogen *Candida auris* for up to 30 days, whether in freshwater or marine water. Additionally, *C. auris* could transfer from plastic beads to beach sand while retaining its pathogenic properties. In the current investigation, the presence of a substantial layer of extracellular polymeric substances (EPS)

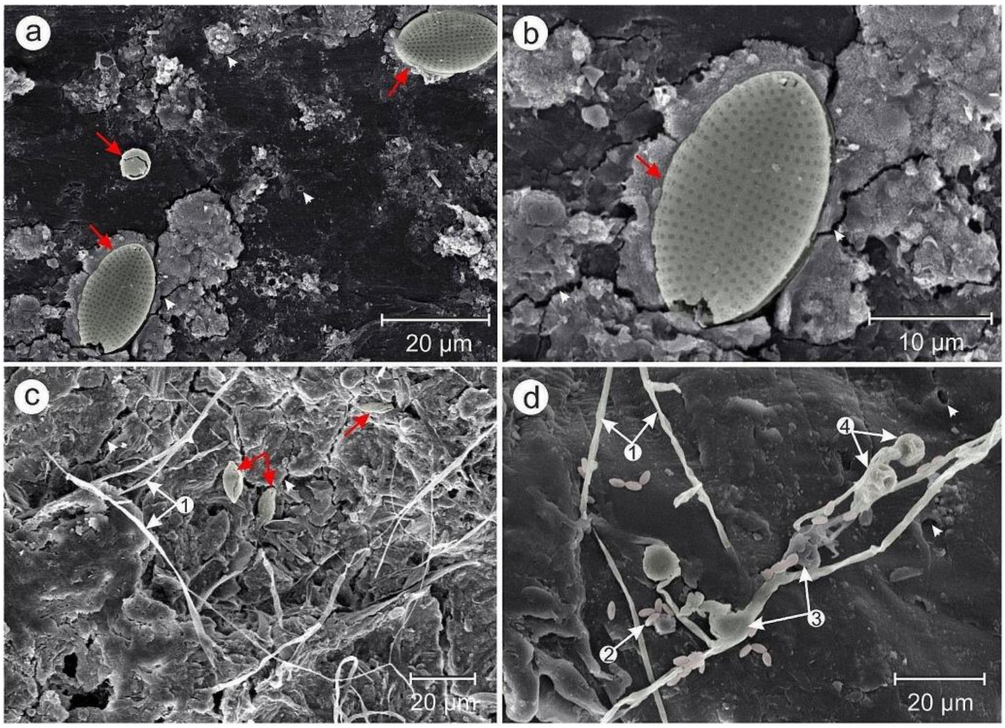

Fig. 3 Surface characteristics and microbial colonisation of microplastic particles observed using SEM: **(a and b)** Microplastic displaying a rough surface with evident cracks, crevices, and holes (white arrowheads), hosting diatom colonisation (red arrow); **(c and d)** Microplastic featuring fungal colonisation, showcasing intricate mycelial networks comprising hyphal filaments (1), clusters of conidia (2), compact mycelia (3), and conidiophore structures (4)

forming a biofilm is apparent on the plastisphere, along with distinct filamentous fungal structures such as vegetative and reproductive hyphae (Fig. 3c and d). As noted by Priyadarshanee and Das (2023), EPS consists of proteins, nucleic acids, and carbohydrates that are recognised for promoting microbial growth and keeping them clustered in the biofilm. Abundant conidia, the asexual fungal spores, are observed surrounding the fungal hyphae (Fig. 3d). Additionally, there were compact mycelia (Fig. 3d) and fungal hyphae distributed across the surface or forming filamentous networks. The presence of conidiogenous hyphae (conidiophore) was also noteworthy, indicating efficient fungal growth and reproduction within the plastisphere (Fig. 3d). Our results support an earlier study conducted on soil samples obtained from disposal areas within the city confines of Siaya, Western Kenya (Gkoutselis et al. 2021). These sites receive a significant influx of plastic waste and sewage water from both terrestrial and river sources. While the specific identification of the fungus was not possible in our study, it is reasonable to assume the potential presence of opportunistic human pathogenic fungi in these environments. Therefore, we suggest that future studies should focus on isolating and characterising the microbial community associated with plastispheres, particularly multi-drug resistant pathogens capable of enduring transitions across diverse environmental matrices, thereby possessing the potential for widespread dissemination within the landscape (Junaid et al. 2022a, b; Metcalf et al. 2022b; Khare et al. 2024). This would assist in reducing the possible risk imposed by these biofilms-associated microorganisms on human health.

Earlier studies have indicated that the microbial colonisation of microplastics is influenced by the type of polymer (Oberbeckmann et al. 2015; Frère et al. 2018). For example, marine bacteria exhibit a preference for polystyrene (PS) polymer, while algal microbes tend to favor polyvinyl chloride (PVC) surfaces (Eich et al. 2015; Miao et al. 2021), and diatoms are more prevalent on PS foam (Carson et al. 2013). In a previous investigation, Frère et al. (2018) found that microbial communities on polyethylene (PE) were more diverse than those on PS and polypropylene (PP). However, our observations revealed more variability, such as the colonisation of fungal hyphae and diatoms on the surface of PP, while PE and PS showed predominant colonisation by fungal hyphae and diatoms, respectively (Fig. 2a and d). However, more investigation is required to fully comprehend the processes driving the preferential colonisation of microorganisms on a certain kind of microplastic polymer.

Conclusions and Future Prospective

The results of the current study indicate that microplastics create distinct microenvironments that support a diverse range of microorganisms in the marine ecosystem, including diatoms and fungal species. The ageing of microplastics enhances microbial colonisation, and the type of microplastic polymer influences microbial colonisation patterns. Diatoms and fungal hyphae display diverse interactions with microplastic polymers. Notably, microplastics are hosts for various fungi reproductive stages, as evidenced by filamentous networks, mycelia, and conidiophores. However, the presence of potentially harmful bacteria and fungi within these biofilms necessitates further investigation. The prevalence of microplastics as microbial hosts may facilitate disease transmission, posing increased risks to human health and wildlife. Our study highlights the intricate relationship between microplastics and marine microorganisms, emphasising potential implications for ecosystems, human health, and animal welfare. Further research is imperative to fully understand these interactions and develop strategies to mitigate their adverse effects. Future investigations should prioritise three key areas: (1) employing molecular techniques like metagenomics to identify the microbes residing on microplastics; (2) assessing the ecological and epidemiological impacts of the plastisphere, and (3) investigating the influence of microplastic polymer type and morphology on biofilm formation and uptake by marine organisms.

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Declarations

Ethical Approval Not applicable.

Conflict of 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.

Competing Interests The authors declare no competing interests.

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Chapter 6

Biological invasion in aquatic ecosystem

Biological invasions have become a critical global issue, with aquatic ecosystems being particularly vulnerable to the spread of invasive species. Human activities, such as global trade and habitat modifications, have broken down natural biogeographic barriers, allowing non-native species to establish themselves in new environments. While many introduced species do not thrive, those that do can have significant ecological and economic impacts. Invasive species often disrupt native communities by altering food webs, introducing new predator-prey dynamics, and changing habitat structures. These disruptions are especially concerning in aquatic ecosystems, where strong trophic interactions make the effects of invasive species more profound, potentially leading to biodiversity loss and ecosystem degradation. Effective assessment and management of invasive species are essential to prevent these ecological disruptions. Modern techniques, such as environmental DNA (eDNA) analysis and iEcology, provide valuable tools for early detection and monitoring of invasive species. These methods offer increased accuracy and speed, allowing for timely interventions and better management strategies to mitigate the spread and impact of invasive species. By using eDNA analysis, researchers can detect the presence of invasive species through genetic material in the environment, even when the organisms themselves are not observed. Such methods are becoming indispensable in managing biological invasions and maintaining ecosystem balance.

In this chapter, two studies are presented focusing on the identification of the invasive fish species Perccottus glenii (Chinese sleeper) in Czechia. The first study marked the initial discovery of P. glenii in the Elbe River basin. The species was identified using iEcology methods when a fisherman posted an image of the fish on social media. This prompted further investigation, leading to the confirmation that the species had been present in interconnected ponds and streams for nearly a decade. P. glenii poses a serious ecological threat due to its predatory nature, capable of significantly altering the ecosystems it invades. Early detection and control measures are critical to prevent its further spread across Czech waters.

To support the management of this invasive species, a second study developed a species-specific environmental DNA (eDNA) assay for rapid detection of P. glenii. This assay targeted a specific genetic marker (COI region) and demonstrated high sensitivity in detecting the presence of the species in water samples. Tests from various water bodies, including the Klabava Reservoir and Karásek Pond, successfully amplified P. glenii eDNA, confirming the species' presence. However, no evidence of P. glenii was found in Loukotovský Pond. These findings suggest an expanding invasion range in Czechia and highlight the importance of continuous monitoring. The eDNA-based method proved to be an effective and reliable tool for early detection, facilitating the timely implementation of management strategies. This method has potential applications across Europe for monitoring invasive species, reinforcing the importance of proactive measures to protect aquatic ecosystems from the negative effects of biological invasions.

