

1 **Assessment of ecotoxicity and total volatile organic compound (TVOC) emissions from**
2 **food and children's toy products**

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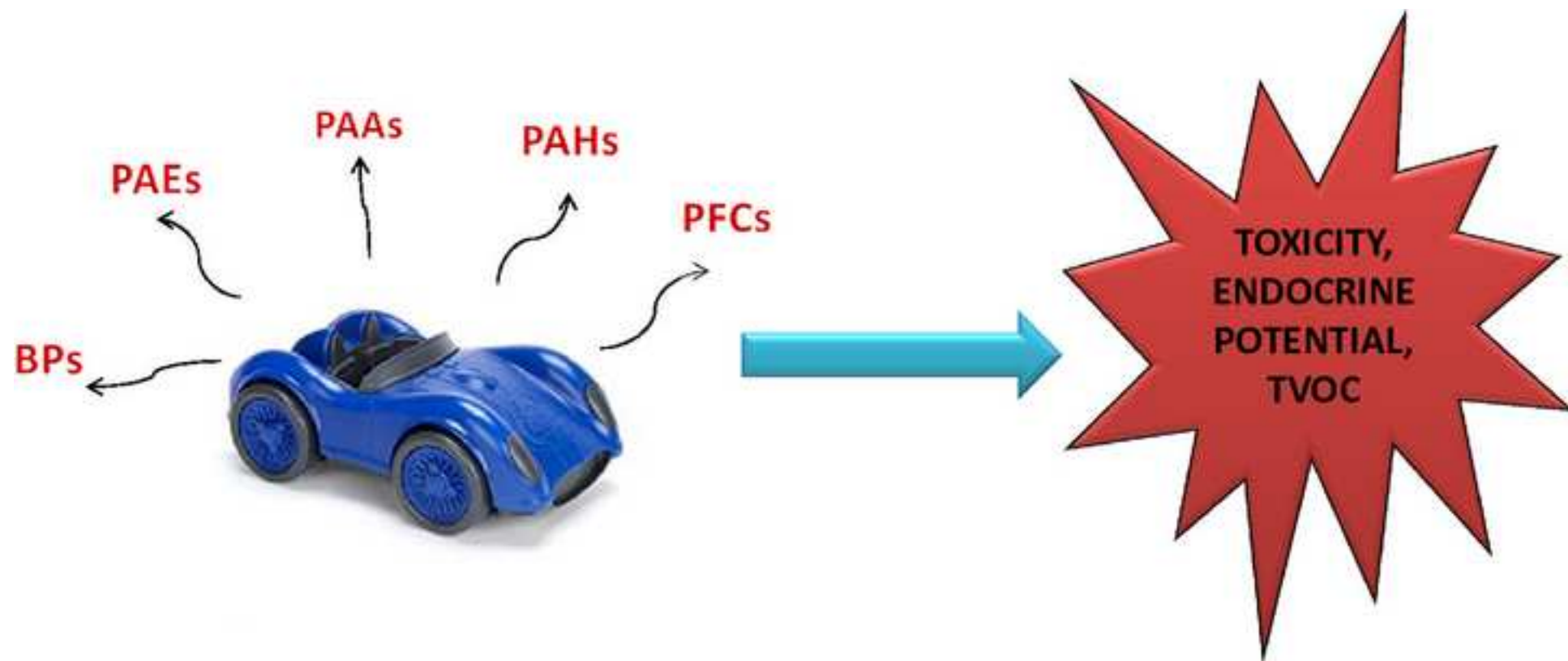
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Highlights

- The assessing studies of total volatile organic compounds emissions from plastic materials were performed;
- The impact of time on toxicity of extracts of product devoted for children was investigated;
- The endocrine potential of extracts of investigated objects was **assessed**;
- The correlation between **total volatile organic compounds** emission and toxicity of children's products was estimated;
- The mathematical assessment approach was applied in the field of ecotoxicity modeling studies

