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ECOTOXICITY STUDIES OF E-LIQUIDS FOR E-CIGARETTES

BADANIA NAD EKOTOKSYCZNOŚCIĄ E-PŁYNÓW DO E-PAPIEROSÓW

Abstract: E-cigarette has gained popularity as a "healthier" alternative to receiving nicotine, which reduces the side effects of smoking traditional cigarettes. Consequently, the question arose how solutions for filling the electronic cigarettes (e-liquids) impact on the environment. They contain, among others, nicotine, propylene glycol and/or glycerin and flavorings. The aim of this study was to evaluate the impact and toxicity degree of selected e-liquids on microorganisms come from two different aquatic ecosystems. Studies were conducted using 96-well microtiter plates where a medium (peptone from soybean) containing the tetrazolium hydrochloride and selected for testing e-liquids with the nicotine concentration gradient were applied. An inoculum was the water from the Brynica River and an effluent was from a waste water treatment plant. The results were evaluated after two days incubation at 30°C. As a measure of the ecotoxicity degree of tested samples used the value of microbial toxic concentration (*MTC*) which was determined using the MARA® procedure (NCBI, Scotland). All tested e-liquids triggered off an inhibition of microorganism growth only after two or eightfold dilution. It was found that obtained *MTC* values were slightly varied for base components and individual e-liquids.The amount of nicotine in the e-liquids had no influence on the microorganisms growth.To a greater extent, only some flavorings might have had an impact on microorganisms.

Keywords: ecotoxicity, MARA, e-cigarettes, e-liquids, microorganism

Introduction

Electronic cigarettes were designed to provide nicotine vapor without toxic compounds, which are present in tobacco smoke [1] and mimic behavior associated with smoking. They gained popularity as a less harmful alternative to conventional cigarettes.

Electronic cigarettes use heat to transform e-liquids into a vapor inhaled by a user. E-liquids mainly consist of base components - propylene glycol and glycerin, nicotine and flavorings [2]. Propylene glycol and glycerin are mostly combined in a ratio of 4:1 or 7:3, respectively. They can also contain polyethylene glycol (PEG) or diethylene glycol (DEG) as a substitute of base components. The maximum concentration of nicotine permitted currently in Poland is 20 mg/cm^3 [3], but "free-of-nicotine" e-liquids are also available. Other used additives are preservatives (e.g. ethanol, up to 5%) and substances regulating viscosity (e.g. water, ethanol). Generally, e-liquids differ in various flavorings, for example, vanilla, fruity or tobacco flavor and a percentage composition of main ingredients [4]. The appearance on the market of new products such as e-liquids used in e-cigarettes arises the questions how they influence a human health, as well as an environment.

There are many available tests evaluate an ecotoxicity of test agent or substance to microorganisms in different taxonomic groups. Microbiological tests are useful in an assessment of an aquatic environment pollution degree and introduction effects of a specific pollutant in the biosphere. In a case of samples collected from aquatic environment (water or sediments), a presence of a harmful or toxic substance is observed as a vital functions and activity inhibition of test microorganisms. This phenomenon displays

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inter alia as a growth speed (cells or biomass), luminescence bacteria *Photobacterium phosphoreum* (*Vibrio fisheri*) or an activity of enzymes (ATPases, LDH dehydrogenases, nitrate reductase).

An example of bioecotoxicological test is MARA® test (Microbial Assay for Risk Assessment Test). This biotest is mainly a screening test in an assessment of surface waters (fresh, salty and salt water), ground waters, treated and untreated waste waters and leachate from landfills toxicity. As a multispecies toxicity test includes 10 different bacteria in taxonomic terms and yeast. These organisms are placed in a 96-well microtiter plate. In addition, a medium and a dye (2,3,5-triphenyltetrazolium chloride) are added to each well. The changes of the dye from colorless to red are observed only in healthy microorganisms by reduction reaction. Introduction of a toxic substance (in concentration gradient) to each microorganism strain may result in their growth inhibition and display as a lack of characteristic color of the reduced dye. After an incubation microtiter plate is scanned and resulted picture is analyzed using MARA® software.