6.1. First record of highly invasive Chinese sleeper *Perccottus glenii* Dybowski, 1877 (Perciformes: Odontobutidae) in the Elbe River Basin, Czechia

Aquat Ecol
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First record of highly invasive Chinese sleeper *Perccottus glenii* Dybowski, 1877 (Perciformes: Odontobutidae) in the Elbe River Basin, Czechia

Marek Šmejkal · Ondřej Dočkal · Kiran Thomas · Chandani R. Verma · Pradeep Kumkar · Lukáš Kalous

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Abstract The Chinese sleeper (*Perccottus glenii*) has invaded European freshwaters, and we present evidence of its first documented occurrence in the Elbe River basin in Czechia. The individual fish was caught by a fisherman and posted on social media. After immediately contacting the person in question, we obtained a live fish from him. The Chinese sleeper appears to have been present in interconnected ponds and streams for ten years and may have spread over a larger area. We recommend that eradication measures be implemented to prevent further spread.

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Statement of Significance The Chinese sleeper has invaded many European countries in the last 50 years and is expected to invade western Europe because of the favourable conditions for its establishment. This finding indicates that it could spread in the Elbe River Basin, which could have serious impacts on floodplain and wetland ecosystems. To prevent this scenario, eradication measures should be implemented.

M. Šmejkal (✉) · K. Thomas
Institute of Hydrobiology, Biology Centre of the Czech Academy of Sciences, Na Sádkách 7, České Budějovice 370 05, Czech Republic
e-mail: marek.smejkal@hbu.cas.cz

O. Dočkal
Nature Conservation Agency of the Czech Republic, Regional Office Olomoucko, Litovelské Pomoraví, Czech Republic

Keywords Invasive species · Culturomics · iEcology · Biodiversity crisis · Rotan · Amur sleeper

Introduction

Introduced invasive alien fish species (IAS) are one of the greatest threats to freshwater diversity, affecting ecosystems through novel interactions of predation, competition, or parasite/disease transmission (Reshetnikov 2004; Dextrase and Mandrak 2006). The Chinese sleeper (*Perccottus glenii* Dybowski, 1877) is a significant threat to wetlands and ponds in Europe and is currently listed among the priority IAS in the European Union (European Commission 2016). The Chinese sleeper occurs naturally in eastern Eurasia, ranging from the Amur Basin in Russia through north-eastern China to the north of the Korean Peninsula (Reshetnikov 2004).

The Chinese sleeper was introduced from its original range into various European freshwaters

K. Thomas
Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

C. R. Verma · P. Kumkar · L. Kalous
Department of Zoology and Fisheries, Czech University of Agriculture in Prague, 165 21 Prague 6-Suchdol, Czech Republic

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(Reshetnikov 2010). The history of its invasion in Europe starts with its release from aquaria in Saint Petersburg and Moscow in 1916 and 1940, respectively (Reshetnikov 2004). Subsequently, in the last 50 years, it has spread mostly due to human interventions in Belarus, Ukraine, Lithuania, Poland, Latvia, Hungary, Slovakia, Romania, Serbia, Bulgaria, Estonia, Moldova or Croatia (reviewed in Reshetnikov 2004, 2010). Last reported advancement is to localized area of Danube River Basin in Germany (Nehring and Steinhof 2015) and Finland (Pihlström et al. 2022). As the Chinese sleeper has been detected in three out of four neighbouring countries of Czechia, a high-risk potential for this country was assumed.

Chinese sleeper may become a dominant species in stagnant waters of floodplains and lentic waters with macrophytes and has serious impacts on insects, fish, and amphibians. Considerable declines were recorded in insect diversity due to Chinese sleeper predation on dragonflies and beetle larvae or adult aquatic beetles (Reshetnikov 2003). Its impact on amphibians is alarming, as some species of newts (*Triturus cristatus*, *Lissotriton vulgaris*) and frogs (*Rana* sp., *Pelophylax lessonae*) have been reported to disappear from the invaded sites. In addition, their feeding niche overlaps with the European mudminnow (*Umbra krameri*), crucian carp (*Carassius carassius*) and sunbleak (*Leucaspius delineatus*), already threatened species with very similar preferred habitat (Koščo et al. 2008; Glińska-Lewczuk et al. 2016), resulting in interspecific competition between native species and the Chinese sleeper. Another described interaction between the Chinese sleeper and native fishes is predation on developing fish eggs and smaller size classes of fish (Reshetnikov 2001), which can effectively eliminate other fish species in small water bodies. Therefore, any introduction of this IAS into previously pristine areas should be monitored with great awareness, and eradication campaigns are recommended to control its spread (Nehring and Steinhof 2015; Pihlström et al. 2022).

For the Chinese sleeper, there are generally three typical pathways for human-caused introduction, which include recreational angling, the ornamental fish trade, and aquaculture. The beginning of its invasion history in Europe is associated with aquarium fish breeding in Moscow (Reshetnikov 2004). However, the further spread is more likely due to stocking for aquaculture, with the first major event occurring in Ukraine (Reshetnikov 2010). Another pathway, as with other highly invasive fishes, as it is the transport of bait fish for angling (Kalous et al. 2013). Finally, source populations may naturally spread downstream, especially during elevated floodplain river levels where surrounding unconnected pools serve as source populations for the colonization of downstream areas (Reshetnikov 2013).

Because of the difficulty of monitoring freshwaters and the high cost compared to surveys in terrestrial environments, harnessing available sources of information collected from the public can help monitor biodiversity with particular attention to IAS in freshwater (Jarić et al. 2020). Jarić et al. (2021) state that images posted on social media are important sources for detecting IAS and that images can be used to identify species and detect new introductions or range expansions. For example, the use of culturomics (human interaction with nature), citizen science (use of data collected by citizens for research/conservation), or iEcology (use of internet data generated for other purposes to study nature) can help reveal advances in the range of IAS. Because angling is a widespread outdoor activity, especially in the western world, posting on social media or direct involvement of anglers in IAS monitoring can help track and suppress IAS (Jarić et al. 2021).

The present note describes the first occurrence of a Chinese sleeper in the Czech Republic within the Elbe River Basin, which was identified when posted by angler on social media.

Materials and methods

The picture of a fish appeared in the Facebook group "Ryby, které mizí z našich vod. Karas obecný, Slunka obecná a další naše …" [Fishes disappearing from our waters. Crucian carp, sunbleak and other ours…] on January 10, 2023, together with the question whether it is "hlaváčkovec glenův", which is the Czech name of the Chinese sleeper (Fig. 1a). We contacted the Facebook user to confirm the location, date and other circumstances of the discovery and to get permission to share his photograph for publication purposes. The person kept the fish alive until it was picked up by authors on January 13, 2023, and brought to the laboratory of the Institute of Hydrobiology, Biology

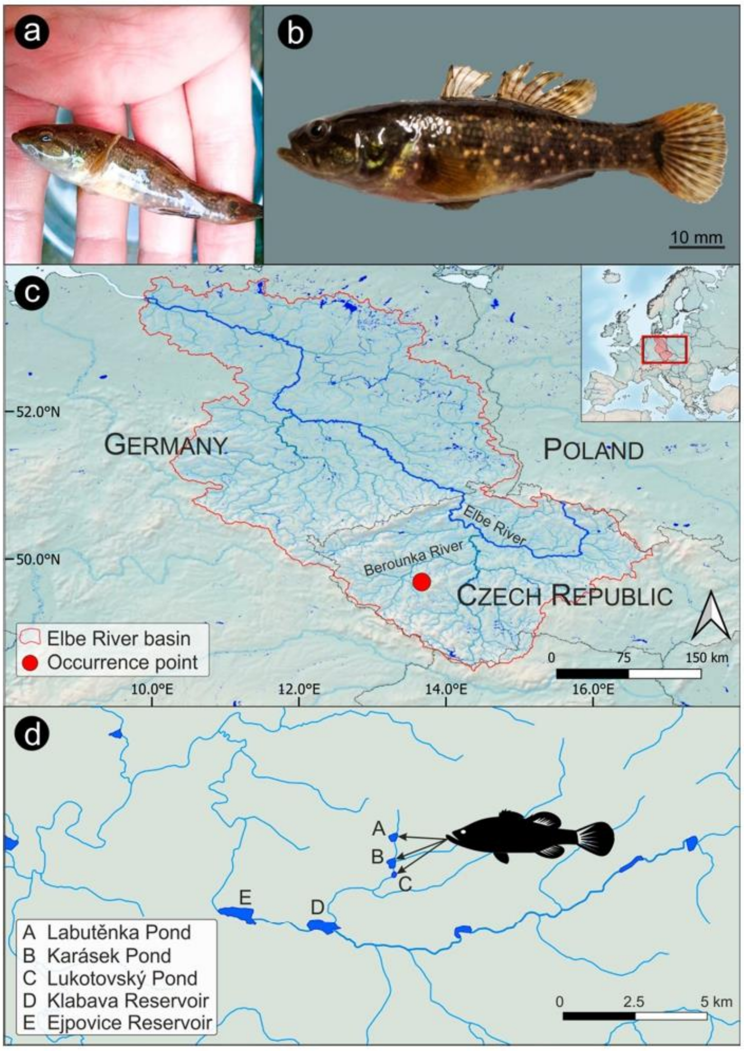

Fig. 1 First record of the Chinese sleeper (*Perccottus glenii*) in the Elbe River Basin and Czechia: **a**—the picture posted on social media, **b**—the picture of the specimen NMP6V 146359 taken before conservation (total length 79 mm), **c**—the position of the record in the Elbe River Basin, **d**—the system of three ponds interconnected with stream, where Chinese sleeper presence is reported by citizens, and surrounding larger water bodies. Maps were drawn using the QGIS (QGIS Development Team 2018) using layers of the State Administration of Land Surveying and Cadastre (CUZK 2023)