14 **Abstract**

15

16 The development of new methods for identifying a broad spectrum of analytes, as well as
17 highly selective tools to provide the most accurate information regarding the processes and
18 relationships in the world, has been an area of interest for researchers for many years. The
19 information obtained with these tools provides valuable data to complement existing
20 knowledge but, above all, to identify and determine previously unknown hazards. Recently,
21 attention has been paid to the migration of xenobiotics from the surfaces of various everyday
22 objects and the resulting impacts on human health. Since children are among those most
23 vulnerable to health consequences, one of the main subjects of interest is the migration of
24 low-molecular-weight compounds from toys and products intended for children. This
25 migration has become a stimulus for research aimed at determining the degree of release of
26 compounds from popular commercially available chocolate/toy sets. One of main objectives
27 of this research was to determine the impact of time on the ecotoxicity (with *Vibrio fischeri*
28 bioluminescent bacteria) of extracts of products intended for children and to assess the
29 correlation with total volatile organic compound emissions using basic chemometric methods.
30 The studies on endocrine potential (with XenoScreen YES/YAS) of the extracts and showed
31 that compounds released from the studied objects (including packaging foils, plastic capsules
32 storing toys, most of toys studied and all chocolate samples) exhibit mostly androgenic
33 antagonistic behavior while using artificial saliva as extraction medium increased the impact
34 observed. The impact of time in most cases was positive one and increased with prolonging
35 extraction time.

36 The small-scale stationary environmental test chambers - μ -CTE™ 250 system was employed
37 to perform the studies aimed at determining the profile of total volatile organic compounds
38 (TVOCs) emissions. Due to this it was possible to state that objects from which the greatest
39 amounts of contaminants are released are plastic containers (with emission rate falling down
40 from 3273 to 2280 ng/g of material at 6 h of conditioning in elevated temperature).

41

42 **Keywords:** TVOC; ecotoxicity; endocrine potential; chemometric methods; children's toys



43 1. Introduction

44

45 For parents around the world, the health and wellbeing of their children is an issue of
46 paramount importance. Still, despite numerous legislative and procedural difficulties for
47 producers of substandard toy and food producers, several accidents occurred that forced the
48 scientific community to search for new ways to respond to this threat. Promisingly, research is
49 being conducted to combine biological, instrumental and chemometric studies to assure
50 holistic assessment of the quality of products intended for children and determine the impact
51 on human well-being.

52 To rapidly obtain screening data about the estimated amount of volatile organic compounds
53 (VOCs) released from the surfaces of various types of products into indoor air, research is
54 carried out to assess the values of total volatile organic compounds (TVOCs) in a defined
55 medium. According to published information, TVOC parameter is determine as the sum (total
56 amount) of wide spectrum of organic compounds eluting between the defined analytical
57 window - retention times from n-hexane to n-hexadecane on an appropriate gas
58 chromatography capillary column (non-polar or slightly polar stationary phases) employing
59 flame ionization detection (GC-FID) and quantified as toluene equivalents (ECA, 1997;
60 Formela et al., 2017, 2016; Ghaffar et al., 2014; Hakkarainen, 2010; Kaykhahi and Linford,
61 2017; Liu et al., 2012; Massold et al., 2005). Chemical compounds such as aromatic
62 hydrocarbons, aliphatic hydrocarbons, monoterpenes, alcohols, aldehydes and ketones
63 (excluding formaldehyde) have a significant impact on the final value of the TVOC
64 parameter. Depending on the object investigated, the intensity of the impact of a selected
65 group of organic compounds on the final TVOC value varies. Preliminary determination of
66 TVOC values allows researchers to obtain screening information on the quality of studied
67 materials and make preliminary comparisons. In addition, the TVOC parameter consists of
68 chemical compounds defined by the International Agency for Research on Cancer (IARC) as
69 carcinogenic such as benzene (Group 1A), probably carcinogenic (Group 2A), or possibly
70 carcinogenic like styrene or ethylbenzene (Group 2B) (IARC, 2016). Therefore, assessing
71 TVOC values enables researchers to perform preliminary evaluations of the impact of studied
72 objects on human health or the environment.