In our study, we used a modified MARA® procedure [5] to evaluate ecotoxicity of 8 selected e-liquids, propylene glycol and glycerin. As a source of microorganisms, we used water from the Brynica River and an effluent from waste water treatment plant. This new approach enabled to gain preliminary information about influence e-liquids on natural ecosystem using fast and inexpensive method.

Materials and methods

Reagents

Eight kinds of commercially available e-liquids were selected for the study. The main selection criteria were as follows: 1) the products came from different manufacturers, 2) e-liquids had various nicotine concentration and 3) one manufacturer but e-liquids had different additives/flavorings. The characteristics of the e-liquids used in the experiments are presented in Table 1.

Table 1

The characteristics of the investigated e-liquids

Trade name of e-liquid	Manufacturer	Nicotine content [mg/cm^3]	Batch No.	Abbreviation in text
Coffee Gold	Volish, Poland	18	01/2014	VCG
Coffee	Mild, PRC	18	BOGE30032013	MK
Caffe Latte	Liqueen, PRC	18	PI05010014	LCL
German Hazelnut	Liqueen, PRC	18	PI05006014	LGH
Blackcurrant	Liqueen, PRC	11	AI-85/07/2013	LBC
New Zealand Peach	Liqueen, PRC	11	AI-85/04/2013	LNZ
Italian Cherry	Liqueen, PRC	11	AI-85/04/2013	LIC
Ice Mint	Liqueen, PRC	24	130217	LM

Propylene glycol (POCh, Poland, p.a. grade) and glycerin (POCH, Poland, p.a. grade) were used as a reference. Before ecotoxicity determination selected liquids, propylene glycol or glycerin was mixed with redistilled water in the ratio 1:1 (v/v). To estimate the e-liquids ecotoxicities to microorganisms present in the collected water samples, an aqueous medium containing peptone from soybean meal (2% w/v; Becton, Dickinson &

Co. San Jose, CA, US) and 2,3,5-triphenyltetrazolium chloride (TTC; 0.01% w/v, POCh, Poland, p.a. grade) was used.

Test microorganisms

In the research the following test microorganisms were used: unselected microorganisms took from the water stream of heavily polluted the Brynica River (Poland) and microorganisms presented in effluent from waste water treatment plant (WWTP) in Sosnowiec-Zagorze (Poland).

Assessment of toxicity of the investigated samples

To assess the ecotoxicity of investigated samples to microorganisms, the 96-well microtiter plates were used in experiments. Firstly, 0.15 cm^3 of growth medium (2% w/v peptone from soybean with 0.01% w/v TTC) was filtered with a sterile filter (Nalgene Syringe Filter, Sterile SFCA membrane, 0.2 μm, Thermo Scientific) and added to each well in rows A-F.