Centre of the Czech Academy of Sciences in České Budějovice. There, the specimen was killed by an overdose of anaesthetics (MS -222) and morphometry (standard length, total length in mm, weight in g) and meristic counts were performed according to Horvatić et al. (2022) following the guide for first fish records (Bello et al. 2014). DNA was isolated from the finclip using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer and further used in PCR to amplify the desired mtDNA cytochrome oxidase subunit I (cox1) gene with primers FishF1 and FishR1 (Ward et al. 2005) with an annealing temperature of 53 °C and sent to Macrogen, Inc. Korea for sequencing. A picture was taken (Fig. 1b), and the specimen was fixed in 4% formaldehyde solution and stored in the depository of the National Museum in Prague under collection number NMP6V 146359.

On January 13, 2023, we contacted the company managing the pond to obtain more information on the possible origin of the Chinese sleeper and other related facts about its presence in the fish stock. In addition, we analysed the potential spread pathways downstream from the map and contrasted them with the information obtained from the company, as emptying the pond is an important source of Chinese sleeper to streams downstream (Nehring and Steinhof 2015).

Results and discussion

The fish comes from Karásek Pond in Osek u Rokycan municipality (N 49° 46′ 20″ E 13° 35′ 17″), altitude 392 m.a.s.l. The pond with an area of 8.2 ha is situated in western Bohemia on the Osecký stream, a third-order tributary of the Berounka River, Elbe River Basin (Fig. 1c–d). The fish was collected on January 8, 2023, during fish harvest in the stream below the dam. The morphometric and meristic characteristics were consistent with the species delimitation according to Horvatić et al. (2022). The specimen had a standard length of 67 mm, a total length of 79 mm, and a weight of 8.1 g. Eight meristic features on the left side of the body matched the species description: six rays in the first dorsal fin, 13 rays in the second dorsal fin, 13 rays in the pectoral fin, 4 rays in the ventral fin, 9 rays in the anal fin, 38 scales in the lateral line, 14 scales in the transverse row, 9 scales in the circumpenducular row (Horvatić et al. 2022). The obtained sequence was deposited under accession number OQ561461 in Genbank NCBI, and the BLAST result showed the 100% similarity with the reference sequences of *Perccottus glenii* deposited in the GenBank under the number MK439912 with the coverage of 100%. To our knowledge, the Chinese sleeper has not yet been detected in Czechia (Chobot and Němec 2017).

According to the fish farm manager, the species identified in this work as Chinese sleeper occurs sporadically in the Labutěnka, Karásek and Lukotovský ponds (Fig. 1d). The fish has been present in these three ponds for at least ten years. The pond manager linked the occurrence of the Chinese sleeper to the stocking of the Labutěnka pond with imported juvenile fish from Hungary. It seems very likely that the Chinese sleeper was present as contamination of the imported stock since it has been present in Hungary since 1997 (Reshetnikov 2010). Subsequent survey conducted by Nature Conservation Agency of the Czech Republic confirmed the presence of Chinese sleeper in stream below Lukotovský Pond.

The risk analysis of a non-native species is of considerable value in terms of analysing the potential negative impacts (ecological, economic, social) it may have on the invaded ecosystem and region (Verreycken 2015). Strong dispersal potential has been predicted in areas of central and western Europe, high climatic suitability, and higher potential for human-mediated transfer (pond aquaculture) place these regions at higher risk of invasion (Verreycken 2015). The Chinese sleeper is defined as an ecologically plastic species that has a high establishment capacity and can tolerate significant fluctuations in water level, temperature, and dissolved oxygen (Reshetnikov 2004). The broad diet of the Chinese sleeper indicates that it is a non-selective, opportunistic predator (Reshetnikov 2013; Verreycken 2015). This highly flexible feeding strategy favours the spread of Chinese sleeper in invaded waters (Grabowska et al. 2009). Considering its invasion history, it has spread remarkably fast over a very large area.

Chinese sleeper is among the animals defined by the European Union as IAS of exceptional interest (European Commission 2016) and is also among the major IAS of animals introduced into Europe for aquaculture and other related activities (Savini et al. 2010). Characteristics such as high resilience, broad habitat requirements, voracity, and ease of reproduction mean that the Chinese sleeper has significant impacts on species composition and especially on threatened species such as amphibians, aquatic insect species, and floodplain fishes (Rechulicz et al. 2015). Its resilience allows it to colonize habitats where other fish predators are unsuccessful due to the lack of oxygen, so the interconnected fauna of these ecosystems are likely not adapted to the change in predation.

Pond aquaculture has a tradition in Czechia, with the most important fish species being the common carp (*Cyprinus carpio*); however, this could provide fertile ground for the Chinese sleeper invasion of Czech waters. Pond aquaculture also produces coarse fish species that are partially flushed from the pond during draining and harvesting, and among them are often small fish species such as topmouth gudgeon (*Pseudorasbora parva*) (Musil et al. 2014). In addition to the release of Chinese sleeper from ponds into streams (Nehring and Steinhof 2015), populations established in streams may be fed by this influx of small prey (Bojarski et al. 2022). Additionally, common carp from pond aquaculture is commonly stocked into fishing grounds in rivers, which emphasises the importance of fish stock control (Kalous et al. 2018). The landscapes filled with ponds and reservoirs are more susceptible to fish invasions (Šmejkal et al. 2023), as has also been shown by the spread of IAS topmouth gudgeon and gibel carp (*Carassius gibelio*) (Lusk et al. 2010).

As for the situation in the identified occurrence area, three kilometres in the southwestern direction downstream is the Klabava Reservoir with an area of 39.2 ha and rich riparian vegetation. The Chinese sleeper prefers waters that either have weak flow or are stagnant, with well-developed aquatic vegetation, floodplains with well-developed vegetation, riparian zones of lakes, marshy waters, and even swamps (Reshetnikov 2004; Horvatić et al. 2022). Reaching this reservoir can support them with a suitable habitat for their establishment, where measures will be very difficult to implement.

The finding was immediately reported to the Ministry of the Environment of the Czech Republic. Although we do not currently know how far Chinese sleeper has spread from the identified area of occurrence, the finding initiated immediate steps to monitor and eradicate Chinese sleeper from the area to prevent it from spreading further downstream in the Elbe River watershed.

Since eradication of this species has proven to be very difficult, a possible action plan should consider a combination of methods such as drying out the invaded ponds for an extended period of time (e.g. one year), intensive electrofishing in the streams, and possible application of ammonia in the streams (Bogutskaya and Naseka 2002). Following this treatment, ponds may host a higher population of piscivorous fish to treat surviving individuals (Verreycken 2015). Because the occurrence of this species is currently very limited, the cost of these measures may prevent much higher eradication costs from being incurred in the near future.

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Authors contribution MŠ contributed to conceptualization, writing—original draft preparation, reviewing and editing, and funding acquisition. OD contributed to writing—original draft preparation, and visualization. KT contributed to data curation, and writing—original draft preparation. PK contributed to sequencing, data curation, reviewing and editing. CV contributed to sequencing, data curation, reviewing and editing. LK contributed to conceptualization, methodology, writing—original draft preparation, reviewing and editing, and funding acquisition.

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Declarations

Conflict of interest The authors declare no competing financial interests.

Ethical approval The field sampling methods and experimental protocols used in this study were performed by the guidelines and permission from the Ministry of Environment of the Czech Republic (OZPZ/2022/IN/1).