73 Without doubt, biological tests are becoming increasingly important in analytical practice.
74 The main advantage of diagnostic tools is their capacity to specify the actual influence of
75 compounds in tested samples on living organisms, taking into account all interactions between
76 them (Wieczerzak et al., 2016). In the literature, there are many examples of studies showing



77 that the coexistence of several pollutants leads to more severe adverse effects than predicted
78 based on the toxicity of individual components (Backhaus, 2012; Pose-Juan et al., 2016;
79 Sexton and Hattis, 2007; Silva et al., 2002). In most cases, such interactions were found in
80 compounds such as bisphenol A and related compounds, phthalates, primary aromatic amines,
81 and heavy metals (Abdul-Ghani et al., 2012; Fic et al., 2014; Michałowicz, 2014; Ramirez et
82 al., 2014; Xu et al., 2014). In a study on the individual and combined effects of bisphenol A,
83 dibutyl phthalate, and cadmium on oxidative stress and genotoxicity in HepG 2 cells,
84 synergistic interactions were noted (Li et al., 2017). In another study, a similar harmful effect
85 was observed between nonylphenol and di-N-butyl phthalate (Hu et al., 2014). These
86 compounds are plasticizers, additives and printing inks also commonly used in the production
87 of toys and other products intended for children (Lv et al., 2015; Szczepanska et al., 2016).
88 Considering these findings, it seems necessary to intensify efforts to accurately estimate the
89 degree of compound mobility and the effects of compound interactions on living organisms,
90 particularly for children who are most vulnerable to the threat. Their tendency to get to know
91 the world with their mouths in the early stages of life combined with weaker detoxicating
92 abilities makes contact with xenobiotics a potential contributing factor to serious health
93 consequences (Damstra, 2002; Mercan et al., 2015).

94 Therefore, the use of bioanalytical methods has become particularly important in research
95 aimed to estimate the degree of xenobiotic migration from object surfaces. It might be
96 observed that there is no information in the literature on the use of such a joint approach in
97 research aimed at estimating the degree of xenobiotic migration from the surface of toys to
98 liquid and gaseous phase. Therefore, the current research will make a significant contribution
99 to knowledge about the degree of compound mobility and the resulting impacts on healthy
100 children.

101 The main aim of the present study was to determine the impact of time on the ecotoxicity of
102 samples extracted from products intended for children and to evaluate the correlation with
103 TVOC emissions using basic mathematical methods performed on appropriate statistical
104 software. The ecotoxicological tests used were Microtox[®] and XenoScreen YES/YAS, while
105 TVOC emission rates were evaluated using a small-scale stationary emission chamber system
106 at the samples seasoning stage. The thermal desorption technique (TD) combined with gas
107 chromatography technique (GC) equipped with flame ionization detection (FID) was
108 employed at the stage of liberation and final determination of VOCs. This mathematical
109 approach is based on best-fit function modeling, with ecotoxicity or TVOC concentration as
110 dependent variable Y and time as independent variable X . As in previous studies on



111 ecotoxicity modeling, the most appropriate model seems to be the polynomial fit of the $Y =$
112 $a_1X^2 + a_2X + b$ type, which accurately describes the experimental data. The weight coefficient
113 a_1 (by value and by sign) can be used to interpret the impact of time. Model validation was
114 performed using R^2 values (multiple correlation coefficients). The research protocol presented
115 in the following paper is an important example of interdisciplinary studies, combining aspects
116 of analytical chemistry, environmental chemistry, ecotoxicology and
117 mathematical/chemometric methods. All of these elements should be considered in seeking
118 novel approaches to evaluating the quality of polymer children's toys and assessing the
119 potential impacts on human health.

