

Article

Floating Microplastics with Biofilm Changes Feeding Behavior of Climbing Perch *Anabas testudineus*

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Abstract

The climbing perch, *Anabas testudineus*, is one of the most widely distributed freshwater amphibious fishes in South and Southeast Asia, inhabiting both natural and artificial water bodies polluted by plastic waste. Current mesocosm experimental study aimed to investigate behavioral responses of wild fish to floating expanded polystyrene (EPS) pellets, with a focus on the biofilm developing on their surface. For biofilm formation, the pellets (diameter 3–4 mm) were exposed for two, six, and fourteen days in an irrigation canal inhabited by climbing perch. Development of an intensive biofilm was observed on days 6 and 14 of exposure, characterized by a high diversity of organisms, including protozoa, cyanobacteria, algae, amoebae, and fungi. Fish feeding behavior was observed in the presence of feed pellets, clean EPS pellets, and three variants of EPS pellets with biofilm developed on their surfaces in the freshwater environment. The fish rapidly grasped and ingested feed pellets compared to all variants of plastic pellets. Climbing perch grasped all types of EPS pellets but always rejected them after oral cavity testing. The time to the first grasp was significantly longer for both clean EPS and EPS exposed for two days compared to feed pellets. Biofilm appeared to function as a taste deterrent for the fish: the duration of oral cavity testing was negatively correlated with the EPS pellet exposure timings in natural conditions. We suggest that floating plastic stimulates foraging behavior in the fish, and the duration of this behavior was significantly longer than that observed with feed pellets. The similarity of positive buoyant EPS pellets to natural food objects may stimulate the fish movements towards the water surface, which likely results in greater energy expenditure and increased risk of predation, without any apparent benefit to the individual.

Keywords: expanded polystyrene; biofilm; foraging behaviour; taste stimuli; natural environments; mesocosm experiments; tropical freshwater bodies



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1. Introduction

Nowadays, ecosystems are widely contaminated by anthropogenic plastic waste around the planet, including water, soil, air, and ice, posing numerous potential ecological and health risks [1–4]. Plastics are often prevalent in aquatic ecosystems, including freshwater environments. In aquatic environments plastic particles serve as habitats for various taxa of prokaryotic and eukaryotic organisms, including bacteria, fungi, algae, crustaceans, and bryozoan communities, collectively referred as the “plastisphere” [5,6]; a closely related term is “biofilm”. The biofilm composition depends on regional and

seasonal conditions across diverse environments [6–8]. Interactions between microplastics and biofilm components present risks to aquatic food webs and may have consequences for energy fluxes and may alter aquatic productivity [8], as well as pose potential hazards to the health of aquatic organisms [6,7].

Plastic ingestion by living organisms can occur in two ways: inadvertently, when the individual cannot identify plastic particles, and deliberately, when plastics are viewed as food items [9]. Plastic particles perceived as food objects can create a “gustatory trap” that prevents fish from distinguishing artificial plastic objects and thereby consuming such particles [10,11]. The likelihood of ingesting plastic fragments with biofilm may increase because organisms inhabiting the surface of plastics are likely to be recognized by the gustatory system of fish as flavorful objects [12]. For example, the surface structure of expanded polystyrene (EPS) pellets, characterized by low density and the ability to absorb water [13,14], promotes the accumulation of foreign additives, including organic substances and microorganisms. Also, the spheric form of EPS fragments provides to its resemblance to natural food objects [9,15].

The ability of fish to avoid plastic consumption may be related to their ecology and food preference [16,17]. The climbing perch *Anabas testudineus* is the most widely distributed freshwater fish in South and Southeast Asia and frequently inhabit areas that have been heavily modified and under persistent anthropogenic pressure, including factors such as plastic pollution. It has been shown that this species successfully avoids ingestion of pristine EPS pellets, which have a size and shape similar to food pellets [18]. Although the fish occasionally grasp plastics and keep them in their oral cavity, climbing perch always reject plastic, possibly because their taste and tactile systems allow them to distinguish plastic from food items. However, we hypothesize that the presence of biofilm on EPS pellets could diminish the climbing perch’s ability to avoid plastics effectively. Similar hypothesis was tested on goldfish *Carassius auratus* [12], which demonstrated that laboratory-induced biofilm over several weeks affected the duration of the fish’s interaction with plastic: the time of captured plastic remained in the oral cavity increased with the aging duration of the microplastics.

In the present study, we performed a mesocosm experiment to test the hypothesis that biofilm formation on microplastics surface in natural conditions could alter fish feeding behavior. Mesocosms allowed us to examine this test under conditions that closely simulated the natural environment. Our research conducted on wild fish that had been in contact with biofilm formed in their native habitat within the tropical climate zone of Central Vietnam. Recent assessments indicate Vietnam as the most plastic-polluted region, with freshwater environments under significant anthropogenic pressure [19,20]. We believe that investigating the features of plastic avoidance mechanisms across different hydrobionts species could help elucidate their adaptational responses and predict the future trends in ecosystems dynamics. The novelty of the study lies in providing a detailed assessment of the feeding reactions and responses of the omnivorous wild *Anabas testudineus* to the presence of floating microplastics covered with biofilm, which forms naturally in their habitat. The study aimed to evaluate the feeding behavior of wild climbing perch, specifically assessing the taste attractiveness of expanded polystyrene pellets, exposed for biofilm formation in native tropical habitat.

2. Materials and Methods

2.1. Sampling Location

The study was carried out in February 2023 in irrigation Am Chua canal (12°17′26″ N, 109°06′04″ E), which inflows into the Cai River near Nha Trang city (Central Vietnam) (Figure 1). The canal flows from the Am Chua Reservoir and is primarily used for agri-

culture, particularly as a water supply for the extensive area of rice fields exceeding 1400 hectares (as calculated in QGIS 3.34.13). The distance between the banks varies from one to twelve meters, with a maximum depth of 2.5 m. The main canal is more than 17 km long, with at least two significant inflows. Throughout its course to the river, the canal contains numerous dams that vary in type and size. Therefore, the occurrence and direction of water flow always depend on the artificial regulation of water discharge through the Am Chua Dam and the smaller dams along the canal. The canal often contains muddy water with high levels of plastic waste pollution due to urban areas disperse along its course. The plastic waste, such as low-density polyethylene, polypropylene, polyethylene terephthalate, and EPS, was often found in the Am Chua canal [18].

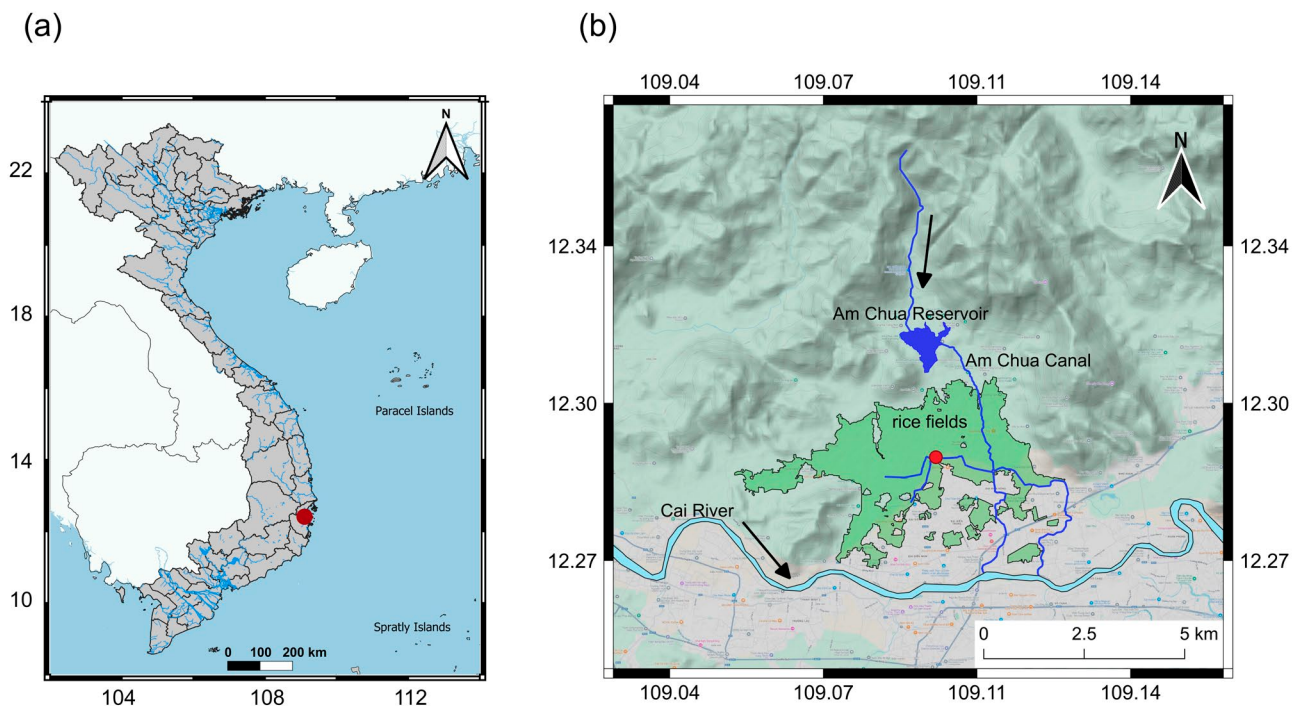


Figure 1. The sampling location (red dots) in the Am Chua canal (a) on a national map of Vietnam and (b) on a detailed scheme. Arrows indicate water flow direction and green area represent lands used for rice fields agriculture (QGIS 3.34.13).