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6.2. Species-Specific eDNA based Detection of the Recently Identified Invasive Chinese Sleeper *Perccottus glenii* (Perciformes) in Czechia

Species-Specific eDNA based Detection of the Recently Identified Invasive Chinese Sleeper *Perccottus glenii* (Perciformes) in Czechia

Chandani R. VERMA[1†], Tushar KHARE[1,2,†], Pradeep KUMKAR[1], Marek ŠMEJKAL[3], Lukáš KALOUS[1,*]

[1] Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Czech Republic

[2] Department of Biotechnology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, India

[3] Institute of Hydrobiology, Biology Centre of the Czech Academy of Sciences, Na Sádkách 7, České Budějovice 370 05, Czech Republic

† First author

***Correspondence**

kalous@af.czu.cz (Lukáš Kalous)

Abstract

Aquatic biodiversity is being threatened by invasive non-native fish species (INFS). Of particular concern in Europe is the Chinese sleeper (*Perccottus glenii*), which has a broad predatory niche and has the potential to modify invaded ecosystems. *P. glenii* is a species that originated in East Asia and has spread quickly throughout Europe, mainly due to human introduction. This work aimed to develop a sensitive and quick eDNA assay for *P. glenii* early detection in freshwater systems in Czechia. A 124 bp amplicon was produced by designing a species-specific primer that targets the COI region. The assay was then optimised to achieve high detection efficiency. *Perccottus glenii* eDNA was amplified positively in environmental samples from Klabava Reservoir and Karásek Pond, indicating the presence of this invasive species; but no amplification was seen in samples from Loukotovský Pond. The findings imply an increasing invasion range in Czech waters and are consistent with recent reports of *P. glenii* presence in the upper Elbe River basin. With its excellent sensitivity and specificity, our eDNA-based approach provides a valuable tool for the early identification and control of *P. glenii* invasions. The method has the potential for broader use in other regions of Europe.

Key Words

Environmental DNA, Invasion, Quantitative PCR, Czech Republic, Rotan

Introduction

Invasive non-native fish species (INFS) belong to one of the greatest threats to freshwater biodiversity. INFS affect ecosystems through predation, competition, and parasite transmission (Clavero & García-Berthou 2005, García-Berthou 2007). One of the INFS threatening the freshwater biodiversity in Europe is the Chinese sleeper (*Perccottus glenii* Dybowski, 1877) (European Union 2017, Verreycken 2015). The natural distribution of this species is in East Asia, ranging from the Amur Basin in Russia through north-eastern China to the north of the Korean Peninsula (Berg 1949, Elovenko 1981). *P. glenii* is a small fish species reaching a maximum total length of 25 cm found in macrophyte-rich stagnant and slow-running waters (Verreycken 2015). Its adaptation to small floodplain pools involves anaerobic metabolism during periods of anoxia and hibernation in mud during droughts of up to several months (Bogutskaya & Naseka, 2002).

Chinese sleeper was introduced from its original range to Saint Petersburg and Moscow in 1916 and 1940, respectively (Reshetnikov 2004), with a likely source of aquaria release. Subsequently, it has spread to Belarus (Lukina 2011), Ukraine (Kvach 2012), Lithuania (Reshetnikov 2010), Poland (Grabowska et al. 2009), Latvia, Hungary (Harka 1998), Slovakia (Koščo et al. 2003), Romania (Nalbant et al. 2004), Serbia (Gergely & Tucakov 2003), Bulgaria (Jurajda et al. 2006), Estonia (Tambets & Järvekülg 2005), Moldova (Mosu 2007), Croatia (Ćaleta et al. 2011), Germany (Nehring & Steinhof 2015, Reshetnikov & Schliewen 2013), Finland (Pihlström et al. 2022), and Czechia (Šmejkal et al. 2023), and the majority of invasion history in more detail is reviewed in (Reshetnikov 2010).

The main pathways for the spread of *P. glenii* are human-induced introductions, including the release of baitfish by anglers (Kalous et al. 2013, Reshetnikov 2013), the ornamental fish trade (Reshetnikov, 2004), and, in particular, accidental introductions with aquaculture stocks (Reshetnikov 2013, 2010). Established populations tend to disperse downstream, especially during periods of high flow (Reshetnikov 2013).

The invasion of *P. glenii* into small wetland ecosystems poses significant threats to biodiversity, primarily due to its broad predatory behaviour across multiple trophic levels (Reshetnikov 2001, 2003). This species' predation has been linked to the local extirpation of amphibians such as newts (*Triturus cristatus*, *Lissotriton vulgaris*) and frogs (*Rana* sp., *Pelophylax lessonae*), and a decline in insect diversity, particularly among dragonfly larvae and aquatic beetles (Reshetnikov 2003). Within EU wetlands, *P. glenii* particularly endangers three native fish species: the European mudminnow (*Umbra krameri*), crucian carp (*Carassius carassius*), and sunbleak (*Leucaspius delineatus*), all of which have seen declines in conservation status at both national and international levels (Carpentier et al., 2008; Freyhof, 2011; Kottelat and Freyhof, 2007; Šmejkal et al., 2024). The presence of *P. glenii* leads to interspecific competition and predation on eggs and juvenile stages of these vulnerable species (Grabowska et al. 2009, Koščo et al. 2008, Reshetnikov 2001, 2008). Additionally, *P. glenii* serves as a vector for non-native fish parasites, potentially exacerbating impacts on native fish populations (Ondračková et al. 2012).

Due to the numerous threats posed by INFS, early detection is vital for effective management. Recently, alongside traditional methods, many researchers have begun using environmental DNA (eDNA) for early INFS detection. Various studies have utilised eDNA samples to detect the presence of *P. glenii* in different European regions (Clusa et al. 2021, Jeunen et al. 2022, Czeglédi et al. 2021), employing metabarcoding approaches for analysis. The eDNA assessment has frequently outperformed traditional ichthyological surveys (Hänfling et al. 2016, Cilleros et al. 2019), and the targeted amplification for active surveillance has demonstrated increased detection sensitivity for rare species compared to metabarcoding (Harper et al. 2018, Bylemans et al. 2019). Early detection of INFS through aquatic eDNA, focusing on targeted species-specific assays, has shown higher detection probability and sensitivity than traditional monitoring methods (Ardura et al. 2015, Dougherty et al. 2016, Simpfendorfer et al. 2016).

To our knowledge, only one study from Canada has focused on species-specific eDNA approaches (Roy et al. 2018), with no similar efforts reported for European waters. Roy et al. (2018) developed a species-specific qPCR assay for detecting *P. glenii* using the Cytochrome oxidase gene. However, considering local species diversity is crucial for designing primers with better specificity. Considering the vitality of the species-specific detection system, we designed a new pair of primers tailored to the fish species found in the Czechia.

The objective of this study is to develop a rapid eDNA assay to facilitate an investigation into the extent of the spread of invasive *P. glenii*, with a view to applying eradication measures and testing this method at the sites of its first occurrence in Czechia.

Materials and methods

Primer designing and screening

The species-specific primers targeting the mitochondrial 'Cytochrome oxidase subunit I' (COI) were designed using the partial COI sequence of *P. glenii* (OQ561461, recently detected invasion in Czech waters). The primers were designed using Geneious Prime 2024 (https://www.geneious.com/). The COI sequences of all fish species in Czechia were also considered to ensure the primer specificity to *P. glenii*. An in-silico assessment using Sequence Manipulation Suite: PCR products (https://www.bioinformatics.org/sms2/pcr_products.html) was performed to ensure the specificity of the designed primers. Primers that amplified other sympatric species were discarded. Details of the final primer pair are provided in Table 1.

In vitro primer testing and optimisation of real-time qPCR

The designed primers were evaluated for specificity and sensitivity using genomic DNA extracted from *P. glenii* tissue. DNA was extracted using the QIAamp® DNA Mini Kit as per the provided protocol. The DNAs' quality and quantity were verified by agarose gel electrophoresis and a Micro UV-Vis Spectrophotometer (Hangzhou LifeReal Biotechnology Co., Ltd), respectively. Specificity testing was conducted via PCR with VWR Taq plus DNA polymerase master mix (VWR Life Sciences, USA) following the manufacturer's instructions. PCR reactions were performed using a CFX 96 Real-Time PCR detection system (Biorad, USA). The optimal annealing temperature was determined using a thermocycler temperature gradient. The PCR protocol included initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, annealing temperatures ranging from 60°C to 46°C for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. PCR products were visualised on a 2% agarose gel. The annealing temperature that yielded a single, sharp band with the highest fluorescence was selected, and the bulk PCR product was obtained using this optimised temperature.

To optimise the qPCR assay, the bulk product was purified using the QIAquick Gel Extraction Kit (Qiagen, Redwood City, CA) as per the manufacturer's instructions and quantified with the Micro UV-Vis Spectrophotometer (Hangzhou LifeReal Biotechnology Co. Ltd). The amplicon copy number was calculated using the DNA concentration and amplicon size with an online calculator (https://www.omnicalculator.com/). The purified DNA was serially diluted to create standards ranging from 10^0 to 10^6 copies per reaction. The limit of detection (LOD) and limit of quantification (LOQ) were determined from the standard curve. The qPCR reactions were conducted with the KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal (Roche; SFUKB) under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 10 seconds and 51.6°C (optimised annealing temperature) for 30 seconds, concluding with a melt curve analysis.

