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Assessment of the bioaccumulation of nicotine and cotinine by the crustacean Daphnia magna

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ABSTRACT

Objectives. This study aimed to investigate the toxicity of nicotine and its metabolite cotinine on crustacean D. magna, and evaluate the quantity of compounds accumulated by D. magna. *Materials and Methods*. The bioassays involved the exposure of D. magna to varying doses of nicotine and cotinine, for 24 h and 48 h. The amount of bioaccumulated nicotine and cotinine was determined by an HPLC-DAD method. *Results*. The study has revealed that nicotine is more toxic than cotinine on D. magna, as the medium lethal concentration (LC50) values were higher for nicotine compared to cotinine. After 24 hours of exposure, D. magna accumulated comparable amounts of nicotine and cotinine. However, after 48 hours of exposure, the crustacean accumulated significantly lower levels of nicotine, which is consistent with the higher toxicity of nicotine compared to cotinine. *Conclusions*. These findings demonstrated that nicotine triggers various alterations in aquatic organism, hence jeopardizing the equilibrium of the aquatic ecosystem within a little timeframe.

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Introduction

In the last decade, it has been suggested that acute tests on invertebrates can be used as a pre-screening method for assessing the acute toxicity of new chemical substances on mammals and humans.

Thus, it has been demonstrated that the biological screening using invertebrate bioassays shows a high degree of correlation with in vivo acute toxicity and can be predictive of cytotoxicity in human cell cultures.

Several studies have shown that the bioassay using Daphnia magna is specific and sensitive for indicating toxicity in rats [1]. For a chemical substance with high toxicity to the D. magna crustacean (LC50 <0.22 mg / l), this biotest provides valuable information, practically providing evidence regarding toxicity in rats.

Considering the Holarctic distribution of the Daphnia magna crustacean in freshwater systems, this bioassay is used as a model for assessing acute and chronic toxicity of aquatic invertebrates [2]. The advantages of using D. magna as a standard test are related to their parthenogenetic reproductive strategy, as well as the simplicity of the test itself (facilitation of handling and observation).

It has been shown that the biological screening by using invertebrate bioassay present a high degree of correlation with the acute in vivo toxicity and it is predictive for the cytotoxicity on human cells cultures.

Several studies demonstrated that D. magna test is more specific than sensitive for an indication of the toxicity to the rat. For a chemical with a high probability of toxicity to D. magna (LC50 < 0.22 mg/L), this bioassay provides valuable information, virtually giving evidence of toxicity to the rat.

The major advantage of using invertebrate bioassays is reduction of the number of mammals required for toxicity testing. In addition, being an in vivo test, taking into account the biotransformation of toxicants and potential integrated effects that occur in the organism as a whole are reasonable. Therefore, the invertebrate bioassays seem to be preferable to in vitro methods applicable to predict the acute toxicity to human. The toxicity of nicotine is well-documented, with nicotine being a highly toxic compound

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that exerts its toxic action practically at the level of every organ. In addition, nicotine has been recognized as a psychoactive drug, acting on different area of brain [3]. Therefore, nicotine is a compound that is commonly consumed by humans as part of their lifestyle and is therefore frequently detected in entire ecosystem.

Daphnia magna, an aquatic crustacean, is a crucial species in the field of ecotoxicology. It is frequently utilized as a test organism to evaluate environmental risks.

Applying aquatic invertebrates for testing offers the benefit of engaging multicellular organisms and is more cost-effective compared to testing on vertebrate animals. In this type of research, it is essential to precisely measure the intake of the test substance by the organism and evaluate the amount of harmful substance that has been assimilated or accumulated by the organism.

The D. magna test is currently used to assess how the nicotine affects the heart rate and behaviour of Daphnia, with the aim of extrapolating the results to humans [4-6]. These extrapolations are based on similarities between Daphnia and humans in terms of the cellular respiratory system, the presence of hemoglobin in their blood to transport oxygen and the heart. In addition, Daphnia is a crustacean with a clear and transparent exoskeleton, which is useful, as researchers can observe ongoing processes in the organism.