We measured the water flow in the canal using the Flow Tracker FP 211 (Global Water Instruments, Yellow Springs, OH, USA). To assess water temperature and mineralization we used a thermometer HI-98501 (Hanna Instruments Nusfalău, Cluj, Romania) and Xiaomi TDS Pen (Beijing, China), respectively. The Sera Aqua-test box (Sera GmbH, Heinsberg, Germany) was used to estimate the water quality on the first and on the last day of field study.

2.2. Formation of Biofilm on Expanded Polystyrene

We used for the experiment expanded polystyrene (EPS) pellets (Nhu Phuong Investment and Manufacturing Co., Ho Chi Minh, Vietnam), which were spherical in shape and measured 3–4 mm in diameter. This type of plastic often had high positive buoyancy due to numerous air bubbles inside, which allowed it to float on the water surface over time [14]. Nine independent containers were constructed for holding the EPS pellets (Figure 2a). Each container had a cylindrical shape, with a height of 8 cm and a diameter of 11 cm. The side walls were made of transparent polyethylene terephthalate (PET) to prevent samples from floating out and allow unobstructed contact between the contents of the container and daylight. The bottom and the top of each container were covered with plastic mesh to

facilitate free exchange of water and air. A lead weight was attached to the bottom mesh to stabilize the container in the water. Each container was secured to a white plastic square ($20 \times 20 \times 0.8$ cm) made of extruded polystyrene (XPS) with positive buoyancy to ensure that the top half of the container remained above the water surface. Our choice of materials was driven by the presence of various types of plastic waste, including PET and XPS, in the irrigation canal [18]. Consequently, different types of plastic waste tend to accumulate in areas with low water flow, creating conditions conducive to biofilm formation. Moreover, microbial community assembly on plastics is often driven by conventional biofilm processes, with the plastic surface serving as a raft for attachment, rather than selecting for the recruitment of plastic-specific microbial colonizers [8,21].

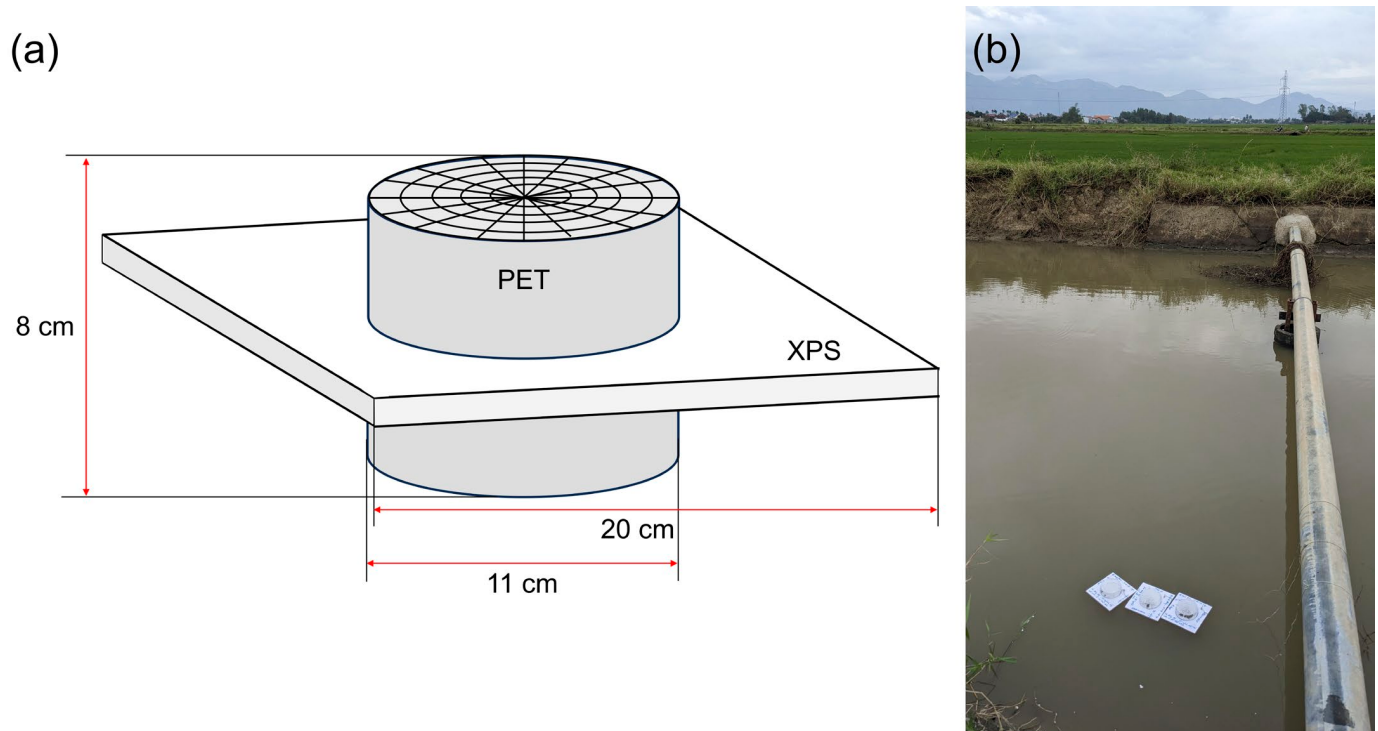


Figure 2. The schematic design of the container for holding EPS pellets (a) and the view of the containers mounted in the Am Chua canal (b).

To promote biofilm formation, approximately 8 cm^3 of pristine EPS pellets was added to each container, ensuring that each pellet had contact with the water, air, and daylight. Constructions were secured near the bank (0.2–1.0 m) in the Am Chua canal using ropes. They were mounted in the morning under full daylight conditions (Figure 2b) for exposure periods of two, six, and fourteen days. (Figure 3). Three containers were used for each exposure duration, in a total of nine construction were used. These exposure durations were chosen to correspond with initial (two days), early (six days) and later (fourteen days) stages of colonization by organisms of the plastic surface, respectively. The specific exposure periods were chosen to reflect the corresponding stages of biofilm development. The duration of microplastic exposure in natural environment was determined based on the particular tropical conditions, such as stable warm water temperatures, intensive insolation, high eutrophication, and low water flow. This is supported by our preliminary observations of biofilm formation rates on EPS pellets in the Am Chua canal.

