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Modulation of levamisole and nicotine toxicity in soil nematodes Caenorhabditis elegans and Caenorhabditis briggsae by moderate heat stress and ambient pH

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Abstract. The influence of moderate heat stress and changes in ambient pH in the range of 6.0 to 8.0 was investigated on two soil nematodes (*Caenorhabditis elegans* and *Caenorhabditis briggsae*). Rise of ambient pH from 6.0 to 8.0 increased the sensitivity of nematodes to agonists of nicotinic cholinoreceptors levamisole (30–120 μM) and nicotine (1–4 mM) by 1.5–5 folds, but not to acetylcholine esterase inhibitor aldicarb (60–240 μM). An increase in temperature up to 30°C caused a steep rise in sensitivity of *C. elegans* and *C. briggsae* to levamisole. The influence of ambient pH on sensitivity of *C. elegans* and *C. briggsae* to levamisole at 30°C was similar to that at 22°C. Therefore, mechanisms of sensitization of *C. elegans* to levamisole toxicity by an increase in pH or by temperature rise are different. The most likely mechanism for the increase in levamisole and nicotine toxicity in *C. elegans* and *C. briggsae* by a rise in ambient pH is deprotonation of levamisole and nicotine. This study shows that it is desirable to conduct the evaluation of the biological activity of toxicants in nematodes in the pH range 6.0–8.0, and not at a single pH.

1. Introduction

Soil is crucial component of terrestrial ecosystems. Soil plays key roles in fertility, decomposition, and the flow of nutrients and energy. These functions in soil are mediated by soil invertebrates and microorganisms. Soils are often contaminated by chemicals used for agricultural and forest management, causing a frequent exposure of soil invertebrates to toxicants. Studies on the negative effect of pollutants on soil organisms may be performed through monitoring the status of communities in natural ecosystems or by carrying out toxicity tests in the laboratory [1].

The most abundant metazoan group in soil are nematodes. Field studies on the effect of pollutants on nematodes meet some difficulties such as (i) the complexity of the soil environment with many factors affecting soil biota, (ii) a great variation in nematode distribution in the soil horizontally and vertically, (iii) rather high genetic variability in a natural population, (iv) high sensitivity of total nematode abundance and community structure to environmental stresses, (v) varying efficiencies of methods of nematode extraction from soil samples, and their consequent effect on the evaluation of pollutants effects [1].

Laboratory tests allow performing experiments under controlled conditions and providing the necessary initial step towards the evaluation of environmental risk. One of the most commonly used nematode species in laboratory assays is *Caenorhabditis elegans*. It has been firstly used as a model for genetic researches, and at present, it is one of the most studied species [1–2]. *C. elegans* is a convenient model animal not only for genetic researches, but also for ecological, toxicological, neurobiological, and pharmacological studies. This nematode is a popular model organism because of its easy laboratory breeding in Petri dishes, small size (less than 1 mm), short-length life cycle (3–4 days at temperature of 20°C), high fertility (approximately 300 offspring from each self-fertilizing hermaphrodite) [2–3].

Nowadays sublethal effects are used as endpoints in ecotoxicology more often than mortality due to their higher sensitivity and accuracy [4–5]. Such sublethal endpoints were used in toxicological experiments with nematodes include the reproduction, ability of larvae to develop to adulthood stage, growth, movement, feeding, respiratory, length of juvenile period, and body length, etc. [5–8]. Different methods were developed for toxicological research with *C. elegans* not only on agar plates but also in liquid media [1, 9]. Most ecotoxicological studies with *C. elegans* are conducted at a constant temperature, optimal for nematodes, and at single ambient pH. It is also known that temperature and pH can alter the toxicity of pollutants for nematodes. Therefore, the aim of our research was to assess the influence of ambient pH and moderate heat stress on the sensitivity of two soil nematodes, namely *C. briggsae,* and *C. elegans*, to the toxic action of nematicides.