Water Sampling and isolation of eDNA

Water samples were collected from three distinct waterbodies located in the Berounka River sub-basin of the Elbe River, western Bohemia, Czech Republic: Karásek Pond (KP) (49.773 N, 13.588 E), Loukotovský Pond (LP) (49.77 N, 13.587 E), and Klabava Reservoir (KR) (49.753 N, 13.559 E) (Fig. 1b & c). Notably, Karásek Pond has recently recorded the presence of *P. glenii* Šmejkal et al. (2023). Samples were collected in sterile 2.5-litre glass bottles, sealed in plastic bags, and transported in an icebox. Samples were processed within four hours of collection. High turbidity samples were prefiltered using a series of 38- and 21-micron plastic filters. Additionally, a water sample from the tank where a single *P. glenii* specimen was maintained for 15 days was taken as a positive control (PC), whereas normal water was used as a negative control (NC).

Each water sample, ranging from 250-500 ml, was filtered through a Nalgene® analytical filter funnel with a pore size of 0.45 μm. DNA was extracted using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany, product code 14900-100-NF) following the manufacturer's instructions. The extracted DNA was quantified using a Micro UV-Vis Spectrophotometer (Hangzhou LifeReal Biotechnology Co., Ltd) and subsequently used as a template for further assays.

qPCR analysis of isolated eDNA samples

The eDNA samples isolated from each location were tested in three technical replicates, along with positive and negative controls. Quantification was performed using the previously optimised qPCR assay. The target DNA copy number in the samples was calculated based on the standard curve generated in earlier steps.

Results

Following the methodological parameters, the primer pair targeting the COI region of *P. glenii* was designed (Table 1), yielding a PCR product with a 124 bp amplicon. Primer specificity tests showed a single distinct band at approximately 150 bp for *P. glenii*, with no bands for non-target species. A standard curve for qPCR optimisation was established using a concentration gradient of copy numbers. This standard curve demonstrated an efficiency of 96.15% and an R^2 value of 0.992 (Fig. 1d). According to the standard curve, a single target copy corresponded to a Cq value of 31.24. The assay's sensitivity determined the limit of detection (LOD) and limit of quantification (LOQ) to be six and twelve copies, respectively.

From the collected water samples, eDNA from Klabava Reservoir and Karásek Pond showed amplified products, consistent with positive controls. However, eDNA from Loukotovský Pond showed no amplification (Fig. 1e). The copy numbers for positive samples exceeded the detection and quantification limits, confirming the presence of *P. glenii*. No amplification was observed in the environmental negative control and the blank template control.

Discussion

Our investigation confirms the ongoing invasion of *P. glenii* in Czechia. A previous study reported its sporadic presence in the Labutěnka, Loukotovský, and Karásek ponds (Šmejkal et al. 2024), and we chose sampling sites in the present study based on these findings.

Our results confirmed the presence of *P. glenii* eDNA in the Karásek pond, indicating that the fish persists at this site despite apparently insufficient control measures implemented by the Nature Conservation Agency of the Czech Republic (NCA). Of greater concern is the corroboration of previously expressed apprehensions by Šmejkal et al. (2024) that *P. glenii* could potentially enter a much larger aquatic habitat downstream. Our survey results confirmed the presence of *P. glenii* eDNA in the Klabava Reservoir. However, whether the fish was already present in the reservoir before its discovery in the Karásek pond in 2023 remains unresolved despite evidence suggesting otherwise. The negative signal for the occurrence of eDNA of *P. glenii* in Loukotovský pond, which is situated downstream of Karásek Pond, may be attributed to the absence of this fish in this water body as a consequence of eradication measures implemented in the year 2023 (NCA, personal communication). Nevertheless, the absence of eDNA does not entirely preclude the possibility of the presence of *P. glenii* individuals. If the fish abundance is significantly reduced, the resulting eDNA concentrations may fall below the detection threshold, thereby explaining the absence of a positive eDNA signal (Takahara et al. 2012). Additionally, environmental conditions such as shallow depth, greater temperature fluctuation, and increased UV light can accelerate the degradation of DNA molecules in the pond water (Strickler et al. 2015, Robson et al. 2016, Buxton et al. 2017, Goldberg et al. 2018).

The rich riparian vegetation of the Klabava Reservoir provides a suitable habitat for *P. glenii* to establish, making its eradication from this site much more challenging, if not impossible. The Klabava Reservoir thus represents a potential source for further spread, mainly because recreational fishing occurs there, and anglers are known to effectively spread non-native species (Kalous et al. 2013).

This method can be effectively used to confirm the presence of *P. glenii* at the mentioned sites and other potential locations in Czechia, as well as in nearby Central European waters. The current study holds significant importance due to its strong focus on regional applicability. The methodology was developed using a *P. glenii* specimen sourced from the location of its initial detection in Czechia, ensuring that our approach is finely tuned to the local fauna and conditions. This region-specific adaptation makes our detection method particularly effective and readily applicable in the areas where this invasive species has recently been detected.

Species-specific eDNA analysis offers a cost-effective and efficient tool for monitoring aquatic habitats in the region and detecting *P. glenii*. Implementing this method could enable early detection and management of this invasive species. Early detection is widely recognised as a critical factor in the successful eradication of invasive species (Hill &Sowards, 2015). Therefore, it is essential to act promptly, as the invasion of *P. glenii* in Czechia is likely expanding but is still at an early stage.

Author Contributions

Conceptualisation and fieldwork: C.R. Verma, T. Khare, P. Kumkar, L. Kalous; laboratory work: C.R. Verma, T. Khare, P. Kumkar. All authors contributed equally to manuscript preparation.

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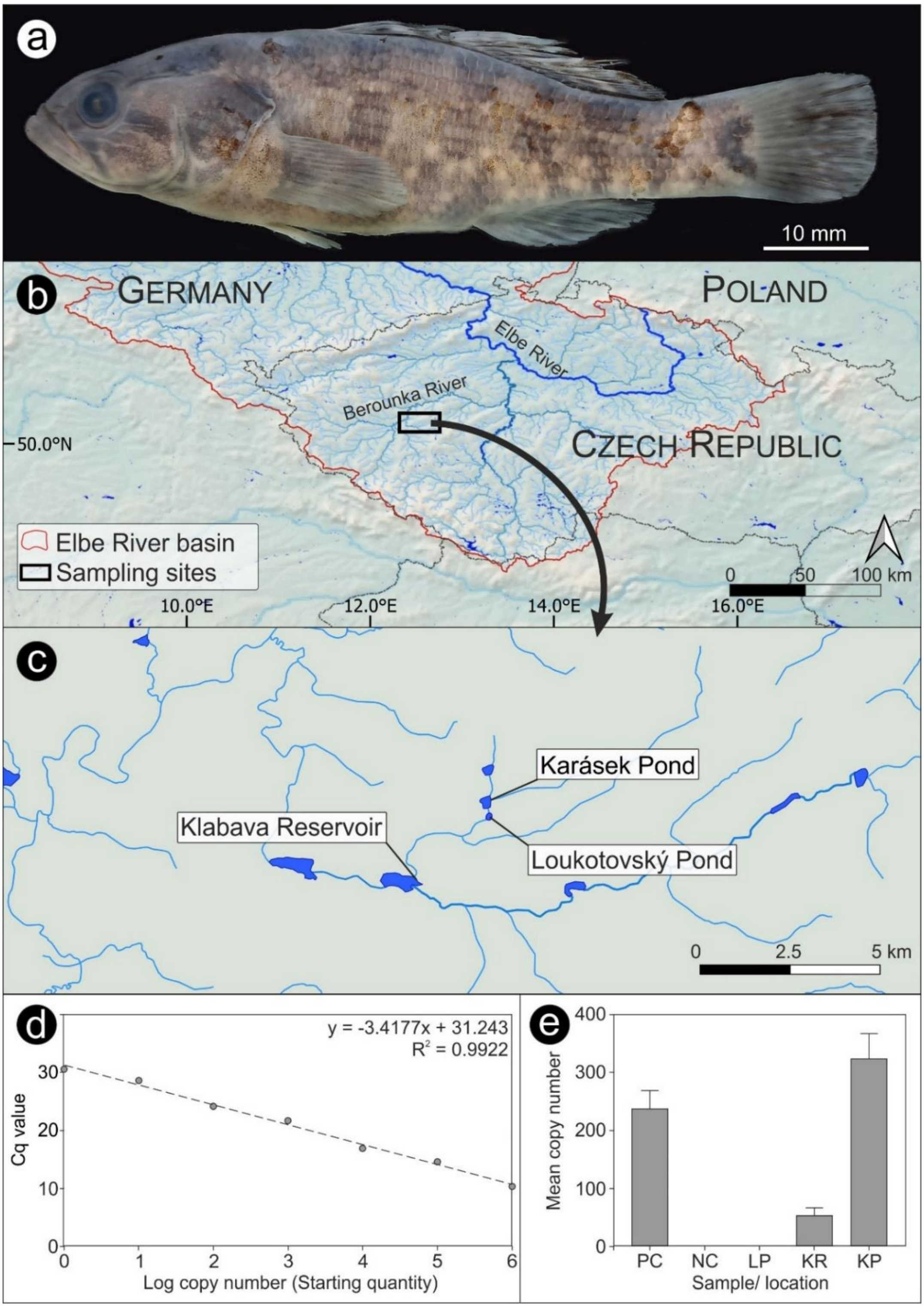
Table 1

Designed and finalised primer pair

Primer	Sequence 5'→3'	Length	Tm (°C)	Amplicon length
PercCOI-154F	TCCTCCTTCACTACTCCTAC	20 bp	52.3	124 bp
PercCOI-277R	GGTAAGGTCTACAGATGCTC	20 bp	51.9	

Figure 1 a. Photograph of a *Perccottus glenii* collected at Karásek Pond; b. Map illustrating the position of the sampling sites in the Elbe River Basin, Czech Republic; c. Specific sampling sites; d. standard curve generated by linear regression from the amplification of tenfold serial dilutions, ranging from 1 to 10^6 copies, with the Cq (crossing point) value representing the cycle threshold for quantification; e. Average initial target DNA copy number per reaction for each sample:, Positive control (PC), Negative control (NC), Loukotovský Pond (LP), Klabava Reservoir (KR) and Karásek Pond (KP).