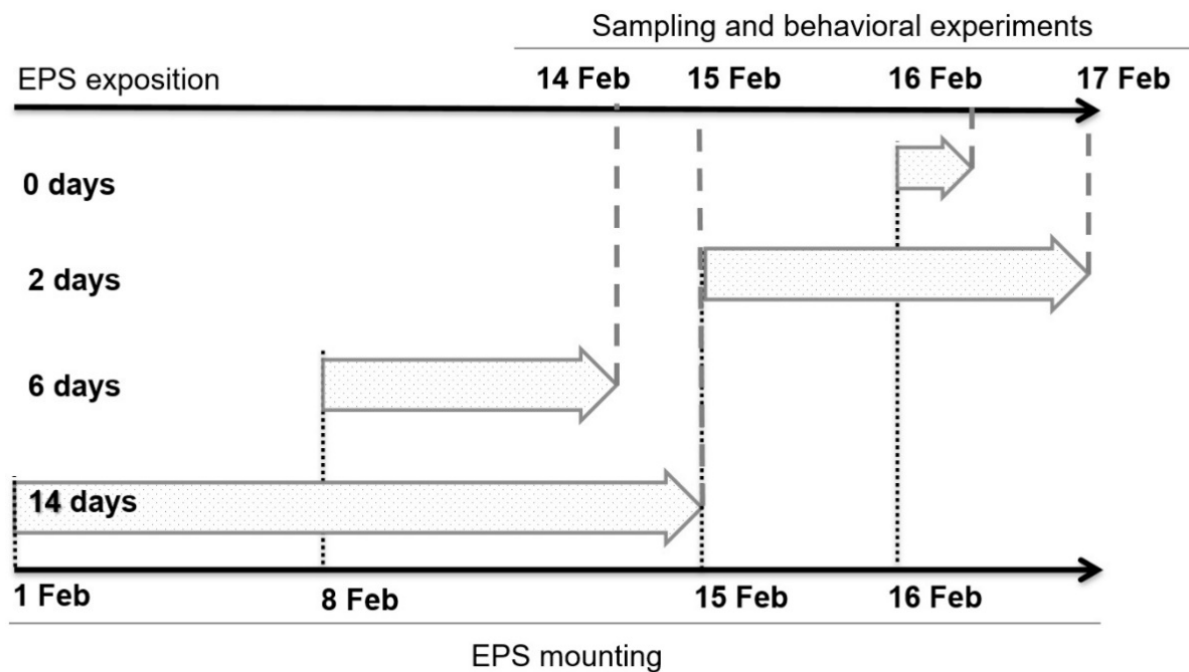


Figure 3. The scheme of biofilm exposure in the Am Chua canal. The arrows illustrate the specific durations of EPS exposure in the environment. Dotted lines indicate the timing of the mounting of EPS containers in the canal, while dashed lines indicate the timing of EPS sampling.

On the day of the behavioral experiments, the exposed EPS pellets were sampled from each container into separate clean PET containers filled with water from the canal and transported to the laboratory. A portion of the biofilm-covered EPS pellets was used for assessment of the feeding behavior of the climbing perch and some pellets were fixed in 4% buffered formaldehyde in 2 mL tubes for subsequent determination of biofouling composition. EPS sampling and behavioral experiments were conducted on the same day.

2.3. Biofilm Assessment

We conducted a basic spatial-temporal analysis of biofilm formation on EPS surfaces in a freshwater environment to assess the diversity of inhabitant organisms and their potential to influence fish taste preferences and, consequently, food chain dynamics. At each time point, five randomly selected EPS pellets were evaluated for biofilm composition. We used a scanning electron microscope (SEM) MIRA 3rd generation (Tescan, Brno, Czech Republic). The study was conducted at the Joint Usage Center «Instrumental methods in ecology» of IEE RAS. Before the analyses, the pellets were carefully washed in distilled water. For SEM analysis, the pellets were dehydrated using a series of increasing ethanol concentrations up to 100%. They were then dried in an automated food dehydrator for 40 min at 50 °C and for 30 min at 40 °C. After drying, the pellets were mounted on aluminum stubs with carbon tape and sputter-coated with a 20–30 nm layer of gold. The samples were examined at a voltage of 5 kV, with a working distance of 9–11 mm and an aperture size of 10–30 mm. The photographs of the organisms were assessed based on their morphological features and classified into taxonomic or morphological groups. To identify the composition of the biofilm at the lowest possible taxonomic level, we utilized primary taxonomic literature [22–26], online resources (e.g., marinespecies.org, westerndiatoms.colorado.edu, arcella.nl, fws.gov, diatoms.org, accessed on 4 July 2025), and consulted an expert in biofilm formation. To ensure the presence of fungi along with the microorganismal community we performed specific staining of another five EPS pellets per time point with Calcofluor White according to a standard protocol. Stained EPS were examined with digital microscopy

using VHX-1000 and Biorevo systems with fluorescent options (Keyence, Takatsuki, Japan). Based on the great diversity of living organisms on plastisphere, our identification was focused on the main structures and filaments, which had clear external distinguishing features in the microphotographs.

Raman spectra of pristine EPS pellets were obtained using a Raman spectrometer RM532 based on optical microscope ADF U300M (Photon Bio, Chernogolovka, Russia) with laser excitation wavelengths of 532 nm, spectral resolution of 4 cm^{-1} , power of 30 mW. Spectral identification performed with Photon Bio Spectra Libraries (Photon Bio, Chernogolovka, Russia).

2.4. Fish Capturing and Maintenance

Adult climbing perch *Anabas testudineus* was caught in the Am Chua canal using a seine net (5 m in length and 5 m in diameter, with a 10 mm mesh size). Fish were collected three times during the study and transferred to the laboratory. To minimize stress, the fish were slowly acclimated to the new water by gradually replacing the water (from the canal) in the transport tanks with the pre-conditioned tap water. Tap fresh water was preconditioned in two 2000 L pools by settling and aeration for 2 weeks. Dissolved oxygen levels in water ranged from 7.0 to 7.2 mg/L (Pro Dissolved oxygen meter MW600, Milwaukee, WI, USA) and mineralization was 200 ppm (Xiaomi TDS Pen, Beijing, China). During the study, the hydrochemical characteristics were periodically monitored using a Sera Aqua-test box.

Three 80 L aquaria ($56 \times 38 \times 38\text{ cm}$; width $\times$ height $\times$ depth) were used to maintain the fish stock. Each aquarium was filled with 50 L of water maintained at $25\text{--}27\text{ }^{\circ}\text{C}$ (HI 98509 Checktemp 1, Hanna instruments Nusfalău, Romania). The sides of the aquaria were translucent white to minimize disturbance to the wild fish, and each was covered with a white plastic sheet. A rectangular hole in the cover accommodated an automatic feeder (Eheim Auto Feeder, Eheim GmbH, Deizisau, Germany). Each aquarium contained no more than fifty fish at any one time. Fish were fed twice daily, at 7:00 and 17:00 (GMT+7), with 6.5 g of Humpy Head dry pellets (Yi Hu Fish Farm Trading, Singapore) per tank. The feeding ration was approximately 2% of the average fish weight per day and was neither related to overfeeding nor long-term starvation, in accordance with CCAC guidelines [27]. The feed pellets exhibited positive buoyancy and remained on the water surface for more than 30 min, expanding to 3.5 mm within the first 10 min. The wild fish began consuming the feed pellets within the first three days of transfer to the laboratory. On the day of the test, the morning feed was skipped in the aquarium from which individuals were taken. That was performed to ensure that only unfed fish were used in the trial.

External filters (Eheim Classic 150, Eheim GmbH, Deizisau, Germany) maintained the water in stock aquaria. The filters were cleaned every five days. Illumination was provided by natural light through laboratory windows and varied throughout the day from 0 to 100 Lx (Lutron LX-1102 light meter, Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan). On average, illuminance in the stock tanks was comparable to the natural conditions in the turbid water of the Am Chua canal, which ranged from 1 Lx (near the bottom) to 250 Lx (near the surface) at 12:00 (GMT+7) (modified Lutron LX-1102 light meter with waterproof sensor) [28].

We followed the protocol outlined by the Organization for Economic Cooperation and Development (OECD) [29] for fish acclimatization before the beginning of experiments according to the statement: 48 h settling-in + 7 days acclimatization = 9 days; batch acceptance required <5% mortality over seven days before testing. To reduce potential confounding factors that could influence feeding behavior, we implemented several control measures, including standardized acclimation periods, consistent lighting conditions, and

a controlled feeding schedule. These measures aimed to minimize variability and isolate the effects of the manipulated variables.

2.5. Behavioral Experiment

Two identical experimental apparatuses were used to estimate feeding behavior of climbing perch. Each test apparatus had a similar size to the aquaria used for stock fish maintenance and was filled with 30 L of water to depth of 15 cm. The sides of apparatuses were also covered with translucent white film. A 30 cm high plastic rectangular parallelepiped without a bottom (box) was placed on top of each experimental apparatus to prevent the fish jumping out. A video camera A10 (SjCam, Shenzhen, China) with remote control was positioned in the top and center of the box.

The trials were conducted from 8:00 to 12:30 (GMT+7) with illumination levels ranging from 40 to 80 lx in the experimental apparatuses. Six randomly selected individuals from the fish stock were placed in each apparatus prior to the start of the trial, followed by the initiation of video recording. After a 20 min acclimatization period for the fish, the video recording was started and 24 pellets were added to each apparatus using Pasteur pipettes. The mixture of pellets from the exposure container at each time point was used in the behavioral experiments, without selective testing of pellets exhibiting different levels of biofilm formation. Each trial used only one of the following five pellet types: (a) feeding pellets (control), (b) clean plastic EPS pellets, (c) plastic pellets after 2 days of exposure in the canal, (d) plastic pellets after 6 days of exposure, and (e) plastic pellets after 14 days of exposure. The plastic pellets had a similar shape and size (diameter 3–4 mm) as the feeding pellets. Before the trial, clean EPS pellets were pre-washed in water from the Am Chua canal to simulate their initial introduction into natural waters. The feeding behavior assessment lasted twenty minutes. The number of each type of remaining pellets in the apparatuses was counted, and any mechanical damage to the pellets was recorded. The water in the apparatuses was changed after each trial to prevent pollution from fish metabolites.