2. Materials and methods

C. elegans from N2 wild type strain and *C. briggsae* from AF16 wild type strain were used for experiments. Both strains were obtained from the Caenorhabditis Genetics Center. Nematodes were bred at 22°C on standard Nematodes Growth Medium (NGM) (2.5 g/L bactopeptone, 17 g/L bactoagar, 3 g/L NaCl, 5 mg/L cholesterol, 1mM $MgSO_4$, 1 mM $CaCl_2$, and 25 mM potassium phosphate buffer at pH = 6.0 or 8.0) [10] in Petri dishes of 100 mm diameter. As a food resource *E. coli* OP50 was used.

Experiments were carried out in NG buffer (1 mM $CaCl_2$, 1 mM $MgSO_4$, 3 g/L NaCl, and 25 mM potassium phosphate buffer at pH = 6.0, 7.0 or 8.0) [10] with synchronized young adults. Before each experiment, worms were washed away from the agar plate with NG buffer (pH = 6.0) into a Petri dish of 40-mm diameter. From this dish worms were transferred using a pipette to a centrifuge tube of 10 mL volume. In this glass tube, worms were rinsed in 10 mL of NG buffer to remove growth medium, bacteria, and metabolites. The supernatant was removed after worms had settled at the bottom of the tubes. The wash procedure was repeated for three times, and a total wash time was approximately of 30–35 minutes. The worms then were transferred to a new 40-mm diameter Petri dish containing NG buffer, and finally worms were transferred individually using a 10-µL pipette into glass tubes containing 1 mL of NG buffer (pH = 6.0, 7.0, or 8.0).

Aldicarb, an inhibitor of acetylcholine esterase (AChE), and agonists of nicotinic cholinoreceptors (nAChRs), levamisole hydrochloride and nicotine hemisulfate (all substances obtained from Sigma Aldrich), dissolved in distilled water, were added to the NG buffer immediately after putting worms into glass tubes. Behavioral disorders caused by the above-mentioned substances were registered using stereomicroscope SMZ-05 at two temperatures, including 22°C and 30°C.

The indicators used to characterize behavior disturbances in worms were as follows: (i) uncoordinated behavior (a lack of coordination of contraction of body muscles which necessary for sinusoidal movements of nematodes body), and (ii) complete paralysis (the total lack of response to mechanical stimulus).

Each variant of the experiment was performed 5 times with 30 nematodes. In each of five independent experiments behavioral disturbances of nematodes were registered after 15, 30, 60, and 90 minutes of exposure to substance under study. For each time point both the proportion of worms with movement disorders and the percentage of paralyzed nematodes were calculated. In Tables 1–3 the mean percentage for 5 experiments along with standard deviation are shown.

3. Results and discussion

Nematodes *C. briggsae* and *C. elegans* are usually bred in laboratories on standard NGM at pH 6.0 [10] and most experiments with these nematodes are also performed at pH 6.0 or 7.0. The soil pH may vary from 3.5 to 9.0 or higher. More than 90% of *C. elegans* individuals can survive in pH ranging from 3 to 10 [11–12] and maintain reproductive power at pH 4.6 [13]. Therefore, in toxicological experiments, it is necessary to evaluate the negative effects of pollutants on nematodes at different pH.

We evaluated the *C. elegans* and *C. briggsae* sensitivity to agonists of nAChRs nicotine and levamisole and AChE inhibitor aldicarb in pH range from 6.0 (the standard nematode growth medium pH) to 8.0 (alkalescent soil pH). This pH range is optimal for most plants and soil invertebrates.

Levamisole in concentrations 60 and 120 μM caused movement disorders in 21 and 48% nematodes respectively after 15 minutes incubation with toxicant at pH 6.0. At pH 7.0, levamisole at 30, 60, and 120 μM concentrations caused 57, 65, and 100% movement disorders in nematodes, respectively (Table 1).

Table 1. The influence of ambient pH on sensitivity of *C. elegans* locomotion to levamisole, nicotine and aldicarb at 22°C.

	The quotient of nematodes with locomotion disturbances after short-term (15-minutes) exposure to nicotine, levamisole and aldicarb, % (Mean±SD)								
	Levamisole, μM			Nicotine, mM			Aldicarb, μM		
	30	60	120	1	2	4	60	120	240
pH=6.0	0	21±3	48±1	0	18±4	52±3	0	24±3	72±2
pH=7.0	57±2	85±1	100	31±3	68±2	80±1	0	28±2	75±1

Table 2. The influence of ambient pH on *C. briggsae* and *C. elegans* sensitivity to toxic action of levamisole, nicotine and aldicarb at 22°C.