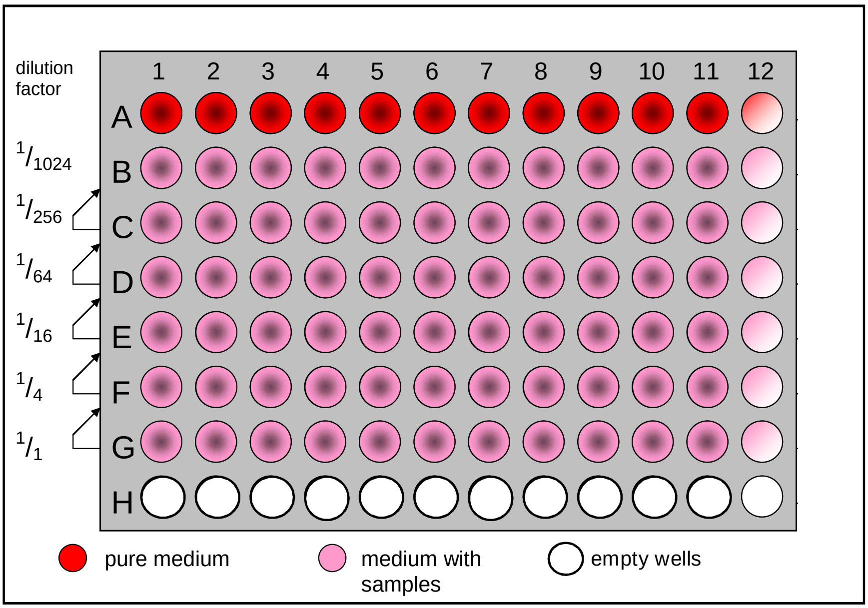

Fig. 1. Procedure of plate preparation

Immediately prior to starting an experiment solid peptone from soybean (2% w/v) and TTC (0.01% w/v) were added to redistilled water solution of selected liquid, propylene glycol or glycerin (samples). Thus prepared samples were also filtered with sterile filter and were added (0.2 cm^3) to each well in a row G. Then, 0.05 cm^3 of the solutions from each well of the row G were transferred to the corresponding wells in a row F. After mixing well contents (medium with sample), 0.05 cm^3 of the solutions from each well in the row F was transferred to wells in a row E. This procedure was repeated to a row B, generating subsequent sample dilutions. To retain a constant volume of the solutions in each well, 0.05 cm^3 of each well content of the row B was collected and discarded. The row A contained 0.15 cm^3 of sterile medium only (positive control). Prepared plates were inoculated with the 0.015 cm^3 of freshly collected water from the Brynica River or effluent from WWTP, except for solutions in the column number 12, which was the cleanliness control. The procedure of plate preparation is presented in Figure 1.

Every sample was repeated 11 times (one plate). Microtiter plates were incubated for 48 h at 30°C. The growth of test microorganisms was observed as a red pellet (*P*) in the wells. The TTC dye changed color (from colorless to red) due to the reduction reaction in viable cells. Thus intensity of pellet color was directly proportional to the growth degree of test microorganisms [6-9].

The minimum sample concentrations inducing visible the growth inhibition of each microorganism expressed as Microbial Toxic Concentration (*MTC*) were determined by the MARA® software (NCIMB Ltd, Scotland) as follows:

$$MTC = C_{min} \cdot d^{\left(P_{tot}/P_0\right)-1}$$

where C_{min} is the lowest concentration in the gradient, *d* is the dilution factor, P_0 is the pellet size in the control well and P_{tot} is the sum of the pellet sizes in all wells that were exposed to the concentration gradient of the samples solutions [10]. The changes on each plate after incubation time were recorded using a scanner (HP Scanjet G4050) with transmitted light. The scans were analyzed using the MARA® software, which calculated the percentage growth and growth inhibition of the microbes in each well relative to the control and the *MTC* values for test microorganisms.

Results and discussion

In this study, we examined the influence of selected e-liquids on unselected microorganisms presented in the Brynica River (Poland) and in the effluent from waste water treatment plant (Sosnowiec-Zagorze, Poland). Additionally, an antimicrobial activity of propylene glycol and glycerin (main ingredients of e-liquids) was investigated. Ecotoxicity degree was determined based on modified MARA procedure and *MTC* value. The highest sample concentration (e-liquid, propylene glycol or glycerin) was 500 g/dm^3. An illustrative test plate is presented in Figure 2.

A plot in Figure 3 shows a corresponding correlation $P_i = f\left(C_i\right)$, where P_i is a pellet size in a well with a sample, C_i is a sample concentration. The percentage microbial growth relative to control is marked on the right axis. The denoted EC_{50} value expresses the concentration of investigated e-liquid that causes 50% inhibition of microorganisms growth.

All results of carried out experiments (*MTC* values) are presented in Table 2 and Figure 4.

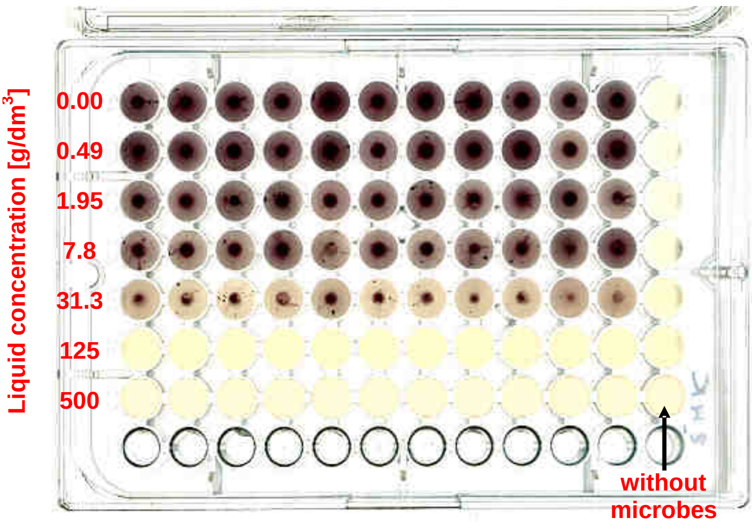

Fig. 2. Scan of the selected plate containing investigated e-liquid (MK) and inoculated with the effluent from WWTP (color intensity is proportional to growth of microorganisms)

