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Dose- and Time-Dependent Behavioural Responses in Fish Following Sublethal Exposure to Emamectin Benzoate

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Abstract
The present study investigated the behavioural alterations in fish exposed to sublethal concentrations of emamectin benzoate (18 µg/L and 36 µg/L) over a period of 96 hours. Behavioural responses were assessed at 24, 48, 72, and 96 hours using a semi-quantitative scoring system to evaluate parameters such as hyperactivity, opercular movement, swimming rate, cornering behaviour, pigmentation changes, mucus secretion, loss of equilibrium, surface gulping, aggregation, and lethargy. The results revealed clear dose- and time-dependent behavioural modifications. At the lower concentration (18 µg/L), fish exhibited moderate alterations, including increased surface gulping, aggregation, and opercular movement, indicating early stress responses. Prolonged exposure led to the appearance of abnormal behaviours such as hypoactivity, reverse swimming, and mucus secretion, suggesting progressive physiological stress. In contrast, fish exposed to the higher concentration (36 µg/L) showed severe and persistent behavioural disturbances throughout the exposure period. Marked lethargy, increased surface gulping, excessive mucus secretion, and disoriented swimming were observed, indicating significant respiratory and neuromuscular impairment. Overall, the findings demonstrate that emamectin benzoate induces pronounced behavioural toxicity in fish, with higher concentrations causing severe and sustained effects. Behavioural endpoints proved to be sensitive indicators of toxic stress and can serve as effective tools for early detection of environmental contamination.

Keywords: Emamectin benzoate, fish toxicity, behavioural alterations, neurotoxicity, respiratory stress, surface gulping

Introduction
Aquatic ecosystems are increasingly threatened by the continuous influx of agrochemicals and veterinary pharmaceuticals, many of which ultimately reach water bodies through agricultural runoff, aquaculture practices, and improper disposal. Among these, emamectin benzoate (EB), a semi-synthetic derivative of avermectins, is widely used as an insecticide and antiparasitic agent, particularly in aquaculture to control ectoparasites such as sea lice (FAO, 2014; Burridge *et al*., 2010) [4, 8]. Although considered effective at low doses, its persistence and bioactivity raise concerns regarding its potential impact on non-target aquatic organisms, especially fish (Gu *et al*., 2023) [11, 12].
Emamectin benzoate exerts its toxic effects primarily by interfering with neurotransmission. It binds to glutamate-gated chloride channels in nerve and muscle cells, leading to increased chloride ion influx, hyperpolarization, and subsequent paralysis in target organisms (Lasota and Dybas, 1991; Woodland, 2024; Pan *et al* 2024) [17]. However, similar mechanisms may disrupt normal neural functioning in fish, resulting in observable behavioural abnormalities. Recent studies have highlighted that EB can induce significant neurotoxic and physiological disturbances in fish, even at sublethal concentrations. For instance, exposure to EB has been reported to impair locomotor activity and disrupt neurodevelopment in zebrafish larvae (Gu *et al*., 2023) [11, 12], while long-term exposure may affect reproductive health and behavioural patterns in subsequent generations (Guo *et al*., 2025) [13]. Additionally, alterations in feeding behaviour, reduced survival, and physiological stress responses have been documented in Nile tilapia following EB exposure (Das *et al*., 2023) [7]. The compound has also been shown to interfere with key enzymatic activities such as acetylcholinesterase, leading to impaired neural transmission and abnormal behavioural responses (2024). Furthermore, its persistence in aquatic sediments increases the risk of prolonged exposure, thereby exacerbating its ecological impact (Taormina *et al*, 2024) [25]. These findings underscore the need for comprehensive evaluation of the behavioural toxicity of emamectin benzoate in fish, as behavioural endpoints serve as sensitive early indicators of environmental stress.
Furthermore, behavioural impairments in fish may have ecological consequences, including reduced predator avoidance, impaired feeding efficiency, and altered social interactions, ultimately affecting survival and population dynamics (Scott and Sloman, 2004; Tierney, 2011) [23, 26]. Behavioural responses are considered sensitive early warning indicators of environmental stress (Hellou, 2010) [14]. Despite its widespread use, limited information is available on the sublethal behavioural effects of emamectin benzoate in freshwater fish species, although recent studies have begun to highlight its neurotoxic potential (Gu *et al*., 2023; Das *et al*., 2023) [7, 11, 12].
Therefore, the present study aims to evaluate the behavioural responses of fish exposed to varying concentrations of emamectin benzoate over different exposure periods. Understanding these changes will contribute to assessing the ecological risk of this compound and provide insights into its mode of action in non-target aquatic organisms.