	The percentage of paralyzed worms after 90-minutes exposure to toxicants, % (Mean±SD)					
	C. elegans			*C. briggsae*		
	pH=6.0	pH=7.0	pH=8.0	pH=6.0	pH=7.0	pH=8.0
Levamisole						
7.5 μM	0	0	0	0	0	13±1
15 μM	0	8±1	28±1	0	18±1	44±2
30 μM	13±1	23±1	52±2	14±1	43±2	85±3
Nicotine						
7.5 mM	0	0	15±1	0	0	27±3
15 mM	10±1	16±1	31±2	15±1	31±2	51±2
30 mM	15±2	24±2	52±1	21±2	48±3	61±3
Aldicarb						
7.5 μM	11±1	12±1	11±1	15±1	14±1	16±1
15 μM	35±2	37±2	36±2	41±2	45±2	44±3
30 μM	75±3	78±3	76±3	85±4	88±4	86±4

An increase in the exposure time of levamisole to 90 minutes led to palsy in nematodes (their full inability to maintain both spontaneous locomotion and mechanical stimulus-induced swimming). As shown in Table 2, the increase in pH from 6.0 to 8.0 increased the toxicity of levamisole both on *C. elegans* and *C. briggsae*. In pH range 6.0–7.0, the sensitivity to toxic levamisole action was stronger in *C. briggsae* in comparison with *C. elegans*, while pH change from 7.0 to 8.0 reduced this difference in sensitivity between *C. briggsae* and *C. elegans* (Table 2).

Table 3. The action of ambient pH and temperature on levamisole toxicity for *C. briggsae* and *C. elegans*.

		The percentage of paralyzed worms after 90-minutes exposure to toxicants, % (Mean±SD)					
		C. elegans			*C. briggsae*		
		pH=6.0	pH=7.0	pH=8.0	pH=6.0	pH=7.0	pH=8.0
	Levamisole						
22°C	3.7 µM	0	0	0	0	0	0
30°C	3.7 µM	0	0	0	0	0	22±2
	Levamisole						
22°C	7.5 µM	0	0	0	0	0	15±1
30°C	7.5 µM	11±1	23±2	41±2	25±2	54±3	85±5
	Levamisole						
22°C	15 µM	0	7±1	29±4	0	19±2	51±3
30°C	15 µM	15±1	48±3	100	18±1	78±3	100

To assess whether an increase in toxicity due to pH change is specific only for levamisole, we investigated the dependence of nicotine toxicity on pH. Nicotine is currently used as an insecticide, but its nematicidal activity is rather low due to its low permeability through nematode cuticle. Nevertheless, nicotine at 2 and 4 mM concentrations caused disturbances of locomotion, induced by strong mechanical stimulus, in 18 and 52% of worms at pH 6.0 and in 68 and 80% nematodes at pH 7.0, respectively (Table 1). High nicotine concentrations (more than 10^{-3} M) caused disturbances in *C. briggsae* and *C. elegans* locomotion (Table 2). These disturbances are the consequence of nAChRs hyperactivation [14–15]. We observed that pH change from 6.0 to 8.0 increased the *C. briggsae* and *C. elegans* sensitivity to nicotine. Therefore, pH as an environmental factor has strongly influenced nematodes' sensitivity not only to levamisole but also to nicotine, another nAChRs agonist.

We earlier observed that the *C. elegans* sensitivity to levamisole could be rised by a moderate increase in external temperature [15]. In the current work, we studied the influence of a moderate increase in external temperature on the *C. briggsae* and *C. elegans* sensitivity to levamisole at different pH. A temperature rise up to 30°C strongly increased the *C. briggsae* and *C. elegans* sensitivity to levamisole (Table 3). This action of ambient pH on the sensitivity to levamisole at 30°C was similar to that observed at 22°C (Table 3). Therefore, the mechanisms of sensitization of *C. briggsae* and *C. elegans* to levamisole toxicity because of the increase in pH or temperature are different. In our previous work, we observed that the thermostability of *C. briggsae* organisms is higher than that of *C. elegans* [16]. Therefore, the increase in sensitivity to levamisole toxicity at temperature increase up to 30°C was higher in nematodes of *C. briggsae* AF16 strain than in N2 *C. elegans* strain both at pH 6.0 and 7.0 (Table 3). Therefore, our experiments show the absence of

correlation between resistance to levamisole toxicity and to damaging action of high temperature in nematodes of genus Caenorhabditis.