Cotinine, the major metabolite of nicotine, is currently used as biomarker for tobacco exposure. Currently, controversy exists regarding the toxic potential of cotinine.

In order to evaluate the acute toxic effects of cotinine comparatively with its parent compound, nicotine, Daphnia magna bioassay has been used. The study also aimed to evaluate the amount of toxic substance accumulated by Daphnia magna. The amount of bioaccumulated nicotine and cotinine was determined by a previously developed HPLC-DAD method.

The method HPLC has found its usefulness in the analysis of the nicotine and its major metabolite, the cotinine. The reported methods in literature have taken into account either the quantification of the nicotine in the type products used in electronic cigarettes [7-10] or in the analysis of the biological samples, especially in the preclinical studies [11]. Generally, few HPLC methods with detection in UV are published for the analysis of the nicotine and cotinine in the biological samples [12-17]. Several HPLC methods have taken into account the dosage of the cotinine in order to use it as a biomarker for smoking, but without quantifying the nicotine [18-21]. Few papers reported HPTLC methods [22], as this technique is simple and economic and has been successful applied for the abuse substances detection [23].

Considering the literature data referring to the analysis of the nicotine and cotinine in biological samples by HPLC, a simple HPLC method with photodiode array detection was developed to simultaneously analyses the nicotine and the cotinine in biological samples.

Materials and Methods

Substances to be tested:(-)- Nicotine (≥ 99%, Sigma-Aldrich) and (-)-Cotinine (≥98%, Sigma-Aldrich). Preparing the solutions. The stock solutions of nicotine and cotinine of concentration 10mM were prepared in DMSO. Then, out of these, they obtain, as the case may be, solutions to be tested, by adequate dilution with culture medium.

Evaluation of the toxicity of nicotine and cotinine on D. magna

Daphnia magna Straus (Figure 1) have been maintained parthenogenetically in Carol Davila University, Department of Pharmaceutical Botany and Cell Biology, since 2012. The bioassay was performed according to the method described by Nitulescu et al. 2013, with some modifications (Olaru et al. 2015) [24,25]. Young daphnids were sorted according to their size. Serial dilutions were made from nicotine and cotinine in order to test concentrations from 0.1 to 10 µM. Each determination was performed in triplicate on 10 daphnids. The lethality was recorded after 24 and 48 h of exposure in a synthetic water at constant condition of temperature and humidity (25°C, 75% RH). Daphnids were considered dead if they did not move their appendages for 30 s during observation.

The lethal concentrations that kill 50% of organisms (LC50) at 24 and 48 h were determined by interpolating on lethality - logarithm of concentration curves using the least squares fit method. 95% confidence intervals of LC50 (CI 95%) and the correlation coefficient (r2) of the curves, were also calculated. All calculations were performed using GraphPad Prism version 5.0 software (USA).

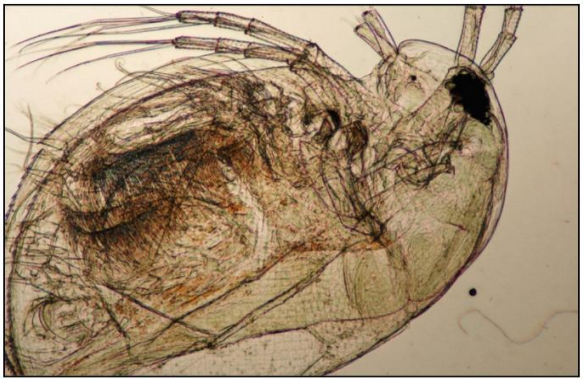

Figure 1. Daphnia magna (40x)