Each fish was used only once in the behavioral experiment. After each session, the body length (TL) and weight of treated fish were measured, and fish were transferred to a separate tank with clean water. In total, two hundred and fifty-two fish with total length (TL) of 70–114 mm (mean = 92 mm, SE = 1.9 mm) and a mass of 7–24 g (mean = 14 g, SE = 0.9 g) was used in the experiment. A total of 42 trials resulting in 14 h of video recording were evaluated second by second. All fish remained alive 48 h after the manipulation. Following a visual assessment of fish health, they were released back into their natural environment.

We analyzed video recording (in 2025) to determine the time it took an individual fish to grasp a pellet and the duration of each pellet's retention in the mouth during the trial. Repetitive grasping of the same pellet was counted as a new event. To assess changes in foraging behavior, we calculated the frequency (time intervals) between two consecutive feeding events by measuring the duration between two grasps.

All experiments adhered to Vietnamese and Russian Regulations and Guidelines, as detailed in the Institutional Review Board Statement.

2.6. Statistical Data Analyses

Statistical data analysis was conducted using Minitab 18.1. Initial assessment of data normality using the Shapiro–Wilk test revealed non-normal distributions for all variables ($p < 0.05$). Statistical significance was defined as $p < 0.05$, with adjustment for multiple comparisons using Holm's sequential Bonferroni procedure.

The Chi-Square test for association was employed to evaluate the similarity in the distribution of pellets grasping events (timings) across the studied fish groups. Mann–Whitney U tests were used for between-group comparisons of the time of the first grasp.

One-way ANOVA, followed by post hoc Tukey tests, was utilized to assess differences in pellets retention times in the fish mouth and frequencies of feeding events among the experimental groups.

3. Results

3.1. Characteristics of the Sampling Location

During the study period, the weather was predominantly cloudy, with occasional intermittent rain occurring during the day, and the average air temperature was 25 °C. At the sampling location, the maximum water level reached 1.2 m. The water flow was 0.2 m per second or less. Water temperatures ranged from 24.7 to 26.3 °C, and the water mineralization was recorded at 58 ppm. The pH of the water was near neutral. Nitrogen levels did not differ significantly between the first and last days of the study (Table A1). The concentrations of chlorides were below the detectable limits of the used Sera test kit. Possibly due to agricultural practices such as irrigation and fertilization of rice fields, the content of phosphates and iron in the water decreased fourfold and sixfold, respectively, while ammonia to ammonium ratio (NH_3/NH_4) decreased twofold over the two-week study period.

3.2. Climbing Perch Feeding Behavior

The fish exhibited behavioral responses indicative of assessment during feeding on both pellet types—feed and plastic—that were exposed to natural conditions for varying durations (0–14 days). Sometimes, fish did not grasp the plastic pellets during the entire trial: 22% of trials involving zero-day exposure and 30% of trials involving six-day exposure.

Climbing perches consistently grasped and consumed all feed pellets within ten minutes in every trial. Occasionally, an individual rejected a feed pellet after grasping it, but the rejected pellet was invariably consumed by the same fish or another individual. Fish often grasped multiple feed pellets simultaneously. The difficulty in swallowing multiple pellets at once likely explains the observed feed pellets rejection. Over 90% of feed pellet grasping events occurred during the first five minutes and all feed pellets were ingested by the tenth minute of the trial (Figure A1).

Fish grasped plastic pellets but invariably rejected them after briefly holding them in their mouths. This feeding behavior feature was observed across all trials, regardless of the pellet exposure duration in natural conditions. At the end of each trial, all plastic pellets remained unconsumed, some of these pellets have mechanical deformation and fragmentation. We did not observe any changes in the internal structure of EPS pellet fragments after breaking, compared to intact EPS pellets, indicating the physical and chemical stability of EPS pellets across all exposure durations (2–14 days) in the natural environment.

Chi-Square test for association indicated that variables related to pellet grasping types (food vs. plastic) are significantly associated and that the foraging behavior of the fish varies depending on the pellet type and exposure duration ($p < 0.001$). Fish were rarely stimulated and grasping of clean plastic pellets occurred four times less frequently compared to feed pellets (Table 1). The presence of biofilm increased the likelihood of plastic pellet grasping with long-term exposure of the pellets associated with a high number of grasping events. The intensity of grasping decreased over the course of the trials, ranging from 20.3% to 52.6% of grasping events compared with the first five minutes of the trial (Figure A1). Therefore, grasping could be observed throughout the entire trial with a maximum of 26 events per trial. This indicates that fish repeatedly tested plastic pellets during the trial.

The first grasping of clean pellets or pellets exposed for two days, occurred later than with feed pellets: Mann–Whitney U test $p = 0.0022$ and $p = 0.0117$, respectively.

Table 1. Feeding behavior of climbing perch *Anabas testudineus*, treated with feed pellets and plastic pellets exposed for two, six and fourteen days in the natural environment.

Parameter	Feed Pellets	Plastic Pellets, 0 Days	Plastic Pellets, 2 Days	Plastic Pellets, 6 Days	Plastic Pellets, 14 days
Pellet grasping, event per fish	2.4	0.6	1.2	1.6	1.9
Maximum number of events (grasping) per trial	19	9	17	26	26
Percentage of events during first five minutes, %	90.4	32.3	20.3	52.6	38.1
Time of the first grasp, minutes M $\pm$ SD (min, max)	0.5 $\pm$ 0.32 ^a (0.2–1.1)	6.1 $\pm$ 5.69 ^b (0.8–16.9)	4.2 $\pm$ 3.19 ^b (0.3–8.9)	2.1 $\pm$ 2.45 ^{a,b} (0.1–6.2)	5.8 $\pm$ 8.09 ^{a,b} (0.1–19.3)
Retention time of the pellet, seconds M $\pm$ SD (min, max)	N/A	4.7 $\pm$ 6.52 (0–36)	2.2 $\pm$ 2.61 (0–10)	1.5 $\pm$ 2.12 * (0–15)	1.9 $\pm$ 2.22 (0–17)

M represents the mean value and SD is standard deviation. Different letters (a, b) indicate significant differences between the values (Mann–Whitney U test, $p < 0.05$). *—Occasional long-term individual retention of a pellet (226 s) was excluded.

One-Way ANOVA with Tukey test showed that the retention duration of clean pellets was longer compared to pellets exposed for two, six and fourteen days in the natural environment ($p = 1.6 \times 10^{-5}$) (Table 1).

The frequency of feeding events was generally shorter in the control group (0.2 ± 0.35 (0–2.1) min) (hereafter, before brackets are the mean value and standard deviation; in brackets are the minimum and maximum). Fish treated with plastic pellets exposed for 0th, 2nd and 6th day in natural environment exhibited longer intervals between feeding events (grasps) compared to the control group: 2.4 ± 2.65 (0–7.9), 1.9 ± 2.76 (0–12.9), 1.2 ± 2.11 (0–8.7) min, respectively (Figure 4). Fish treated with feed showed similar duration between feeding events and those grasping EPS pellets on the 14th day of exposure: 0.8 ± 1.26 (0–8.3) min.

3.3. Biofilm Formation on EPS Surface

Biofilm development on the EPS pellets occurred gradually over the course of two to fourteen days under the natural environment of the Am Chua canal. The color of the EPS pellets changed from white to greenish-brown and became yellowish-green by 14th day of exposure (Figure 5). Biofilm aggregation was more intense on the EPS located near to the walls of the container and spread from the edges toward the center. The sides of the container were also covered with substances consisting of dissolved organic matter and biofilm, which was often more intense than the biofilm observed on the surface of the EPS pellets. The increasing intensity of biofilm formation from center to side is likely due to higher ultraviolet (UV) activity in the center of the container, with reduced UV penetration through the PET walls.