Nematodes can easily adapt either to toxicants or to physical environmental factors. Therefore, one may assume that nematodes, which were grown at different ambient pH, namely 6.0 and 8.0, may possess different sensitivity to levamisole toxicity. Contrary to this hypothesis, our experiments with *C. briggsae* and *C. elegans*, grown at pH 6.0 and 8.0 showed that pH of growth medium had no effect on the levamisole toxicity.

The increase in sensitivity to levamisole due to pH rise in the range of 6.0–8.0 made it possible to hypothesize that ambient pH may also influence the nAChRs sensitivity of the assessed nematodes to acetylcholine. The level of acetylcholine in cholinergic synapses can be increased by AChE inhibition. In this study, we found that pH variation did not influence the toxicity of the AChE inhibitor aldicarb for *C. elegans* and *C. briggsae* (Table 1 and 2).

Two possible mechanisms for the effect of pH on levamisole toxicity in nematodes include:

1. Sensitization of nAChRs to levamisole and other their agonists by an increase in ambient pH.

2. Changes in levamisole and nicotine toxicity at different ambient pH.

We believe that the second mechanism is more likely. Levamisole and nicotine possess slightly hydrolytic properties and may exist in both protonated and deprotonated forms, subject to external acidity. These two forms may differ in their toxicity if deprotonated molecules have better permeability through cuticle or higher affinity to nAChR. Moreover, levamisole hydrochloride is stable in acidic medium and is subject to hydrolysis in neutral and alkaline mediums. It is also known that the absorption rate of nicotine through human biological membranes is pH-dependent, being higher at pH 8.5 than at pH 5.5 [17–18]. Therefore, in our experiments with nematodes, the increase in nicotine toxicity in pH range 6.0–8.0 may be a consequence of higher permeability of uncharged lipophilic nicotine molecules through their cuticle. Data indicative of this explanation may be, (i) in our experiments aldicarb toxicity for nematodes did not change in pH range 6.0–8.0 (Table 2), and (ii) unlike levamisole and nicotine, aldicarb cannot exist in protonated or deprotonated forms. Therefore, the most likely reason for the increase in levamisole and nicotine toxicity in *C. briggsae* and *C. elegans* due to the rise in ambient pH is the high permeability of nematode cuticle for deprotonated forms of these toxicant molecules.

4. Conclusion

Ambient pH may strongly influence the toxicity of nematicides for *C. briggsae* and *C. elegans* depending on the chemical structure of toxicants. A moderate increase in external temperature from 22°C up to 30°C and a rise in ambient pH in the range 6.0–8.0 had a synergistic effect on sensitivity of *C. briggsae* and *C. elegans* to levamisole. Deprotonation of levamisole and nicotine enhances their permeability into the internal environment of nematodes. Consequently, deprotonation of levamisole and nicotine is the most likely mechanism for an increase in their toxicity in *C. briggsae* and *C. elegans* following a rise in ambient pH. The results of our study show that it is desirable to conduct the evaluation of the biological activity of toxicants in nematodes in pH range 6.0–8.0, and not at a single pH. The sensitivity of *C. briggsae* to toxic levamisole action was found to be higher than that of *C. elegans* both under a slight increase in environmental temperature, and a rise in external pH.

Many modern pesticides, such as neonicotinoids, sulfoximines, butenolides, spinosyns, and nereistoxin analogs, target the cholinergic system of invertebrates. The mode of action of these pesticides involves activation or blockage of invertebrate nAChRs. The results of this work allow the presumption that toxicity of the above-mentioned pesticides may depend on external pH. To check this hypothesis additional studies are required.

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