Assessment of the bioaccumulation of nicotine and cotinine by D. magna

Exposure of the Daphnia to nicotine and cotinine was made according to the experimental protocol presented above. Assessment of the bioaccumulation of nicotine and cotinine by the crustacean Daphnia magna. The final concentrations, which the invertebrates were exposed to, were selected on the basis of the study concerning the lethality assessment: 0.25 µM nicotine and 1.0 µM cotinine. These have been the concentrations of the 50%

lethality in 24 h and higher or equal to 50% in 48 h. A group of the Daphnia has been exposed to a mixture of the two substances. After 24/48 h from the exposure, the Daphnia have been washed with distilled water through a filtering funnel provided with ceramic filter (Sigma), then they have been transferred on a paper filter. After having been dried for 2 minutes, the Daphnia were weighed, then they were put into a homogenizer, made of glass, in which 2 mL of distilled water were added. After obtaining the homogenous result, this was centrifugated for ten minutes at 1600 rotations per minute at the room temperature. After the centrifugation, one mL supernatant was taken and submitted to deproteinization with a mixture of equal parts of methanol: acetonitrile. The samples were agitated in a vortex for several minutes, then centrifugated for 5 minutes at 1400 r/per minute. 100 µL supernatant were injected in HPLC.

The quantification of bioaccumulated nicotine and cotinine was performed by a previously developed HPLC method [26]. The separation was achieved on a Hypersil Gold (150 mm length x4.6 mm i.d., 5µm particle size) chromatographic column, using as mobile phase 20 mM triethylamine, acetic acid and ammonia, adjusted to pH 10 and acetonitrile in a ratio of 85:15 (v:v), with a flow rate of 1 mL/min and a column temperature of 25°C. The detection wavelength was set at 260 nm. The method was validated by parameters provided in literature (selectivity/ specificity, linearity, precision, accuracy, limits of detection and quantification), in order to use the bioaccumulation of the nicotine for assessment by the D. magna crustacean.

Devices and instruments: liquid chromatograph Surveyor Plus, with a PDA detector (photodiode array detector) having a special program to determine the spectral purity of the eluated compounds, quaternary pump, with degassifier incorporated and thermostated (Peltier) autosampler and the column compartment.

Results

Investigation of the toxicity of nicotine and cotinine on D. magna. The lethality – logarithm of concentration curves is presented in Figure 2 and Figure 3 and the results of statistical analysis are shown in Table I.

Table I. Toxicity assessment of nicotine and cotinine on *D. magna*

Compound	Moment of determination	LC50 (µM)	CI95% of LC50 (µM)	r2
Nicotine	24 h	2.134	1.204 - 3.781	0.7601
	48 h	0.163	0.084 - 0.316	0.7876
Cotinine	24 h	ND	ND	ND
	48 h	0.959	0.225 - 4.083	<0.6000

ND – not determined; CI95% of LC50 – 95% confidence interval of LC50; r2 – goodness of fit

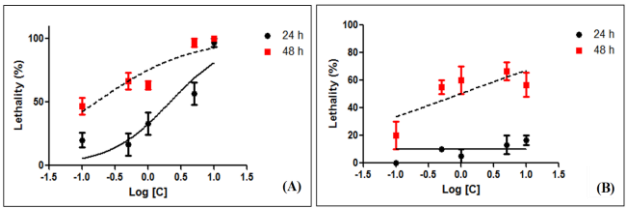

Figure 2. Lethality – concentration curves for nicotine (A) and cotinine (B)

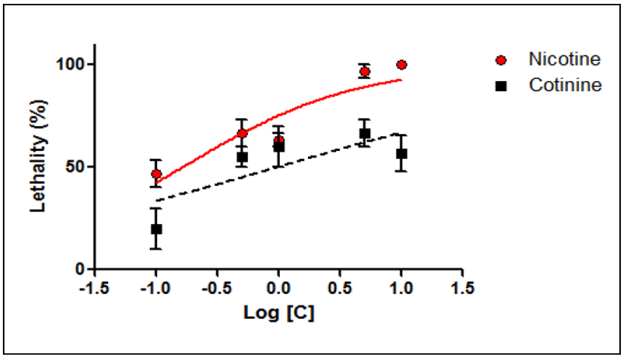

Figure 3. Lethality – concentration curves for nicotine and cotinine at 48h of exposure

Evaluation of bioaccumulation of nicotine and cotinine by D. magna (Table 2).