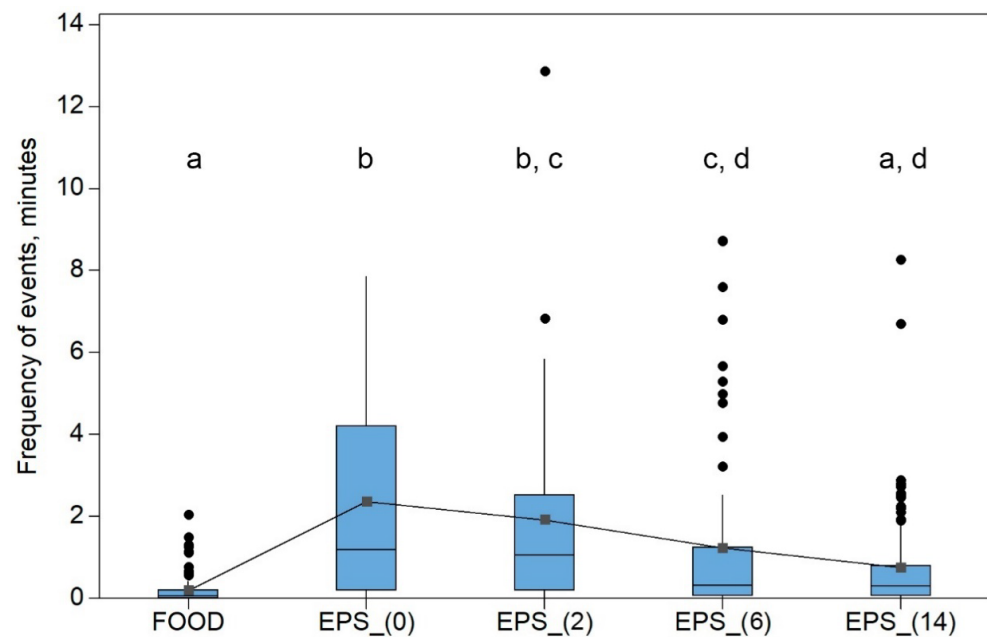


Figure 4. Frequency of feeding events (pellet grasping) by climbing perch *Anabas testudineus*, treated with feed (FOOD) and plastic (EPS) pellets under exposure in natural environment for 0, 2, 6 and 14 days. Different letters (a, b, c, d) indicate significant differences between the values (One-Way ANOVA with Tukey's post hoc test, $p = 1.4 \times 10^{-10}$).

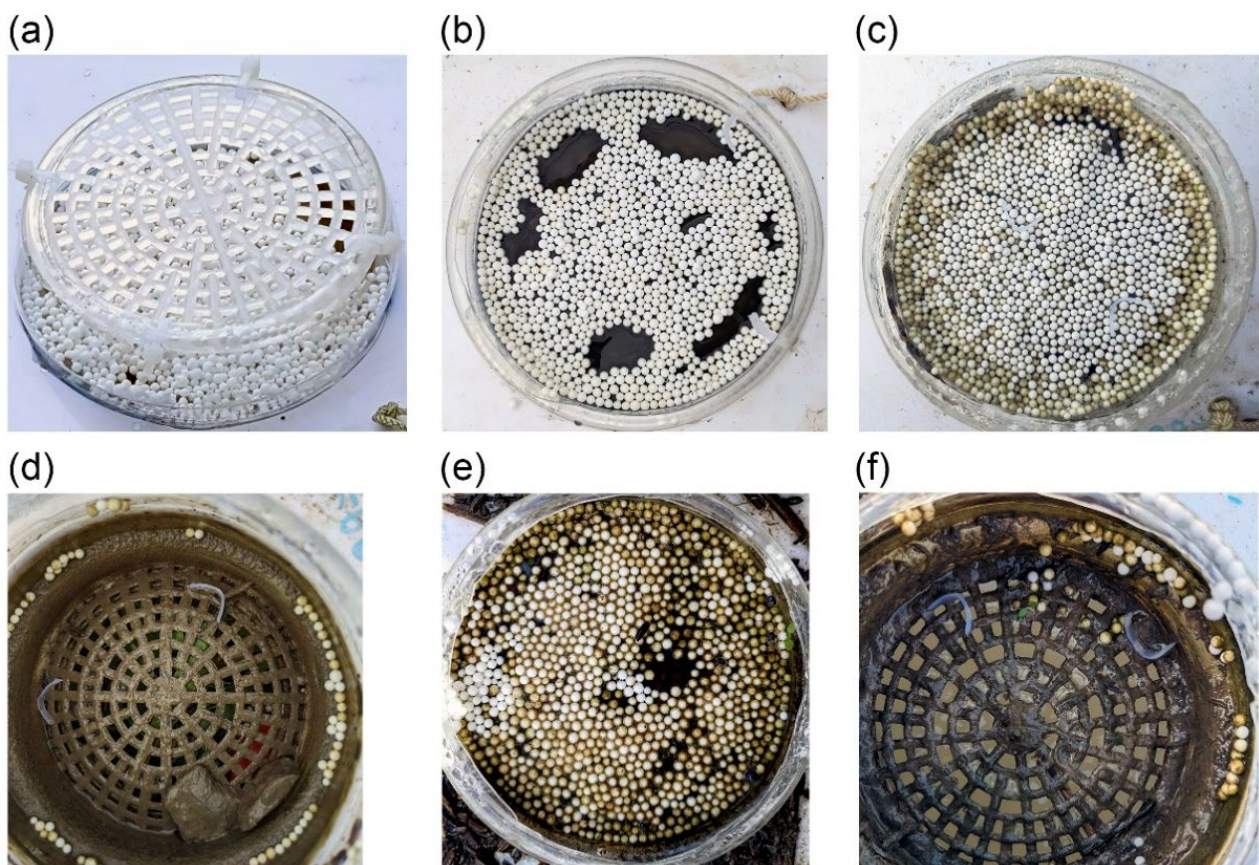


Figure 5. EPS pellets under exposure in the Am Chua canal: (a) before exposure (zero day); (b) after two days of exposure; (c) after six days of exposure; (d) sides and bottom mesh of the container after six days of exposure; (e) after fourteen days of exposure; (f) sides and bottom mesh of the container after fourteenth day of exposure.

Initial colonization of the EPS surface (two days of exposure) was primarily performed by bacteria. *Bacillus* species were frequently observed, presented in low numbers, often forming palisades and streptobacilli arrangements (19 aggregates with 22–240 cells per aggregate across the 26 images analyzed). Single cocci, coccobacilli, and filamentous were observed less frequently. A prominent protozoan colony (*Sarcodina*), comprising 76 cells was identified (Figure 6a,b).