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121 **2. Materials and Methods**

122 ***2.1. Objects studied***

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124 The objects studied were all elements of popular commercially available chocolate/toy sets,
125 presented in Figure 1 for easier visualization. All elements (sealing aluminum foil package,
126 chocolate, plastic package for toy, toy itself) of both the original product and its cheaper
127 equivalent were subjected to both toxicological and TVOC studies in duplicate.

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129 ***2.2. Extraction studies***

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131 Extraction conditions were selected to reflect plausible forms of human contact with the
132 objects of interest. Two liquids were used as extraction media: distilled water and artificial
133 saliva ($\text{NaCl } 0.53 \text{ g/dm}^3$, $\text{KCl } 0.33 \text{ g/dm}^3$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O } 0.15 \text{ g/dm}^3$, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O } 0.76$
134 g/dm^3 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O } \text{g/dm}^3$, $\text{K}_2\text{CO}_3 \text{ } 0.53 \text{ g/dm}^3$, 1 % HCl 0.75 g/dm^3). The experiments were
135 run at room temperature while aliquots of extracts were sampled at 0.5, 1.0, 2.0, 6.0, and 12.0
136 hours. The objects were placed in glass beakers and flooded with proper extraction media
137 (100 cm^3), sealed with Parafilm[®] and shaken for proper extraction times. After each period,
138 the aliquots of extracts were sampled and subjected to toxicological studies. Prior to
139 conducting the studies, pH was measured to verify if it was in the range of 6.5 - 7.5. In
140 addition, the chocolate extracts were filtered to increase the clarity of the sample. Detailed
141 information about the chemicals and reagents used in the research were enclosed in the
142 Supplementary Material for Methodology.

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144 ***2.3. Microtox[®] studies***



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The acute toxicity of extracts was measured with Microtox[®]. The toxicity was assessed as the inhibition of bioluminescence of the indicator organism (refer to Supplementary Figure 1 for details). Appearance of factors that negatively influence the enzymatic activity of the indicator organism inhibits the oxidation of luciferin, which is manifested as reduced luminescence (Parvez et al., 2006). Because these bioluminescent organisms are highly sensitive to the presence of toxic ingredients, they are used in environmental research to control the degree of toxicity in water, sewage, soils, sludge, ionic liquids, and nanoparticles, among others (Mortimer et al., 2008; Rossetto et al., 2014). To perform calculations it was necessary to calculate the bioluminescence inhibition (BI) [%]. Q-Dixon tests were performed prior to conducting respective calculation with eqs. 1-3:

$$R = \frac{I_0 - I_t}{I_0} \quad (1)$$

$$G_t = \frac{I_{0k} - I_{tk}}{I_{0k}} \quad (2)$$

$$\%SB = \frac{I_0 - I_t}{I_{0k} - I_{tk}} \cdot G_t \quad (3)$$

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where:

I_0 – initial bioluminescence of bacterial suspension,

I_t – bioluminescence of bacterial suspension after 30 min of exposure,

I_{0k} – initial bioluminescence of bacterial suspension exposed to control solution,

I_{tk} – bioluminescence of bacterial suspension after $t = 30$ min of exposure to control solution,

R_t – correction factor,

G_t – Gamma coefficient,

%SB – bioluminescence inhibition after time t .

166 **2.4. XenoScreen YES/YAS studies**

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The XenoScreen YES/YAS test was used to determine hormonal potential of extracts solutions with respect to oestrogenic, antioestrogenic, androgenic and antiandrogenic activity samples tested as the test uses genetically modified *Saccharomyces cerevisiae* with human oestrogenic and androgenic receptors (hAR) (ref. to Szczepańska et al. 2016 for details)

172 (Johnson et al., 2013; Kudłak et al., 2015; Ramirez et al., 2014). The test was performed on
173 the basis of manufacturer's instructions with certain modifications. The general diagram of
174 the XenoScreen YES/YAS procedure used for endocrine potential determinations was shown
175 on the Supplementary Figure 2.