Table II. The descriptive statistics concerning the quantity of nicotine and cotinine accumulated by *Daphnia magna*

Sample	N	Quantity(µ/g)			Standard deviation	Standard Error
		Minim	Maxim	Average		
Nicotine (24 h)	3	3.87	4.93	4.27	0.5736	0.3311
Cotinine (24h)	3	3.47	4.54	4.17	0.3394	0.1956
Nicotine in mixture, 24 h	3	3.75	4.76	4.24	0.5056	0.2919
Cotinine in mixture,24 h	3	2.90	3.33	3.10	0.2165	0.1250
Cotinine in nicotine samples, 24h	3	1.17	1.44	1.29	0.1365	0.0788
Nicotine 48h	3	1.84	2.34	2.10	0.2516	0.1453
Cotinine 48h	3	3.43	4.01	3.81	0.3292	0.1900
Nicotine in mixture ,48h	3	1.02	1.56	1.31	0.2730	0.1576
Cotinine in mixture ,48 h	3	2.58	3.30	2.92	0.3611	0.2085
Cotinine in nicotine samples	3	1.87	2.39	2.10	0.2640	0.1524

Table III. Experimental data in terms of the accumulation of nicotine and cotinine by *D. magna*

Substance	Determined Quantity(µg/g) *	
	Exposure 24 h	Exposure 48 h
Nicotine	4.27 +/- 0.331	2.10+/- 0.145
Cotinine	4.17+/-0.195	3.72+/-0.190
Nicotine (determined in the samples exposed to the mixture (nicotine+cotinine)	4.24+/- 0.291	1.31+/- 0.157
Cotinine (determined in the samples exposed to the mixture nicotine+cotinine)	3.10+/-0.125	2.92+/-0.208
Cotinine (determined in the samples exposed to nicotine)	1.29+/-0.078	2.10+/- 0.152
*average +/- standard error		

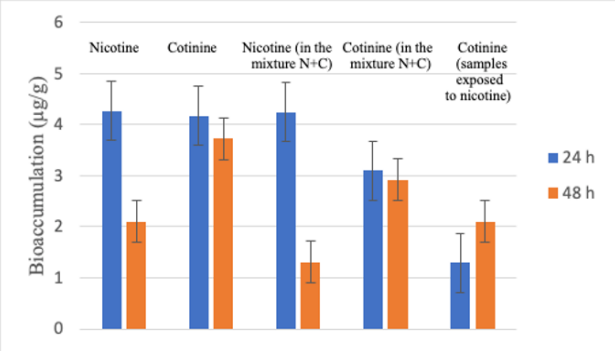

Figure 4. Bioaccumulation of the nicotine and cotinine by Daphnia magna (the daphnia were exposed to nicotine, cotinine and mixture nicotine + cotinine, for 24 h and 48 h; N=nicotine, C=cotinine) ($p< 0.01$ for cotinine/cotinine in samples exposed to nicotine, at 24 h; $p<0.05$ for cotinine/ cotinine in the mixture N+C, at 24 h; nicotine/cotinine, at 48 h; nicotine (at 24 h), nicotine (at 48 h).

Discussions

After 24 hours of exposure, cotinine does not exhibit toxicity, while nicotine had a median lethal concentration (LC50) of 2.13 µM. The lethality induced by cotinine was shown after 48 hours of exposure, with a LC50 of 0.96 µM. The overall results indicate that nicotine has a higher toxicity than cotinine, as the ratio between the LC50 values (cotinine/nicotine) after 48 hour of exposure is of approximately 5.9. At 48 h, lethality curves of both compounds are parallel (fig. 2), indicating similar toxicity effects with different potency.