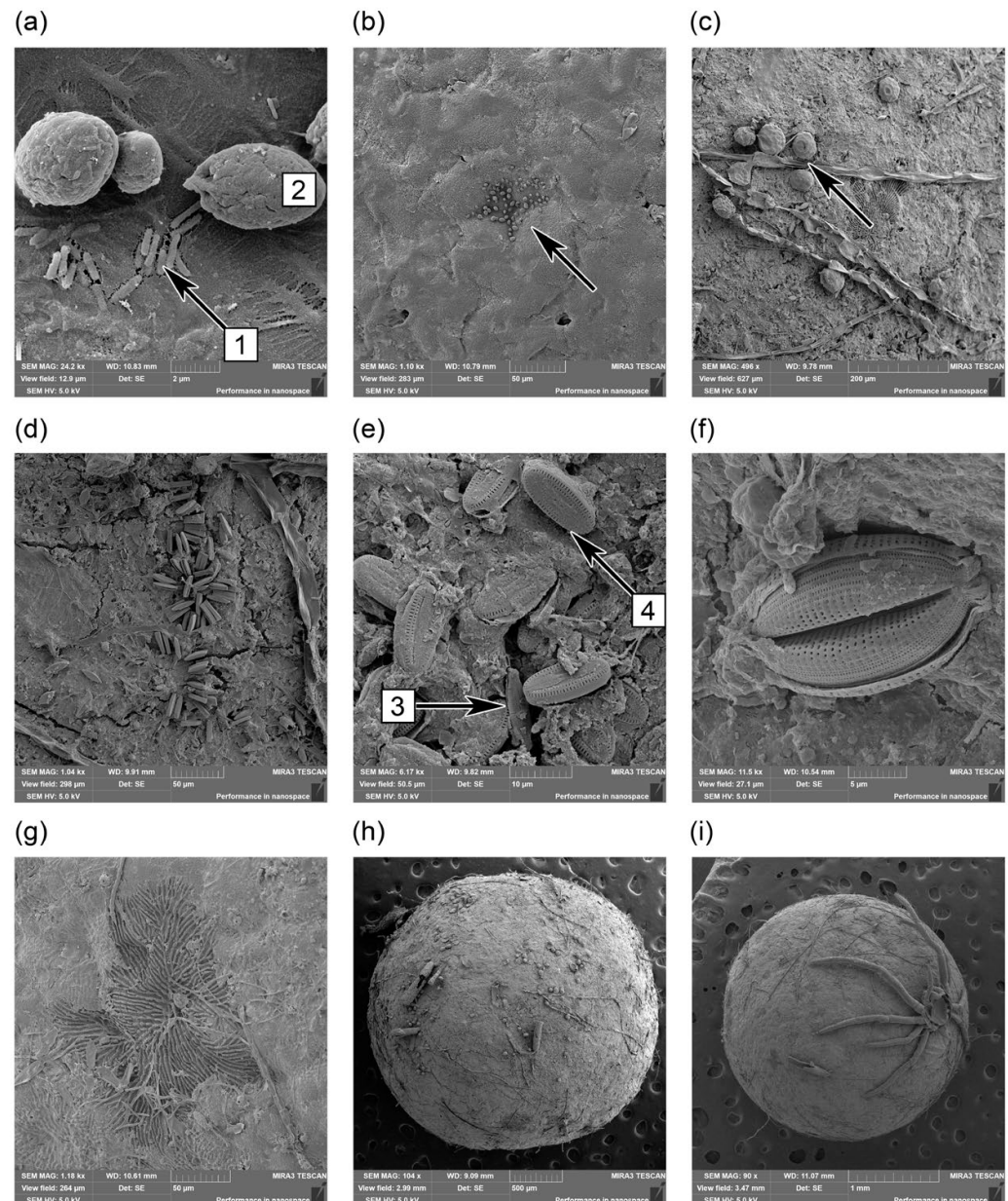


Figure 6. Biofilm on the surface of expanded polystyrene pellets at the 2nd (a,b), 6th (f,g,i) and 14th (c–e,h) days of exposure in the Am Chua canal. (a)—bacteria (1) and *Sarcodina* cells (2); (b)—protozoan colony of *Sarcodina*; (c)—testate amoebae; (d)—colony of pennate diatoms; (e)—diatoms *Navicula* sp. (3) and *Chamaepinularia* sp. (4); (f)—diatom *Amphora* sp.; (g)—colony of specific but unidentified microorganisms; (h)—surface of EPS pellet at 14th day of exposure; (i)—attached spider on EPS pellet.

Both early (6 days) and later (14 days) stages of colonization exhibited similar characteristics, with an abundant and diverse assemblage of organisms and a roughened underlying biofilm matrix. The main differences between earlier and later colonization were the presence of well-developed extensive biofilm matrix at the later stage. This matrix was char-

acterized by a thicker layer of extracellular compounds and an accelerated accumulation of dissolved organic matter, containing numerous intact and fragmented microorganisms and filaments embedded within it. At both time points, plastic surfaces exhibited numerous clusters of bacteria embedded in the matrix. By the 14th day, a greater variety of bacterial morphologies was observed, including single cells such as club-shaped rods, enlarged rods bacilli, cocci, coccobacilli, as well as formations of palisades and streptobacilli distributed along the surface. Based on morphology and the environmental context—a rice fields water collector—the likely genera include bacteria commonly inhabiting water, sediments, and soil, such as *Bacillus* spp. and *Pseudomonas* spp., amongst others. In addition to bacteria, filamentous and hyphae-like structures were present, accompanied by numerous oval or ellipsoidal structures attached to or near the hyphae. These structures, potentially represent spores or conidia. Their shape and arrangement suggest they may belong to various fungal species, such as *Aspergillus*, some *Penicillium* species, and yeasts. The presence of fungi in plastisphere was confirmed via Calcofluor White staining, indicating the presence of chitin. Additionally, filamentous cell morphology consistent with phytoplankton such as actinobacteria (capable of forming filamentous structures as well), cyanobacteria (especially under elevated nutrient levels), and some green algae were observed, particularly in tropical freshwater environments [23,25]. At this stage of colonization became well established with numerous protozoa present including testate amoebae (Figure 6c), rotifers (sessile or attached to a substrate via tubes or gelatinous holdfasts), and presumably ciliates and flagellates. Testate amoebae (shelled amoebae) often observed forming colonies, and were tentatively identified as belonging to the genera *Diffugia* and *Arcella*, both cosmopolitan genera, while single cells were attributed to the genus *Cryptodiffugia* [26]. Pennate diatoms (Figure 6d), affiliated with the genera *Navicula*, *Chamaepinularia* (Figure 6e), *Synedra*, *Amphora* (Figure 6f), *Cymbellales*, *Nitzschia*, were also observed. The analysis revealed numerous colonies of microorganisms exhibiting pillar-like and spindle-shaped structures oriented both vertically and horizontally (Figure 6g). Two distinct types of these structures were observed, each characterized by specific morphologies and surface features. The first type presented as cells embedded within an extensive extracellular matrix, presumed to be bacterial in origin. The second type was distinguished by pillar-like cells interconnected by a complex network of thin, filamentous elements, which could be associated with fungi. By the 6th and 14th days of EPS exposure, the diversity of microorganisms within the biofilm community had increased, along with the presence of organisms that utilized the EPS matrix as habitat and substrate for sustenance (Figure 6h,i).

4. Discussion

We observed some behavioral changes in climbing perch associated with the presence of floating microplastic debris, including plastic particles coated with biofilm on their surface. The examined expanded polystyrene plastic pellets, measuring 3–4 mm, resembled commercial feed pellets that wild climbing perch began to consume earlier. A few days were sufficient for the wild fish to acclimate to this novel artificial food, indicating their behavioral plasticity and demonstrating that climbing perch are often capable of grasping floating objects from the water surface in their natural environment. In natural habitats, the risk of climbing perch contacting EPS pellets is high because these habitats are often subjected to anthropogenic pressure, including extremely high levels of plastic pollution in Vietnamese freshwater ecosystems [18–20,30–32]. It should be noted that our findings have broader implications for assessing the risk posed by floating microplastics to not only for the studied species but also for other omnivorous freshwater fish. Furthermore, we highlight behavioral alterations in the feeding behavior of climbing perch induced by

floating microplastics and discuss potential ecological risks related to reduced fish energy resources and diminished survival prospects of these fish in their natural habitats.

4.1. Feeding Behavior of Climbing Perch

Our experimental results demonstrated that climbing perch rapidly detected feed pellets, approached them, and grasped them. Occasionally, an individual fish rejected a pellet, but after repeated attempts to grasp it, the feed pellet was invariably consumed by this or by another fish during the trial. Most feed pellets were consumed during the first five minutes of the trial.

Despite the presence of feed pellets, clean plastic pellets elicited weaker stimulation in climbing perch to grasp them. The number of behavioral events (grasping) with clean microplastics was approximately four times lower than that for feed pellets, and the time interval between these events was significantly longer. This behavioral pattern aligns with our previous findings indicating that climbing perch less frequently grasp white expanded polystyrene pellets compared to feed pellets [18]. It is possible that fish found it more difficult to recognize these pellets due to their white coloration, which provides weak contrast against the water surface. For instance, dark-colored diets provided better feed efficiency for Nile tilapia *Oreochromis niloticus* larvae compared to light-colored diets, presumably due to the sharper contrast during daylight hours [33]. Additionally, the preference of climbing perch for dark-colored pellets could be related to the predominantly dark coloration of feed objects in their natural habitats. In *Anabas testudineus*, visual recognition of the feed object likely prevails over the olfactory cues, as the fish grasped the clean plastic, which lacked any pronounced smell attractant. Moreover, in our experiment, the used EPS pellets contained no chemical additives other than the main component—polystyrene, as confirmed by Raman spectroscopy with identification accuracy 0.96 (Figure A2). *Anabas testudineus* possesses a morphological adaptation that reduces the likelihood of bottom soil particles suspended in water from entering the olfactory cavity: a small diameter of the tube of the anterior nostril [34]. This feature may facilitate localized olfactory searches or directed assessment of the surrounding odor environment, potentially helping in detection and testing floating objects prior to grasping. However, in our experiment climbing perch did not manifest this potential ability. The ability to successfully search and grasp feed or non-feed objects is often associated with schooling behavior of the fish. Individual goldfish (*Carassius auratus*) and minnows (*Phoxinus phoxinus*) in shoal of conspecifics locate food more rapidly [35]. Climbing perch also exhibits group behavior [36,37], and the likelihood of grasping floating plastic objects may depend on factors such as group size and water transparency, which are necessary for effective group aggregation and simple detection of potential food objects.