176 Positive and solvent control were included in which experiment. Final hormone concentration
177 in positive controls ranged from 0.10 to 100 μ M. The determinations were repeated three
178 times. For the oestrogenic activity, the growth factor (G) and induction ratio (IR) according to
179 the equations 4 and 5:

180
$$\text{---} \quad (4)$$

181
$$\text{---} \quad (5)$$

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183 where:

184 - $A_{690,S}$ and $A_{570,S}$ is absorbance of samples, respectively, at 690 nm and 570 nm;

185 - $A_{690,N}$ and $A_{570,N}$ is absorbance of the solvent control at 690 nm and 570 nm, respectively.

186 It is assumed that the tested sample has agonistic YES/YAS properties if the value of the
187 induction coefficient ≥ 1.5 (for control/calibrators solutions) and shows antagonistic
188 YES/YAS properties if the value of the induction factor $\leq 66.7\%$ of the value obtained for the
189 control/calibrator sample

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191 ***2.5. TVOC studies and FT-IR analysis of studied products***

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193 Estimation of the amount of TVOCs released from the surfaces of studied plastic materials
194 was performed using the four small-scale stationary environmental test chambers - μ -CTE™
195 250 system (Markes International, Inc, Gold River, California, USA). Detailed information
196 about the construction, design, operating range, working conditions and fields of application
197 of the μ -CTE™ 250 system is described in previous scientific literature (Marć et al., 2017,
198 2015; Marć and Zabiegała, 2017; Schripp et al., 2007).

199 In brief, prior to placing the samples inside the chamber, the small polymeric toys were
200 weighed. Average sample weight was 4.3 ± 1.9 g. Afterwards, selected samples of small
201 polymeric toys were placed inside each of four chambers. Then, the chamber lids were closed
202 and samples were seasoned/conditioned under static conditions (without inert gas flushing)
203 under defined conditions of temperature and time (see Supplementary Table 1). After the

204 sample seasoning/conditioning stage, organic compounds emitted from the studied plastic
205 materials into the gaseous phase were purged from the chamber by flushing with inert gas
206 (nitrogen) at the previously defined flow rate. The samples of organic compounds were
207 collected using commercially available cylindrical containers filled with Tenax TA. The TD-
208 GC-FID system was applied to perform liberation and final determination of organic and to
209 assess the TVOC parameter values. More comprehensive information about the working
210 parameters of employed TD-GC-FID system (analytical devices working conditions) is listed
211 in Supplementary Table 1.

212 In order to obtain the general analytical information about the main type of polymer material
213 that was applied at the manufacturing process of studied brand and non-brand products (toys
214 and capsules), the Fourier transform infrared spectroscopy (FT-IR) analysis was performed.
215 The Thermo Scientific Nicolet 6700TM (USA) spectrometer equipped with ATR accessory
216 with diamond crystal (smart iTR) was employed, to carried out the FT-IR analysis of studied
217 samples. The measurements were conducted with 2 cm⁻¹ resolution, in the range from 4000 to
218 650 cm⁻¹. The OMIC ver. 8.0 was used as the main software to analyze the obtained FT-IR
219 results. Detailed information about the FT-IR analysis of plastic materials might be found
220 elsewhere (Marć et al., 205, Formela et al., 2016, Formela at al., 2017).

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222 ***2.6. Quality assurance/quality control***

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224 For quality assurance, the following parameters were set according to manufacturer's
225 guidelines: for Microtox[®] I₀ of bacterial suspension >70 U and CaSO₄ was used as positive
226 control of bacterial response (as well as K₂Cr₂O₇ and 3,5-dichlorophenol routinely considered
227 as positive control/reference solutions) while in case of studies with XenoScreen YES/YAS,
228 the OD₆₉₀ of yeast cultures should be >0.3. In all cases, these requirements were fulfilled.