Material and methods
Fish Collection and Acclimatization
Sixty specimens of freshwater fish, *Cyprinus carpio* (common carp), with an average length of 18 ± 0.5 cm and

body weight of 100 ± 5.5 g, were obtained from the fish farm of the Department of Fisheries, Dr. G.C. Negi College of Veterinary and Animal Sciences (DGCN COVAS), CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, Himachal Pradesh, India. Prior to the experiment, the fish were acclimatized for 10 days in aerated glass aquaria with a 100 L water capacity. The aquaria were filled with dechlorinated bore-well water. During the acclimatization period, important water quality parameters such as pH, ammonia, total hardness, carbonate hardness, nitrite, and nitrate levels were regularly monitored. The fish were fed commercial pelleted feed once daily, and the aquaria were regularly cleaned to maintain suitable water quality and ensure the health of the fish.

Table 1: Physicochemical parameters of water in control and emamectin benzoate-exposed groups (18 µg/L and 36 µg/L) at different time intervals (24, 48, 72, and 96 h)

Parameter		24 h	48 h	72 h	96 h
1.DIssolved oxygen (ppm)	Control	6.33 ± 0.57	6.66 ± 0.57	6.33 ± 0.57	6.66 ± 0.57
	Solvent control	7.00 ± 1.00	7.33 ± 0.57	6.33 ± 0.57	7.00 ± 1.00
	18µg/L	1.50 ± 0.00	2.66 ± 0.28	1.66 ± 0.28	1.50 ± 0.00
	36µg/L	1.33 ± 0.28	2.33 ± 0.28	2.00 ± 0.50	1.66 ± 0.28
2.Carbon dioxide (ppm)	Control	13.66 ± 0.57	13.33 ± 0.57	13.00 ± 0.00	13.66 ± 0.57
	Solvent control	14.00 ± 1.0	14 ± 1.00	14.66 ± 0.57	13.66 ± 1.15
	18µg/L	34.00 ± 2.00	26.00 ± 2.00	28.66 ± 1.15	36.67 ± 1.15
	36µg/L	30.67 ± 3.60	32.00 ± 5.29	22.00 ± 2.00	32.00 ± 2.00
3.Total hardness (ppm)	Control	49.66 ± 1.15	49.66 ± 0.57	49.33 ± 0.57	50.66 ± 0.57
	Solvent control	50.00 ± 1.00	50.33 ± 2.08	50.00 ± 0.00	51.00 ± 1.00
	18µg/L	32.66 ± 1.15	26.00 ± 2.00	28.66 ± 1.15	36.66 ± 1.15
	36µg/L	30.66 ± 1.15	32.66 ± 1.15	30.66 ± 1.15	33.33 ± 1.15
4.Alkalinity (ppm)	Control	49.33 ± 1.15	51.33 ± 1.15	52.66 ± 1.15	51.33 ± 1.15
	Solvent control	52.66 ± 1.15	51.33 ± 1.15	52.00 ± 0.00	51.67 ± 2.89
	18µg/L	30.66 ± 1.15	30.00 ± 0.00	30.00 ± 0.00	30.00 ± 0.00
	36µg/L	30.00 ± 0.00	30.00 ± 0.00	30.00 ± 0.00	30.00 ± 0.00
5.Total dissolved solutes (ppm)	Control	50.00 ± 1.00	50.00 ± 1.73	49.00 ± 1.00	50.00 ± 1.00
	Solvent control	50.66 ± 0.57	51.33 ± 1.52	50.66 ± 0.57	50.67 ± 1.15
	18µg/L	59.67 ± 2.31	63.67 ± 3.79	62.33 ± 6.51	46.66 ± 1.52
	36µg/L	63.67 ± 3.51	56.33 ± 1.52	55.33 ± 3.06	52.33 ± 1.52
6.Ammonia (ppm)	Control	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
	Solvent control	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
	18µg/L	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
	36µg/L	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
7.pH test	Control	7.77 ± 0.12	7.73 ± 0.17	7.74 ± 0.79	7.74 ± 0.12
	Solvent control	7.52 ± 0.16	7.54 ± 017	7.49 ± 0.13	7.54 ± 0.13
	18µg/L	6.55 ± 0.08	6.63 ± 0.03	6.50 ± 0.08	6.52 ± 0.08
	36µg/L	6.44 ± 0.05	6.56 ± 0.07	6.35 ± 0.06	6.55 ± 0.06
8.Temperature	Control	9.33 ± 0.57	9 ± 0.00	9 ± 0.00	9.33 ± 0.57
	Solvent control	9 ± 0.00	9 ± 0.00	9.33 ± 0.57	9.33 ± 0.57
	18µg/L	9 ± 0.00	9.33 ± 0.57	9 ± 0.00	9.33 ± 0.57
	36µg/L	9 ± 0.00	9 ± 0.00	9.33 ± 0.57	9 ± 0.00

Chemical

The pesticide Emamectin Benzoate (5% SG formulation; Katyayani Organics) was used as the test chemical. A stock solution was prepared in dimethyl sulfoxide (DMSO) following the procedure described by Guo *et al*. (2025) [13]. Two sublethal concentrations were selected based on previously reported LC_{50} values for *C. carpio* (Benson *et al*., 2017) [2]. The experimental concentrations corresponded to 1/10 LC_{50} (18 µg/L) and 1/5 LC_{50} (36 µg/L).