Plastic pellets with surface biofilm more frequently stimulated contact and grasping by the fish. Moreover, the duration of plastic exposure under natural conditions positively influenced the likelihood of fish grasping the pellets. There was no significant difference in the time to the first grasp between feed pellets and plastic pellets exposed for six and fourteen days. The presence of biofilm likely increased the detectability of the plastic pellets. However, after grasping, fish identified biofilm-covered pellets as non-feed objects more rapidly compared to clean plastic, as evidenced by significantly reduced retention time of the pellet with biofilm in the oral cavity. This indicates that biofilm, which naturally forms on plastics in the native habitat of climbing perch, acts as a repellent, resulting in increased rejection of the plastic pellet. In other words, we observed two opposing effects of microplastics with biofilm on fish feeding behavior: it enhanced the likelihood of detection and grasping due to improved visual contrast in water, yet it decreased the likelihood of consumption by reducing the external palatability of the plastic pellet.

The prolonged retention times observed for pristine pellets compared to biofilm-laden variants may reflect the absence of readily detectable chemosensory cues [38], forcing the fish to rely predominantly on mechanosensory information to assess the edibility of the item. Possibly, the mechanosensory response to testing clean EPS pellets, which involves mechanical contact, requires more time than the chemosensory response to EPS pellets with biofilm. Therefore, the external palatability associated with aged biofilms might indicate the presence of easily recognizable repulsive chemical signals in the biofilm, which facilitate rapid discrimination. It should be noted that our findings contrast with those reported by Mitsuharu et al. [12], which indicated that the time spent in the oral cavity capturing plastic increased with the aging duration of microplastics in *Carassius auratus*. Both results suggest that different fish species may vary in their response to biofilm, and that the composition of biofilm and plastic type could have opposite effects on fish feeding behavior. It is important to note that the response of goldfish was observed on laboratory-induced biofilm, which is likely not represent conditions in natural environments. Despite this, our mesocosm experiment indicates that wild climbing perch do respond to biofilm formed naturally in their habitat.

As mentioned above, fish grasped feed pellets more rapidly, and the frequency of their feeding events was significantly lower compared to fish that grasped plastic pellets. Additionally, the total number of feeding events was lower in the control group as all feed pellets were ingested. It appears that feeding activity, including the foraging period, decreased when fish are consuming actual food: the absence of any pellets in the aquarium may reduce their response. However, this process was likely not interrupted in fish treated with plastic pellets, as intervals between their feeding events remained prolonged throughout the entire trial. The multimodal integration of visual and chemoreceptive signals from food-like objects seems to exacerbate an evolutionary trap, leading to repeated plastic consumption, as observed earlier [8,10,11]. In nature, this behavioral trait could result in increased energy expenditure during foraging and a higher risk of predation. Unsuccessful attempts to search for food and starvation can induce stress, increased movement activity associated with foraging behavior, and concomitant energy mobilization [39,40]. For example, odor signatures from both biofouled plastic and food elicit olfactory responses associated with foraging in captive schools of anchovy (*Engraulis mordax*) [10]. It seems that any distraction by non-food objects, including floating microplastics, causes fish to spend more effort toward locating real feed objects.

To grasp floating objects, climbing perch must rise to the surface, where they face a higher risk of predation. The frequency of fish rising to the surface may increase significantly with the number of floating objects, such as plastic particles. Climbing perch is an amphibious species that naturally rises to the water surface to gulp air [41,42]. However, in habitat where food is scarce and numerous floating microplastic particles are present on the surface climbing perch may predominantly move toward the surface, which greatly increasing the risk of predation and offers no benefit to the fish. Moreover, starvation can reduce their aversion to deterrent substances, which explains why starving fish may consume new objects they would typically refuse or only occasionally consumed [38]. Despite the fact that the climbing perch in our study were starved for a short period (up to 18.5 h), they repeatedly tested the plastic particles.

Although climbing perch in the present study avoided ingesting EPS, the presence of plastic particles in the digestive tracts of various animal taxa, including fishes, is well-documented, highlights the widespread nature of this problem [11,12,43]. Microplastics can enter the organism inadvertently either directly through accidental ingestion, as observed in filter feeders such as bivalves and forage fishes [44], or via trophic transfer, whereby predators consume prey contaminated with microplastics [8,45]. In the current study, we

assume a deliberate pathway of plastic grasping by climbing perch, likely prompted by the similarity in shape and color between EPS pellets and feed pellets [14]. This similarity, along with an increasing number of incorrect grasps, intensified as the duration of plastic pellet exposure in the water extended. By the fourteenth day of plastic exposure, climbing perch could scarcely distinguish between plastic and food visually, as the frequency of feeding events for both items was similar.

Our results reveal a nuanced interaction between climbing perch and non-food object (EPS), wherein biofilm mature presence modulates the plastic's initial attractiveness by visual or chemical cues, while pre-ingestive sensory processing dictates subsequent behavioral responses—avoidance. We hypothesized that the protective mechanism against microplastic ingestion in climbing perch and allied species encompasses a hierarchical decision-making process involving sequential (1) visual assessment of the object as food, (2) olfactory evaluation upon approach and contact with the particle, and (3) grasping combined with intraoral testing comprising both mechanoreception and chemoreception. The simultaneous integration of neurophysiological sensory information from diverse receptor types ensures the fish fidelity of feeding behavior [46]. The chemoreceptive capacity enables fish to identify food sources based on the presence of key trigger molecules (proteins, sugars, or lipids parts), whereas tactile mechanoreception is deployed to manipulate and assess the properties of potential food items, including texture, density, elasticity, and size. The observed rejection of EPS during intraoral testing, despite passing initial visual and olfactory screening phases, underscores the critical importance of mechanoreception in mediating plastic avoidance. This assertion is further supported by the control of the fish behavior, which exhibited selective retention and occasional rejection of artificial feed pellets based on intraoral assessments of nutritional quality, thereby emphasizing the role of sensory feedback in modulating feeding decisions. It is important to note that the sensory ecology of feeding may differ significantly among opportunistic fish species, potentially leading to divergent feeding behavior driven by different sensory preferences and ecological adaptations not captured in the present study. Comparative investigations of feeding behavior across a range of fish species are necessary to elucidate the broader ecological significance of these sensory-mediated decision-making processes.

4.2. Some Ecological Features of Biofilm Formation on Plastic Surfaces in Natural Environments

While much of the current literature on plastic surface colonization focuses on marine environments and the microbial communities that develop there [6,47–49], a significant knowledge gap remains concerning community assembly over time and the specific microbial taxa involved in the different stages of plastisphere succession, particularly under freshwater conditions [6,50,51]. However, several studies have demonstrated that environmental conditions and local factors substantially influence the composition of plastic-associated microbial communities. For instance, Hoellein et al. [52] demonstrated that water exchange (e.g., river vs. pond) serves as a primary driver of biofilm community composition, as well as that significant differences exist in biofilm composition and metabolism depending on the substrate, with biofilms on organic substrates (cardboard and leaves) exhibiting lower gross primary production compared to those on hard substrates (glass, plastic, aluminum, and tiles). Given the vital importance of high-quality freshwater systems in supporting human health and societal well-being, this disparity highlights the need for a more comprehensive understanding of plastic pollution in freshwater ecosystems. These environments can harbor complex and diverse microorganisms assemblages that exhibit varying sensitivities to environmental and anthropogenic pollution [49,53].

The timing of biofilm formation and taxonomy richness could vary depending on seasonality (dry or wet season) and nutrient levels [8]. Our investigations are conducted

during the dry season and conditions of high nutrient levels in the water body. The Am Chua irrigational canal, receiving runoff from adjacent rice fields, presents conditions highly conducive to the colonization of plastic surfaces. These conditions include elevated concentrations of nutrients, particularly nitrogen and phosphorus, classifying the canal as a eutrophic environment characterized by reduced water transparency and intense solar irradiance. The tropical freshwaters of Vietnam harbor a diverse assemblage of microorganisms, including bacteria, archaea, fungi, viruses, algae, protozoa, etc. [23,25,26]. Consequently, plastic debris exhibiting positive buoyancy tends to remain near the water surface, predisposing it to prolonged exposure to solar radiation and dissolved organic matter, thereby increasing the opportunity for biofilm colonization. In our study, the formation of biofilms on EPS surfaces occurred rapidly, as evidenced by a color transition from white to yellowish-green or greenish-brown within 14 days of exposure. We also observed that the distribution of different organisms may exhibit spatial heterogeneity across the EPS surface. Consistent with previous research on early plastisphere development, bacteria and phototrophic eukaryotes as initial colonizers, suggesting their fundamental role in establishing the microbial community on plastic surfaces [5,6,47,54]. Unfortunately, our estimation of the diversity of organisms inhabiting the biofilm is insufficient to determine which organisms or their complex could give the repulsive chemical signals of biofilm affecting climbing perch. More studies are needed to clarify these interactions.