Both substances have LC50 values calculated at 48 hours of exposure that indicate a very high level of toxicity. The toxicity is significantly higher than that of colchicine, which had an LC50 of 13.9 µM [24]. However, the toxicity of the substances is approximately 1.5 times lower than that of potassium dichromate, which had an LC50 of 0.62 µM [27].

According to literature data, nicotine at a dosage of 100 µg/L (0.617 µM) negatively impacted the reproductive capacity of the crustacean D. magna by reducing the number of young produced by females. Conversely, it was demonstrated that doses of 10 µg/L (0.0617 µM) or greater stimulated the generation of males, suggesting that nicotine acts as a slight endocrine juvenoid component in D. magna [28]. This study reported a no-effect concentration (NOEC) value of 1 µg/L for nicotine on D. magna.

Evaluation of bioaccumulation of nicotine and cotinine by D. magna

Extensive research of recent years referring to the environmental micropollutants allowed to clear up the mechanisms of absorption and their accumulation in the environment organisms [29,30]. The mechanism proposed for absorption is the one of the passive diffusions through the cell membranes and the established samples are based on the physicochemical properties, as for example the partition coefficient octanol/water (log P) in order to describe and predict the concentration of xenobiotic in the environmental organisms [31,32]. New mechanisms like ion retention, the carrier-mediated transport and the distribution in the non-lipidic compounds (protein binding) were also proposed to acquire residues of pharmaceutical substances in the environment [33-35]. Because most of the researches were focused on vertebrate animals, like fish, the bioaccumulation of the xenobiotics in invertebrates is not fully known. The guide OCDE 305 is largely used to estimate the factor of bioconcentration (BCF) or the factor of bioaccumulation (BAF) in fishes and was also applied to invertebrates, like bivalve and amphibious mollusks [36-38]. In spite of all these, recent investigations have showed that the OCDE model led to significant disparities for the measured data in the case of invertebrates [39]. Among the causes of these disparities, it is generally the fact that the biotransformation is not taken into account in the bioconcentration studies [40].

The assessment the toxicity of the nicotine on D. magna crustacean has presented significant differences of toxicity between the two compounds. The cotinine is known as the main metabolite of the nicotine identified in the human plasma and urine. Considering all these aspects, we aimed to investigate the accumulation of the two substances by D. magna. The Daphnias were exposed, according to the experimental protocol presented, in nicotine and in the mixture of the two substances.

The descriptive statistics on the quantity of nicotine and cotinine accumulated by Daphnia magna under the circumstances of exposure described in the experimental protocol is presented in the Table I.

The results show that after 24 h of exposure, the Daphnias accumulate similar quantities of nicotine and cotinine, but after 48 h of exposure, the quantity of nicotine

accumulated is significantly less (p= 0.021) than the one in the cotinine (Table III). These differences could be interpreted in the context of the values CL50, significantly lower in the case of the nicotine, this showing toxicity labeled after 48 h of exposure (6 times higher than in the case of the cotinine). After 24 h of exposure, the Daphnia accumulate higher quantity of cotinine (similar to the nicotine), we can correlate the reduced toxicity after 24 h, in the context of which the differences of partition coefficient octanol/water are significant (nicotine is much more hidrophobe, having a value of logP of 1.17, and the cotinine 0.07), and the most frequent mechanism proposed for absorption is the one of the passive diffusions through cell membranes.

The quantity of cotinine accumulated after 24 h is significantly statistically higher than the one accumulated by exposure to the mixture of nicotine and cotinine (p=0.016), suggesting the possible influence of the nicotine and its lethal effect on the Daphnia. The quantity of nicotine, accumulated after 48 h of exposure is statistically significantly lower (p=0.021) than the nicotine on D. magna. While the quantity of cotinine accumulated at the two testing moments does not differ significantly statistically, the quantity of nicotine accumulated after 24 h is significantly statistically higher, at the limit of statistical significance (p=0.044) than the one in the nicotine accumulated in 48 h, in accordance with the higher toxicity of the nicotine after 48 h, reflected by CL50.