Chapter 7

Summary Discussion

The environmental pressures in aquatic ecosystems play a critical role in shaping the morphological adaptations in fish, particularly in response to dietary requirements and competition for resources. Environmental factors, such as prey availability and interspecies competition, drive specific structural adaptations in fish, enabling them to occupy unique niches and reduce resource overlap (Peterson and Winemiller, 1997). For instance, certain carnivorous fish species, including the scale-eating or lepidophagous varieties, have evolved to exploit unique food sources by consuming parts of other fish, such as scales, skin, or fins (Sazima, 1980). This adaptation is particularly evident in *P. khavalchor*, a predatory catfish whose morphological and anatomical features have evolved to support scale-feeding, potentially as a response to competitive pressures from other predators in its environment (Gosavi et al., 2018).

In *P. khavalchor*, specialized adaptations like the ventrally positioned, wide mouth, and outward-facing premaxillary teeth facilitate efficient scale-eating. This unique dentition pattern, different from typical inward-facing teeth observed in other catfish species like *Pangasius macronema* and *Hypostomus pantherinus*, highlights an evolutionary shift towards lepidophagy (Mercy & Pillai, 1985; Diogo & Diogo, 2007; Pereira & Zanata, 2014; Zawadzki et al., 2021). Such morphological changes, including the asymmetric jaw structure (where the upper jaw is longer than the lower jaw), enhance the fish's ability to scrape scales from prey, underscoring the influence of ecological pressures on the development of functional anatomical traits (Peterson & Winemiller, 1997). This adaptation in *P. khavalchor* aligns with a broader evolutionary trend, where specialized feeding strategies such as lepidophagy are conserved within select fish families across freshwater and marine environments. As noted by Gosavi et al. (2018), these modifications are not only morphological but are also supported by behavioral and physiological changes, illustrating how species like *P. khavalchor* optimize both their structure and feeding techniques to exploit specific dietary niches.

Parasites are integral components of aquatic ecosystems, significantly influencing host physiology, behavior, and ecosystem dynamics. Representing one of nature's most prevalent modes of life, parasites have evolved across various taxa, adapting to exploit specific biological traits of their hosts (Poulin & Morand, 2000). In aquatic environments, the complex food web interconnects organisms, making them particularly susceptible to parasitic infections. These infections can alter host physiology and behavior, thereby affecting population dynamics, community structures, and even ecosystem health (Dobson et al., 2008; Hechinger et al., 2011). Parasites often serve as bioindicators of environmental health due to their sensitivity to ecological stressors, such as pollution and habitat changes (H.W.Palm, 2011). Furthermore, global environmental changes, including warming, have impacted parasite-host interactions, sometimes leading to increased parasitic loads that add further stress to aquatic organisms (Marcogliese, 2001; Cable et al., 2017). In our study of loach fish species from the northern Western Ghats of India, we observed a noticeable incidence of nematode infections, specifically *Contracaecum* nematodes, in loaches since 2010. This trend aligns with observations by Kumar et al. (2019), who reported increased *Contracaecum* infections in pelicans in southern India. Environmental pollutants, including heavy metals and organic waste from domestic sources, which are prevalent in the study area (Abhyankar et al., 2020; Gosavi et al., 2020; Nawani et al., 2016), may contribute to this rise in parasitic infections. Heavy metals and organic waste can disrupt the ecological balance, making organisms more vulnerable to parasitic infections (Marcogliese & Cone, 2001; Schludermann et al., 2003; Williams & MacKenzie, 2003).

Physiologically, the presence of *Contracaecum* nematodes in loaches was associated with oxidative stress, as indicated by elevated antioxidant enzyme activities and increased lipid peroxidation in infected fish. These findings suggest that the fish host is experiencing oxidative stress, a condition known to compromise immune defenses and overall health (Kiron, 2012). Histological examinations further revealed muscle damage in infected loaches, which could impair their swimming efficiency and increase vulnerability to predators. Similar effects have been documented in other studies, where muscle damage by parasitic infections reduced host mobility and survival rates (Ackman & Gjelstad, 1975). This impact on muscle function could also interfere with the loaches' migratory behavior, potentially affecting their breeding success. Our research also provided the first molecular data on *Contracaecum* from the Indian subcontinent, with phylogenetic analysis indicating a distinct clade within the genus, possibly an undescribed endemic strain. *Contracaecum* nematodes have been reported in various fish species across marine, brackish, and freshwater environments globally (Chaturvedi & Kansal, 1977; Aydogdu et al., 2011; Sood, 2017). However, this is the first report of *Contracaecum* in the family Nemacheilidae, suggesting a previously unrecorded host-parasite relationship within this region.

The public health implications of *Contracaecum* infections are also noteworthy, as consumption of raw or undercooked infected fish can lead to anisakidosis in humans, a painful condition caused by nematode larvae (Buchmann & Mehrdana, 2016). In Maharashtra, where loaches are consumed by local tribal communities, the risk of human infection may be heightened due to traditional cooking practices that may not eliminate parasitic larvae effectively. This underscores the importance of monitoring parasitic infections, both for the health of aquatic ecosystems and for public health, particularly in rural areas where awareness and healthcare resources may be limited.

Pollutants, particularly microplastics (MPs), heavy metals (HMs), pharmaceuticals, and personal care products (PPCPs), pose substantial threats to aquatic organisms by impacting their behavior and physiology (Horký et al., 2021; Cerveny et al., 2021). These contaminants, primarily derived from urban and industrial activities, accumulate in rivers and coastal areas, creating significant stressors for the fauna. In our study, we found that microplastic contamination was widespread in sediment samples from the Ulhas River and in beach sediments along the Maharashtra coast. The presence of MPs, alongside various chemical pollutants, raises serious concerns as these contaminants disrupt the biological functions and behaviors of aquatic organisms.

Microplastics have become pervasive in aquatic ecosystems, with ingestion by aquatic organisms leading to various adverse physiological and behavioral effects. In our study, MP contamination levels in the Ulhas River varied significantly across sampling locations, with the highest concentration recorded near heavily industrialized zones. The Ulhas River, recognized as one of Maharashtra's most polluted rivers, showed MP concentrations as high as 600 particles per kilogram of sediment in some sub-basins, reflecting the extensive pollution from industrial and residential waste (Raut et al., 2019; Kumkar et al., 2021). Similarly, MP contamination in coastal sediments displayed variability, with the highest concentrations observed at Suruchi Beach, likely due to its proximity to the Ulhas River's mouth where pollutants from the river are discharged into the sea. Ingested MPs can physically damage the digestive tracts of aquatic organisms and may create a false sense of satiation, thus reducing feeding efficiency and nutritional intake. Moreover, MPs act as vectors for additional pollutants, including heavy metals and PPCPs, due to their high affinity for hydrophobic chemicals, which adhere to their surfaces. Studies have demonstrated that MPs can absorb HMs and PPCPs from their surroundings, leading to bioaccumulation in organisms that ingest these MPs (Atugoda et al., 2021; Liu et al., 2021). This is particularly concerning as MPs with adsorbed toxicants, such as bisphenols and phthalates, can cause endocrine disruption, reproductive toxicity, and other physiological effects in aquatic organisms (Khalid et al., 2021; Wagstaff et al., 2022). Our study revealed that MPs collected from Maharashtra's northern coastal zone contained plasticizers like B2EHP and DBP, compounds known for their potential to disrupt hormonal regulation and developmental processes in marine life (Net et al., 2015; Zhang et al., 2018).