Experimental Design

The sublethal toxicity experiment was conducted in accordance with OECD Test Guideline 204 (OECD, 2019) [20]. Healthy fish of uniform size and free from visible pathological symptoms were randomly selected for the experiment.

Following acclimatization, the fish were randomly distributed into four groups, each consisting of 20 fish, and maintained in triplicate:

Group I: Control group (without chemical exposure)

Group II: Solvent control group (DMSO only)

Group III: Fish exposed to 18 µg/L emamectin benzoate

Group IV: Fish exposed to 36 µg/L emamectin benzoate

Behavioural Alterations

Behavioural responses of fish were carefully monitored in both the control and treatment groups at 24, 48, 72, and 96 hours of exposure. Various behavioural parameters were recorded, including hyperactivity, opercular movement, swimming rate, cornering behaviour, pigmentation changes, mucus secretion, loss of equilibrium, surface gulping, aggregation, lethargy, response to external stimuli, and scale erosion.

Fish in each experimental group were observed twice daily (morning and evening) throughout the exposure period. Observations were carried out by a trained observer to ensure accurate identification of subtle behavioural changes. Behavioural responses were assessed using a semi-quantitative scoring system, where (+) indicated mild alteration, (++) moderate alteration, and (+++) severe alteration. This scoring system facilitated comparison of

behavioural responses between the control and exposed groups.

Results

Behavioural responses of fish exposed to sublethal concentrations of emamectin benzoate (18 µg/L and 36 µg/L) showed clear dose- and time-dependent alterations (Tables 1 and 2).

At the low concentration (18 µg/L), fish exhibited moderate behavioural changes. During the initial exposure period (24–48 h), surface gulping, aggregation, and corner behavior, was observed, indicating early stress responses. As exposure progressed, independent movement increased, along with operculum movement and mucus secretion. Notably, hyperactivation and hypoactivation appeared at 96 h, suggesting a shift from initial stress to altered neuromuscular activity. Abnormal behaviours such as reverse swimming and jumping were also recorded at later stages.

In contrast, the high concentration (36 µg/L) induced severe behavioural disturbances throughout the exposure period. Lethargic behaviour and corner behaviour were consistently high, indicating strong stress and loss of normal activity. Independent movement was initially absent but gradually increased over time. Operculum movement and surface gulping were markedly elevated, especially at 72 h, reflecting significant respiratory distress. Additionally, hypoactivity, reverse swimming, and mucus secretion were prominent, particularly during prolonged exposure.

Overall, the results indicate that low-dose exposure induces moderate and adaptive behavioural responses, whereas high-dose exposure leads to pronounced and persistent behavioural impairment. The progressive increase in abnormal behaviours such as lethargy, surface gulping, and disoriented swimming confirms the toxic impact of emamectin benzoate on fish behaviour.

Table1: Heat map representation of behavioural alteration in fish exposed to low dose concentration 18µg/L

Parameters	24 h	46 h	76 h	96 h
Lethargic behaviour	++	-	-	++
Independent movement	-	++	++	++
Operculum movement	-	+	+	++
Corner behaviour	+	-	+	+
Aggregation	+	-	++	++
Hyper activation	-	-	-	++
Hypo activation	-	+	+	-
Surface gulfing	++	++	+++	+
Reverse swimming	-	+	+	+
Jumping	-	-	+	+
Mucus secretion	-	+	+	++

Table2: Heat map representation of behavioural alteration in fish exposed to High dose concentration 36µg/L

Parameters	24 h	46 h	72 h	96 h
Lethargic behaviour	++	+++	+++	++
Independent movement	-	-	+	++
Operculum movement	-	-	++	++
Corner behaviour	+++	+++	+++	+++
Aggregation	+++	+++	++	++
Hyper activation	-	-	-	+
Hypo activation	-	++	++	+
Surface gulfing	+	+	+++	++
Reverse swimming	-	-	++	++
Jumping	-	-	+	+
Mucus secretion	+	+	++	++

Discussion

The present study demonstrates that exposure to emamectin benzoate (EB) induces pronounced dose- and time-dependent behavioural alterations in fish, highlighting its potent neurotoxic and respiratory effects. Behavioural endpoints such as surface gulping, opercular movement, lethargy, abnormal swimming, and loss of equilibrium are widely recognized as sensitive and rapid indicators of toxic stress in aquatic organisms (Scott and Sloman, 2004; van der Oost *et al*., 2003; Kane *et al*., 2005) [15, 23, 27]. These behavioural changes often precede biochemical and histopathological alterations, making them reliable early warning biomarkers of environmental contamination (Little and Finger, 1990; Schreck *et al*., 2001) [18, 22].