The analysis of the correlations (Person, Kendall and Spearman) did not highlight correlations between the quantities of nicotine and cotinine depending on the two moments of exposure and the test conditions.

The results have shown the presence of the cotinine in the samples which have been exposed to nicotine only, suggesting the biotransformation of the nicotine into cotinine by means of the D. magna crustacean (Table III). The quantity of cotinine after 24 h is statistically significantly higher than the one determined in the samples exposed only to nicotine (p=0.007), indicating that the nicotine is partially biotransformed into cotinine after 24 h.

Recent researches have shown that some invertebrates (ex. Gammarus Pulex, an amphibious crustacean) have the power to biotransform a large range of organic micropollutants [41-43]. The conservation of the enzymes of the cytochrome P450 was noticed at invertebrates, and some active medicine substances have proved that they undergo oxidizing and conjugation reactions [42,44]. However, there are limited data regarding the xenobiotic's transformation by the invertebrates.

The aquatic crustacean Daphnia magna is an important species for the ecotoxicology study and is often used as a testing organism to assess the risk on the environment. However, the mechanism of the metabolizing the xenobiotics by D. magna has not been studied in detail.

They have reported a recent toxicokinetic model which describes the bioconcentration and the biotransformation of the diazinon organophosphoric repellent by the Daphnia magna crustacean to the inactive compound 2-izopropil-6-metil-4-piridimol (resulted by the hydrolysis of the organophosphoric ester) as well as to the active metabolite diazinon (resulted by oxidative desulphurisation) [45].

They have also demonstrated that the cytochrome P450 (CYP) and some other enzymes which take part in the conjugation reactions (ex: sulphoconjugation) are important in the biotransfer of the xenobiotics by D. magna. Thus, they have shown that D. magna can metabolize the pyrene of the hydrosoluble metabolites [46], as well as the l-hydroxipyrene (resulted by hydroxilation on the way of the cytochrome P450, the biotransformation being significantly inhibited by SKF-525 A, an inhibitor of the cytochrom P450. They have also demonstrated that the hydroxilated metabolite underwent other reactions in the second phase, respectively sulphoconjugation.

Conclusions

The investigation of the nicotine toxicity on the crustacean Daphnia magna has shown that the higher toxicity of the nicotine compared to cotinine, proved by the values of the average lethal concentration (CL 50) has been obtained after only 48 h of exposure.

The assessment of the nicotine and cotinine bioaccumulation by the Daphnia magna has shown that the Daphnia accumulate similar quantities of nicotine and cotinine after 24 h of exposure, but significantly less quantities of nicotine after 48 of exposure, in correlation with the toxicity of the nicotine compared to the one of the cotinine.

The cotinine has been quantified in the samples exposed to nicotine indicating the biotransformation of the nicotine into cotinine by the Daphnia magna.

The results obtained provide valuable data for investigating the toxic potential of the nicotine and cotinine in different experimental models on invertebrates.

Further studies are necessary to deepen the mechanism of the nicotine and cotinine toxicity, as well as their influence over the metabolism Daphnia magna.

Contributions

Conceptualization: B.D.L., V.A.M., O.O.T, Data curation: B.D.L., O.O.T., G.D.; Formal analysis: B.D.L.; Investigation: V.A.M., B.D.L., O.O.T.; Methodology: G.D., N.G.V.; Project administration: B.D.L., O.O.T., V.A.M.; Resources: O.O.T, B.D.L.; Supervision: B.D.L., G.D.; Validation: B.D.L., V.A.M., G.D.; Visualization: O.O.T., B.D.L., V.A.M.; Writing – the initial draft: B.D.L., V.A.M., O.O.T.; Writing – revision and editing: B.D.L., V.A.M.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. Informed consent was obtained from all subjects involved in the study.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

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