Pharmaceuticals and personal care products (PPCPs) are increasingly prevalent in aquatic environments (Grabicová et al., 2020), largely due to high usage rates and inadequate wastewater treatment facilities. In our research, we detected six PPCPs in the coastal waters of Maharashtra, including metoprolol, tramadol, venlafaxine, triclosan, bisphenol A, and bisphenol S. Among these, metoprolol a commonly used beta-blocker—was found in concentrations considerably higher than reported in other regions, highlighting the scale of pharmaceutical pollution along the Indian coast (Maszkowska et al., 2014; Gu et al., 2012; Godoy et al., 2015). Pharmaceutical contaminants can interfere with various physiological processes in aquatic organisms, including behaviors related to predator avoidance, reproductive cycles, and feeding. For instance, metoprolol impacts cardiac function and energy expenditure in fish, potentially reducing their ability to evade predators. Similarly, tramadol, observed at concentrations higher than those reported elsewhere, can disrupt the nervous system, potentially altering behavior, mobility, and environmental responsiveness (Edmondson et al., 2012; Rúa-Gómez & Püttmann, 2013). Triclosan, an antimicrobial agent widely found in consumer products, was recorded at high levels in our study and is known to interfere with endocrine function in fish, disrupting thyroid hormones essential for growth and metabolism (Ramaswamy et al., 2011; Wang, 2024). Heavy metals (HMs) constitute another major class of pollutants with severe ecological implications. While HMs are naturally occurring elements, industrial activities have significantly increased their concentrations in aquatic environments. Although our study did not find significant differences in HM concentrations across various zones along the Maharashtra coast, the presence of HMs in conjunction with MPs and other pollutants highlights the potential for combined, deleterious impacts on aquatic life (Foshtomi et al., 2019). HMs, such as lead, mercury, and cadmium, disrupt essential biochemical processes in fish, causing oxidative stress, immunosuppression, and neurotoxicity. Oxidative stress arises from an imbalance between reactive oxygen species (ROS) production and the organism's antioxidant defenses, leading to cellular damage. Fish exposed to both HMs and MPs may experience elevated ROS production, which weakens immune responses and makes them more susceptible to infections (Di Giulio & Meyer, 2008; Folgueira et al., 2019). Moreover, HMs can attach to MPs, facilitating the entry of toxic metals into the food web when aquatic organisms ingest contaminated plastics, with implications for higher trophic levels.

The presence of pollutants, such as MPs, HMs, and PPCPs, often results in behavioral and physiological changes in aquatic organisms. Pollutants can impair critical behaviors, including mobility, predator evasion, and feeding. Exposure to neuroactive compounds like tramadol and metoprolol may alter swimming patterns and reduce alertness, thus increasing the likelihood of predation (Bretaud et al., 2000; Rúa-Gómez & Püttmann, 2013). Triclosan disrupts endocrine signaling, leading to impaired reproduction and developmental abnormalities in fish (Wang, 2024). The combination of these pollutants in aquatic ecosystems creates a highly stressful environment where organisms exhibit altered behaviors that may reduce their chances of survival and reproductive success. The findings from our study along the Maharashtra coast illustrate the extensive and varied impact of pollution on aquatic organisms, ranging from oxidative stress due to heavy metals to endocrine disruption caused by plasticizers and PPCPs. The heterogeneous distribution of pollutants, with hotspots near urban and industrial areas, highlights the need for targeted pollution management strategies. For example, the elevated MP levels observed at Suruchi Beach, likely due to its proximity to the polluted Ulhas River, emphasize the importance of monitoring riverine inputs into coastal systems.

The presence of pollutants, such as microplastics (MPs), phthalates, and other chemical contaminants, exerts significant physiological and behavioral impacts on aquatic fauna. In our study, the examination of mudskipper fish revealed a high prevalence of microplastic ingestion, with 74% of sampled fish containing MPs in their digestive tracts. The average number of plastic microparticles per fish ranged from approximately 3.75 to 6.11 particles. This frequency of plastic ingestion is notably higher than previous studies, such as the findings of Robin et al. (2020) along the southwest coast of India, where only one fish had ingested four particles. The morphological adaptations in the oral cavity of mudskippers, specifically in the Boleophthalmus genus, seem to play a role in their selectivity, as only fibrous types of MPs were observed in their guts. These findings suggest that unique anatomical features, such as the pharyngeal structure and specialized dentition, allow mudskippers to selectively filter and retain certain microplastic forms, thus shaping their exposure and potential impact from MPs (Farrell & Nelson, 2013; Naidoo et al., 2016; Polgar et al., 2017).

Pollutants also interfere with predator-prey interactions, as evidenced by studies on *L. thermalis*, a loach species exposed to diethyl phthalate (DEP). This phthalate significantly alters the chemical cues involved in predator recognition and avoidance behavior. Under typical conditions, *L. thermalis* utilizes chemical cues, including kairomones and alarm signals, to detect native predators such as the dwarf snakehead (Tapkir et al., 2017, 2019; Gosavi et al., 2020). However, exposure to DEP was found to impair these chemosensory capabilities, resulting in diminished anti-predator responses. Such impairment in detecting and reacting to predatory threats heightens the vulnerability of exposed fish, potentially disrupting the ecological balance by altering predator-prey dynamics (Kaplan et al., 2013; McIntyre et al., 2012). Similar studies have reported the chemosensory disruption caused by phthalates and other pollutants in various fish species, affecting their ability to make critical ecological decisions, such as shoaling and predator evasion, thereby increasing predation risk (Tierney et al., 2010; Sovová et al., 2014).

The impact of DEP on invertebrate physiology, particularly in freshwater planarians, provides further insights into pollutant effects on aquatic organisms. Planarians are often used as model organisms due to their regenerative capabilities and sensitivity to pollutants. In our study, DEP exposure in planarians caused a reduction in regeneration abilities, attributed to the suppression of pluripotent stem cells known as neoblasts, which are essential for tissue repair and growth. When pollutants disrupt the functioning of these stem cells, planarians experience diminished regenerative capacity, which may result in a compromised response to environmental injuries or stressors. Furthermore, DEP exposure leads to oxidative stress, marked by the overproduction of reactive oxygen species (ROS) and a consequent increase in antioxidant enzyme activity as the organism attempts to neutralize free radicals. This heightened ROS activity can impair cellular functions and exacerbate tissue damage, revealing a cascading effect of pollutants on organismal health (Kumkar et al., 2022).

The role of plastispheres biofilms formed on the surfaces of microplastics highlights another dimension of pollutant impact. Microplastics in the Maharashtra coastal waters exhibited microbial colonization, leading to the formation of biofilms that include diverse microbial communities, such as autotrophs, bacteria, and algae (Oberbeckmann et al., 2015; Frère et al., 2018). The formation of these biofilms not only affects the microplastics' physical properties but also potentially alters their ecological role, as plastispheres can facilitate the transport of harmful microbes and increase the ingestion risk for aquatic species. Studies indicate that the type of polymer influences microbial colonization patterns, with polystyrene (PS) favoring bacterial growth and polyvinyl chloride (PVC) favoring algal communities (Eich et al., 2015; Miao et al., 2021). This microbial colonization can transform MPs into vectors for pathogenic organisms, further complicating the environmental and health risks associated with microplastics in aquatic ecosystems.

In summary, pollutants such as MPs, DEP, and other contaminants exert multifaceted effects on aquatic organisms, ranging from physical and chemical disruption to alterations in behavior and ecological interactions. These findings underscore the importance of assessing the complex interactions between pollutants and aquatic fauna, especially in regions like Maharashtra's coastal waters, where contamination levels are alarmingly high. Continued research into the effects of pollutants on physiology and behavior will be crucial for understanding the long-term ecological consequences of contamination in aquatic ecosystems.

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Chapter 8

Conclusion

This thesis provides a comprehensive analysis of how environmental factors, parasitic infections, pollution, and biological invasions influence the morphology, physiology, and behavior of various aquatic organisms. By examining fish species in the Western Ghats of India and invasive species in Central Europe, this work sheds light on the complexity of aquatic ecosystems and the numerous pressures impacting them.

The study's osteological examination of *P. khavalchor*, an endemic lepidophagous catfish, revealed unique structural adaptations that support its scale-eating behavior. These include the outward orientation of premaxillary teeth and specialized dentition, which help the species efficiently consume the scales of other fish, reducing competition for food. The lack of detailed information about the osteological aspects of the Horabagridae family limited comparative analysis. Nevertheless, this research establishes a foundational understanding of the morphological adaptations in *P. khavalchor*, showing how evolutionary pressures shape anatomical features to support unique feeding strategies. This is especially relevant in environments with high competition for resources, where ecological adaptations enable species to exploit specific niches effectively.

Parasitic infections are another significant factor influencing the health and behavior of aquatic species. The study documented *Contracaecum* nematode infections in hillstream loaches, which are commonly consumed by local communities in the Western Ghats. Histological and genetic analyses identified the nematodes in muscle tissues, causing oxidative stress and muscle damage that could impair the mobility and survival of infected loaches. This finding raises potential health concerns, as these fish are often consumed without degutting, posing a risk of human infection. The gradual increase in parasite infections observed in recent years could be attributed to anthropogenic stressors that degrade riverine habitats. This study underscores the importance of monitoring parasitic infections, as they not only impact fish health but also pose indirect risks to human populations that rely on these fish as a food source.