At the lower concentration (18 µg/L), fish exhibited initial hyperactivity, aggregation, and increased surface respiration during the early exposure phase (24–48 h), indicating acute stress and elevated metabolic demand. Such hyperactive responses are commonly associated with stimulation of the nervous system and increased energy expenditure under toxicant exposure (Little and Finger, 1990; Tierney, 2011) [18, 26]. The observed increase in surface gulping and opercular movement further suggests an attempt to compensate for reduced oxygen availability or impaired gill function (Fernandes and Mazon, 2003) [10]. As exposure progressed, enhanced independent movement and mucus secretion were recorded, reflecting adaptive or compensatory mechanisms aimed at maintaining physiological homeostasis. Excess mucus production, in particular, has been linked to protective responses against toxicants by forming a barrier over gill surfaces, although prolonged secretion can impair respiration (Olson, 2002; Aisyah & Andriani, 2024) [1, 21]. Similar patterns of initial behavioural excitation followed by adaptive responses have been reported in fish exposed to pesticides and pharmaceutical contaminants (Das *et al*., 2023; Gu *et al*., 2023; Kumar *et al* 2020) [7, 11, 12, 16].

In contrast, exposure to the higher concentration (36 µg/L) resulted in severe and persistent behavioural impairments throughout the experimental period. Fish displayed pronounced lethargy, reduced locomotor activity, and increased cornering behaviour, indicating central nervous system depression and loss of normal behavioural patterns. Such hypoactivity is often attributed to neuroinhibitory effects of toxicants, leading to reduced responsiveness and impaired coordination (Kane *et al*., 2005; Tierney, 2011) [15, 26]. Emamectin benzoate, a macrocyclic lactone, is known to interfere with neurotransmission by modulating γ-aminobutyric acid (GABA)-gated chloride channels, resulting in altered neuronal excitability and neuromuscular dysfunction (Bloomquist, 2003; Campbell, 2012) [3, 6]. The marked increase in surface gulping and opercular activity at higher concentration further indicates severe respiratory distress, likely due to gill damage, reduced oxygen uptake, and disruption of ion regulation (Fernandes and Mazon, 2003; Evans *et al*., 2005) [10].

Additionally, abnormal behaviours such as reverse swimming, erratic movement, and disorientation observed during prolonged exposure reflect impaired sensory-motor integration and neurotoxicity. These findings are consistent with previous studies reporting behavioural disruption in fish exposed to EB and other neurotoxic pesticides, where alterations in swimming patterns and activity levels were

linked to oxidative stress and neuronal damage (Singha *et al*., 2022; Guo *et al*., 2025; Peter *et al* 2021) [13, 24]. The progressive shift from hyperactivity at lower exposure durations to hypoactivity at higher concentrations and prolonged exposure highlights a transition from initial stimulation to eventual physiological exhaustion and toxicity.

Overall, the observed behavioural responses clearly demonstrate that EB exerts significant toxic effects on fish, with severity increasing with both concentration and exposure duration. The consistent alterations in key behavioural endpoints such as locomotion, respiration, and social behaviour confirm their utility as sensitive biomarkers for assessing aquatic toxicity. These findings underscore the ecological relevance of behavioural studies in evaluating the impact of pharmaceutical and pesticide contaminants on aquatic organisms.

Mechanistically, EB acts on γ-aminobutyric acid (GABA)-gated and glutamate-gated chloride channels, leading to increased chloride ion influx, neuronal hyperpolarization, and disruption of synaptic transmission. This results in an initial hyperexcitation phase, followed by neuromuscular paralysis and hypoactivity (Lasota and Dybas 1991 avermectin mode of action; Campbell 2012 avermectin neurotoxicity) [6, 17]. Recent evidence further suggests that EB exposure induces oxidative stress, acetylcholinesterase inhibition, and neurodevelopmental toxicity, which collectively contribute to behavioural dysfunction (Das *et al*., 2023; Gu *et al*., 2023; Marine Pollution Bulletin, 2023) [7, 12, 13].

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