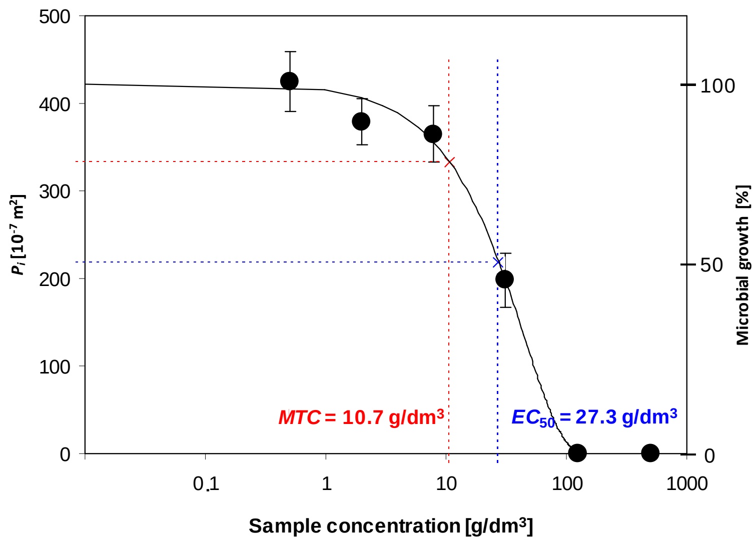

Fig. 3. Effect of the e-liquid concentration (MK) on the pellet size of test plate (left axis) and on percentage microbial growth (right axis) inoculated with the effluent from waste water treatment plant. *MTC* and EC_{50} values are marked on the plot

Table 2

The *MTC* values ± SD [g/dm^3] determined for samples in relation to test microorganisms

Sample	Water from the Brynica River	Effluent from the WWTP
Propylene glycol	31.6 ± 2.7	29.0 ± 5.7
Glycerin	48.8 ± 7.6	20.8 ± 7.4
VCG	37.2 ± 3.3	22.5 ± 6.1
MK*	27.5 ± 2.5	10.7 ± 3.2
LCL	36.4 ± 4.5	20.5 ± 6.9
LGH	34.6 ± 1.2	25.4 ± 3.7
LBC	56.1 ± 8.4	33.1 ± 1.2
LNZ	38.5 ± 4.2	31.7 ± 3.1
LIC	35.1 ± 1.4	32.4 ± 2.0
LM	32.0 ± 6.3	23.4 ± 1.5

* the most ecotoxic e-liquid (mean *MTC* values ± SD determined for this e-liquid were lower than values determined for propylene glycol and glycerin in all experiments)

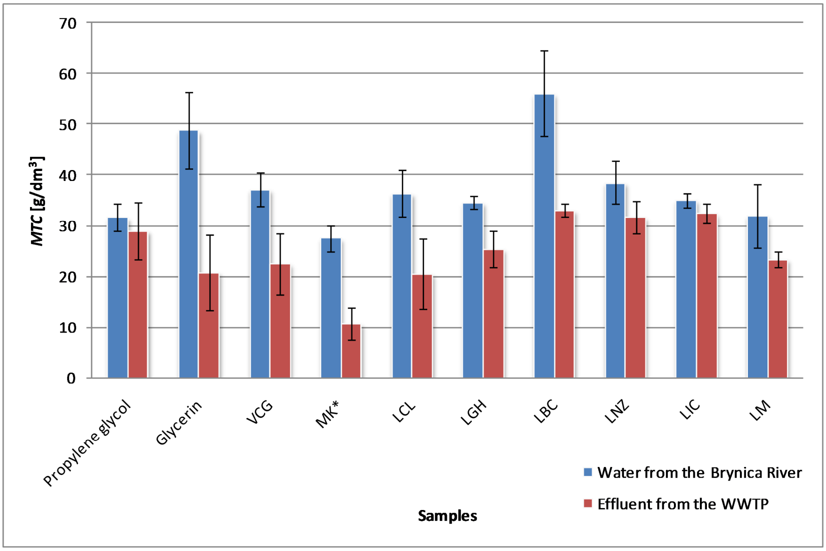

Fig. 4. Ecotoxicity of the investigated e-liquids relative to microorganisms from the inoculum selected for study