Pollution, especially from microplastics (MPs), heavy metals (HMs), pharmaceuticals, and plasticizers, has emerged as a major stressor in aquatic environments. This thesis presents the first comprehensive data on MP contamination in the Ulhas River, identifying hotspots along the river where pollution control measures should be prioritized. Microplastics in coastal regions were found to interact with other contaminants, acting as carriers for HMs and plasticizers, thereby intensifying the risks these pollutants pose to aquatic fauna. MPs have also shown bioaccumulation potential, posing serious long-term health risks for both marine life and human consumers of seafood. The study's findings on the occurrence of emerging contaminants along the Maharashtra coast reveal high levels of pharmaceutical pollutants, such as metoprolol, tramadol, and triclosan, which are present in concentrations exceeding those reported in other parts of the world. These compounds impact different trophic levels, from algae to fish and crustaceans, and highlight the alarming ecological risks posed by pharmaceutical pollution. The potential for bioaccumulation and the observed synergistic effects of pollutants emphasize the need for stricter regulation and management strategies to protect coastal and marine ecosystems. Furthermore, the high contamination levels found in mudskippers, a popular food fish, indicate the risk posed to human health, especially among local populations with a diet reliant on seafood.

The study also documents the invasion of the Chinese sleeper (*P. glenii*) in European waters, a species capable of significantly altering the local ecosystem structure due to its broad predatory niche. Using eDNA techniques, the research successfully detected the presence of *P. glenii* in the Elbe River Basin, providing a tool for early detection and control of invasive species. This work underscores the importance of rapid-response management strategies in addressing invasive species that threaten biodiversity and ecosystem stability. The rapid spread of *P. glenii* in European waters highlights the need for vigilant monitoring and proactive measures to prevent further ecological damage.

This thesis illustrates the interconnectedness of environmental, biological, and anthropogenic factors in shaping the lives of aquatic organisms. The findings emphasize the need for integrated approaches to conservation that address both natural and human-induced stressors. The adaptation of *P. khavalchor* to a scale-eating diet, the physiological cost of parasitic infections in loaches, the multifaceted impact of pollution on aquatic species, and the spread of invasive species all point to the complexities and vulnerabilities of aquatic ecosystems in the face of global environmental change. For policymakers and conservationists, this research provides critical data to inform strategies for managing aquatic biodiversity. Measures such as improved pollution monitoring, habitat protection, and early detection of invasive species are essential for mitigating the effects of these stressors. The implications for public health, particularly with regard to the contamination of fish consumed by local populations, highlight the urgent need for effective pollution control and regulatory policies. Moreover, this research suggests that collaborative efforts involving local communities, governments, and industry stakeholders are crucial for achieving sustainable ecosystem management and protecting both biodiversity and human health.

Overall, this thesis advances our understanding of how aquatic species respond to and are impacted by a range of ecological pressures, offering insights that are crucial for preserving biodiversity and ecological balance in freshwater and marine ecosystems. Continued research in these areas, coupled with targeted conservation efforts, will be essential for safeguarding aquatic environments and the diverse life forms they support.

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11. Appendix: List of publications and unpublished works

1) scientific publications with IF

1. Kumkar, P., **Verma, C.R.**, Gosavi SM., Lexa M., Kharat, SS., Rinn R. & Kalous, L. (2024) Microplastic contamination in the Aquaculture Icon Oreochromis mossambicus: Prevalence, Characteristics, and Comprehensive Overview. *Environmental Toxicology and Pharmacology*.
2. **Verma, C. R.**, Khare, T., Chakraborty, P., Gosavi, S. M., Petrtýl, M., Kalous, L., & Kumkar, P. (2024). Impact of diethyl phthalate on freshwater planarian behaviour, regeneration, and antioxidant defence. *Aquatic Toxicology*, 107110.
3. **Verma CR.**, Pise M., Khare T., Kumkar P., Kalous L. (2024) Social media and genetic evidence demonstrate the expansion of an invasive fish in India. *Journal of Vertebrate Biology*.
4. **Verma, C.R.**, Pise, M., Hýsek, Š., Źółtowska, S., Kumkar, P., Kalous, L. & Gosavi SM. (2024) Occurrence and Distribution of Plastispheres in Coastal Sediments and Waters along the Maharashtra Coast, India. *Thalassas*.
5. Kumkar, P., **Verma, C. R.**, Hýsek, Š., Pise, M., Źółtowska, S., Gosavi, S. M., ... & Kalous, L. (2023) Contaminants and their ecological risk assessment in beach sediments and water along the Maharashtra coast of India: A comprehensive approach using microplastics, heavy metal(loid)s, pharmaceuticals, personal care products and plasticizers. *Science of The Total Environment*, 164712.
6. Šmejkal, M., Dočkal, O., Thomas, K., **Verma, C. R.**, Kumkar, P. & Kalous, M. (2023) First record of highly invasive Chinese sleeper *Perccottus glenii* Dybowski, 1877 (Perciformes: Odontobutidae) in the Elbe River Basin, Czechia. *Aquatic Ecology*.
7. **Verma, C. R.**, Gosavi, S. M., Pise, M., Kalous, L., & Kumkar, P. (2023) Effect of diethyl phthalate on predator–prey chemo-ecology in Lepidocephalichthys thermalis. *Aquatic Ecology*.
8. **Verma C. R.**, Kumkar P., Shendage T., Shinde P., Kumar V., Kharat S., Khare T. & Kalous L. (2023) Carbon nanofibers caused oxidative stress and disrupted anti-predator responses in common spiny loach. *Toxicological & Environmental Chemistry*.
9. Kumkar, P., Pise, M., **Verma, C. R.**, Khare, T., Petrtýl, M., & Kalous, L. (2022). Micro-contaminant, but immense impact: Source and influence of diethyl phthalate plasticizer on bottom-dwelling fishes. *Chemosphere*, 306, 135563.
10. **Verma, C. R.**, Pise, M., Kumkar, P., Gosavi, S. M., & Kalous, M. (2022) Microplastic Contamination in Ulhas River Flowing Through India's Most Populous Metropolitan Area. *Water, Air, & Soil Pollution*, 233.
11. **Verma, C. R.**, Kumkar, P., Khare, T., Pise, M., Kalous, L., & Dahanukar, N. (2022). Contracaecum nematode parasites in hillstream loaches of the Western Ghats, India. *Journal of Fish Diseases*, 2022, 1–10.

12. Pise, M., Gosavi, S. M., Gorule, P. A., **Verma, C. R.**, Kharat, S. S., Kalous, L., & Kumkar, P. (2022). Osteological description of Indian lepidophagous catfish Pachypterus khavalchor (Siluriformes: Horabagridae) from the Western Ghats of India. *Journal of Vertebrate Biology*, 71(22021), 22021-1.
13. Kumkar, P., Gosavi, S. M., **Verma, C. R.**, Pise, M. & Kalous, M. (2021) Big eyes can't see microplastics: Feeding selectivity and eco-morphological adaptations in oral cavity affect microplastic uptake in mud-dwelling amphibious mudskipper fish, *Science of The Total Environment*, 2021, 147445.
14. Kumkar P., Pise, M., Gorule, P.A., **Verma, C.R.**, Kalous, L. (2021) Two new species of the hillstream loach genus Indoreonectes from the northern Western Ghats of India (Teleostei: Nemacheilidae). *Vertebrate Zoology*, 71, 517-533.
15. **Verma, C. R.**, Kumkar, P., Gosavi S. M. & Kalous, L. (2021) Nothing in excess is good: Double pelvic fin malformation in the wild-caught hill stream loach, Indoreonectes evezardi (Day, 1872) from biodiversity hotspot of India. *Journal of Applied Ichthyology*, 37(2), 326-330.

2) conferences

1. Pise, M., Kumkar, P., **Verma, C. R.**, & Kalous, M. (2023) Protected areas may fail to protect fishes in the future: A case study of *Nemachilichthys ruppelli* from the Western Ghats, India. In Book of abstract, XVII European Congress of Ichthyology (ECI 2023), CZU, Prague, Czech Republic, 4-8 Sept. 2023.
2. Kumkar P., Pise M., **Verma C. R.** & Kalous L. (2021) Sand extraction for growing cities destroying habitat for benthic fishes: a case study from northern Western Ghats, India. in Kubík & Barták (ed) Proceedings of the 13th Workshop on Biodiversity, Jevany (18-26).
3. **Verma C. R.**, Kumkar P., Pise M. & Kalous L. (2021) Shelter or Danger?Benthic fishes hiding behind plastic waste. in Kubík & Barták (ed) Proceedings of the 13th Workshop on Biodiversity, Jevany (71-79).

3) Other (Unpublished – under review)

1. Kumkar, P., Pise, M., **Verma, C. R.**, & Kalous, L. Protected areas may fail to protect fishes in the future: A case study of Nemachilichthys ruppelli from the Western Ghats, India
2. Kumkar, P., **Verma, C. R.**, Patoka, J., Raghavan, R., Dahanukar, N., & Kalous, L. From tropical streams to glass tanks: rising demand for Indian loaches in the global aquarium trade.
3. Kumkar, P., **Verma, C. R.**, Kharat SS., Khare, T., & Kalous, L. eDNA-Based detection of invasive South American loricariid catfishes (*Pterygoplichthys* spp.) upper tributaries of Krishna River system, India.