5. Conclusions

The temporal dynamics of biofilm formation on EPS in the eutrophic freshwater environments of Central Vietnam elucidate the complex feeding behavior of *Anabas testudineus*, emphasizing the primary role of surficial biofilm in stimulating initial attraction and subsequent behavioral responses. However, intraoral assessment—mediated by mechanoreception and chemoreception—typically leads to rejection, suggesting a protective mechanism against the ingestion of non-nutritive materials. We observed several features in the feeding behavior of climbing perch upon contact with floating microplastics: a delayed and prolonged response to clean plastic pellets compared to feed pellets; incorrect visual detection of plastics as prey objects, leading to an increase in grasping events as the extent of biofilm development on the plastic surface progresses; and a negative correlation between the duration of plastic testing in the oral cavity and the amount of biofilm on the surface of the plastic pellets. Consequently, floating plastics stimulate foraging behavior in climbing perch and prompt their upward movement toward the water surface, likely resulting in energy expenditure and increased predation risk without any obvious benefit to the individual. Thus, the current study indicates that the mere presence of microplastic debris can induce behavioral changes in hydrobionts without the necessity of plastic ingestion.

While this study provides valuable insights into the feeding ecology of *Anabas testudineus*, it is important to recognize that sensory-mediated feeding behaviors may vary significantly among different fish species. The complex interplay between biofilm dynamics and fish feeding behavior highlights the necessity of considering both biotic and abiotic factors that influence the fate and impact of microplastics in aquatic ecosystems. Additionally, the type of plastic waste, its size and buoyancy potential, along with the duration of exposure in the water ecosystem, could differently influence the responses of various fish species. Furthermore, comparative studies examining feeding behaviors across diverse fish species, along with detailed characterization of biofilm composition and toxicity, are essential to fully understand the risks that plastic debris pose to aquatic food webs.

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Institutional Review Board Statement: All experiments were conducted in accordance with the Vietnam National Regulations on the Use of Animals in Research [55], the Guide for the care and use of laboratory animals [56], the Guidelines for the treatment of animals in behavioral research and teaching [57], and the recommendations of the Bioethics Commission of the Institute of Ecology and Evolution A. N. Severtsov of the Russian Academy of Sciences (<https://sev-in.ru/en/komissia-po-bioetike>, accessed on 5 November 2021).

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Abbreviations

The following abbreviations are used in this manuscript:

EPS Expanded polystyrene
 PET polyethylene terephthalate
 XPS extruded polystyrene

Appendix A

Table A1. Chemical parameters of water in the Am Chua canal on the first day (1 February 2023) and the last day (15 February 2023) of the field study.

Parameters	First Day	Last Day
pH	6.5	6.7
Nitrites (NO ₂) mg/L	0.5	0.5
Nitrates (NO ₃) mg/L	10.0	10.0
Ammonia/Ammonium (NH ₃ /NH ₄)	1.0	0.5
Phosphates (PO ₄), mg/L	1.1	0.25
Iron (Fe), mg/L	6.0	1.0
Chlorides, Cl, mg/L	0	0

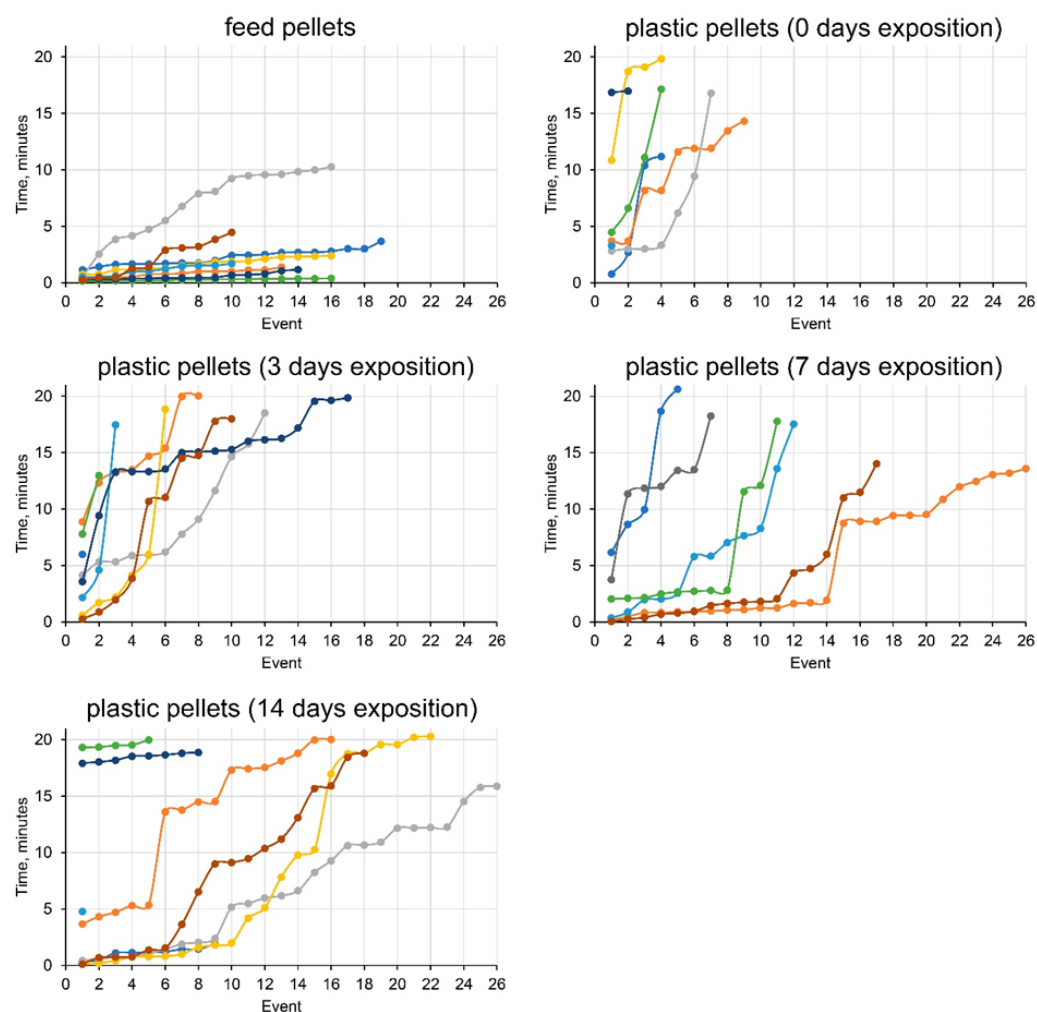


Figure A1. Behavioral events related to different pellet grasping by climbing perch *Anabias testudineus* during the trials. Each line or point (if representing only one event), colored in a special color, indicates a single trial.

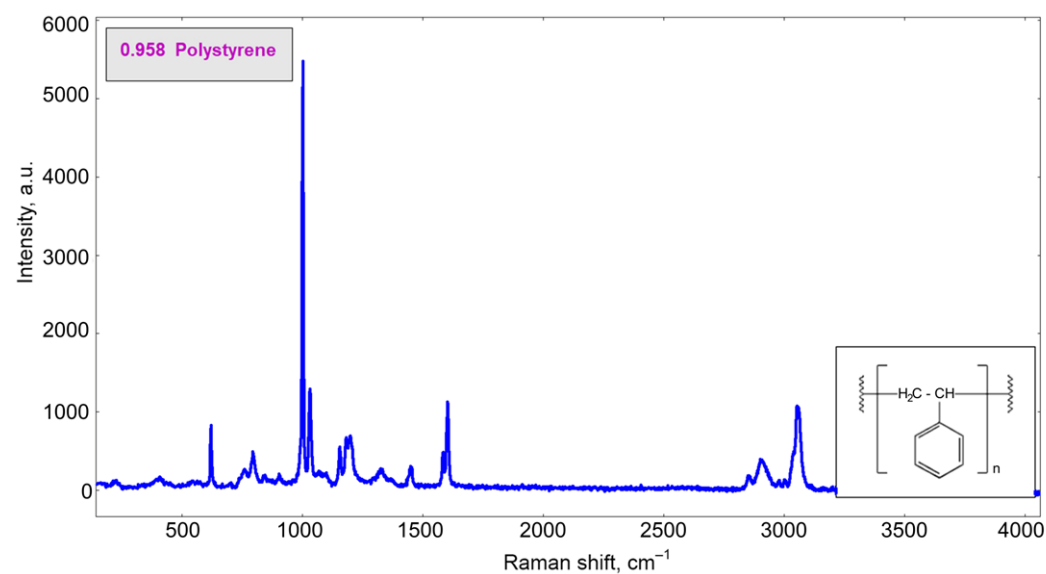


Figure A2. Raman spectra of pristine EPS pellets. The blue line represents the tested microplastic pellet.

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