Obtained *MTC* values indicate that propylene glycol, glycerin, and selected e-liquids appear to inhibit microbial growth in both tested water samples used at a concentration > 50 g/dm^3. Such high concentrations of the investigated substances and e-liquids should not exhibit negative influence on microorganisms in the natural environment. Slightly higher *MTC* values were noted for the water from the Brynica River. Water from this river can be classified as highly contaminated, mainly industrial pollution, which may indicate its high electrical conductivity [11]. The above data suggest a greater resistance of microorganisms present in this water to the impurities.

The lowest *MTC* value, thus the highest ecotoxicity, was obtained for the coffee flavored e-liquid (MK). Due to the high average *MTC* values for the base components (propylene glycol and glycerin), their effect on growth inhibition of microorganisms can be probably eliminated. In addition, there was no correlation in the increase in nicotine concentration in e-liquid with the decrease of *MTC* values. Based on the above observations, it can be assumed that the greatest ecotoxicity of coffee flavoured e-liquid (MK) may be driven by the presence of flavors.

Conclusions

We found that propylene glycol and glycerin- e-liquid base components as well as selected e-liquids used in electronic cigarettes, inhibit the growth of microorganisms present in water samples, but only in the examined concentrations of > 50 g/dm^3. This ecotoxicity effect may be due to a rheological change of the medium. However, such high concentrations of investigated substances and e-liquids should not exhibit negative influence on microorganisms in the natural environment. Based on analysis of the *MTC* values, e-liquids are more ecotoxic than propylene glycol or glycerin. Furthermore, the ecotoxicity of e-liquids does not depend on the concentration of nicotine in e-liquid. Thus, it can be assumed that the flavors added to e-liquids are responsible for observing inhibition of microbial growth.

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BADANIA NAD EKOTOKSYCZNOŚCIĄ E-PŁYNÓW DO E-PAPIEROSÓW

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Abstrakt: E-papieros zyskał popularność jako „zdrowsza” alternatywa przyjmowania nikotyny, która ogranicza skutki uboczne palenia tradycyjnych papierosów. W związku z tym pojawiło się pytanie, jak wpływają na środowisko roztwory służące do napełniania e-papierosów (e-liquidy). Zawierają one m.in. nikotynę, propanodiol i/lub glicerynę oraz substancje smakowe. Celem pracy była ocena wpływu i stopnia toksyczności wybranych e-liquidów na mikroorganizmy pochodzące z dwóch różnych ekosystemów wodnych. Badania przeprowadzono z użyciem 96-dołkowych płytek mikrotitracyjnych, na których aplikowano pożywkę (pepton sojowy) zawierającą chlorowodorek tetrazoliny i gradient stężeń wytypowanych do badań e-liquidów. Inokulum stanowiła woda z rzeki Brynicy oraz efluent z oczyszczalni ścieków. Wyniki oceniano po inkubacji trwającej dwie doby w temperaturze 30°C. Jako miarę stopnia ekotoksyczności badanych próbek wykorzystano wartość mikrobiologicznego stężenia toksycznego (MTC), wyznaczanego za pomocą programu procedury MARA® (NCBI, Szkocja).Wszystkie badane e-liquidy powodowały inhibicję wzrostu mikroorganizmów jedynie po dwukrotnym lub ośmiokrotnym rozcieńczeniu. Stwierdzono, że uzyskane wartości MTC były zróżnicowane w niewielkim stopniu dla składników bazy i poszczególnych e-liquidów. Ilość nikotyny w e-liquidach nie miała wpływu na wzrost mikroorganizmów. W większym stopniu na mikroorganizmy mogły oddziaływać jedynie niektóre z dodatków smakowych.

Słowa kluczowe: ekotoksyczność, MARA, e-papierosy, e-płyny, mikroorganizmy