229 The assessed values of TVOC emissions were corrected based on the value of the blank/zero
230 sample, which was evaluated for the complete protocol under the conditions used in the
231 analysis of the selected polymeric samples. The analytes recovery from the applied sorption
232 medium - Tenax TA, was performed taking into account the studies in which a well-defined
233 amount of the internal standard solution – toluene d₈, was injected directly onto the previously
234 conditioned and purified sorbent. Next, the Tenax TA stainless steel tube with a defined mass
235 (deuterated derivative of toluene - 40 ng per tube) of standard solution was analyzed
236 employing the same analytical protocol as for the polymeric samples. Considering the
237 obtained results of analytes recovery studies, recovery values range from 96 to 104%. Making



238 an allowance for the equation of the calibration curve, the numerical values of method
239 quantification limit (MQL) of the employed analytical protocol was estimated at 0.45 ng/g
240 with toluene d₈ as a reference compound. More detailed information about the QA/QC
241 protocol might be found elsewhere (Marć et al., 2017; Marć and Zabiegała, 2017).

242 The mathematical approach is based on best-fit function modeling, with ecotoxicity or TVOC
243 concentration as dependent variable Y and time as independent variable X . As in previous
244 studies on ecotoxicity modeling, the most appropriate model seems to be the polynomial fit of
245 the ' $Y = a_1X^2 + a_2X + b$ ' type, which accurately describes the experimental data. The weight
246 coefficient a_1 (by value and by sign) can be used to interpret the impact of time. Model
247 validation was performed using R^2 values (multiple correlation coefficients).

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249 **3. Results and Discussion**

250 **3.1. Toxicological studies**

251 **3.1.1. Impact of time on toxicity of aqueous extracts**

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253 In Table 1a, the values of a_1 , a_2 , b and R^2 are presented for all 10 models (refer to Figure 1, 10
254 different objects of study – parts of the original brand product, numbers in front of object
255 names denote extraction time). In almost all cases (9 out of 10 objects) a_1 is negative, which is
256 an indication that, in general, toxicity in water decreases with time. The complex nature of the
257 toxicity/time relationship, however, is indicated by the appearance of local maxima and
258 minima in the time dependence model. Strong time dependence is observed for
259 objects/samples 1 Foil B, 2 Foil B, 6 Foil B, 12 Foil B, 1 Foil NB, and 2 Foil NB, with low
260 time dependence for 0.5 Foil B, 0.5 Foil NB, 6 Foil NB and 12 Foil NB (foil and chocolate;
261 sample 0.5 Foil NB showed very low time impact). Model adequacy is acceptable and
262 especially high for samples 1 Foil B, 2 Foil B, 6 Foil NB and 12 Foil NB. In Table 2, the
263 ecotoxic effects in water for all cases and time periods are presented. The highest toxicity is
264 observed among the “chocolate” cases, while the lowest is observed for case 1 Foil NB (the
265 strong time impact found for this case is related to the significant changes of effect from the
266 starting time to the final time).

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268 **3.1.2. Impact of time on toxicity of artificial saliva extracts**

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270 In Table 1b, the values of a_1 , a_2 , b and R^2 are presented for all 10 models (10 different objects
271 of study) for the artificial saliva medium. For this medium, the models are less adequate. As

272 seen in Table 2, the ecotoxicity values are much lower than those in water (with the exception
273 of cases 6 Foil NB and 12 Foil NB – extracts of “chocolate” objects that correspond almost
274 entirely to the models in the water medium). All other cases (0.5 Foil B, 1 Foil B, 2 Foil B, 6
275 Foil B, 12 Foil B, 0.5 Foil NB, 1 Foil NB, 2 Foil NB) differ both in absolute value of ecotoxic
276 effects and time trends. Samples 12 Foil B and 2 Foil NB do not indicate a significant time
277 trend. Models of low adequacy are found for samples 0.5 Foil B, 1 Foil B, and 6 Foil B. For
278 the remaining cases, two (2 Foil B and 0.5 Foil NB) show a local minimum of toxicity at 2
279 hours of treatment while one (1 Foil NB) shows local maximum at 2 hours of treatment. Thus,
280 no reliable interpretations of time impacts are reasonable. However, one interesting finding is
281 the response of the “chocolate” extract samples. The toxicity data for artificial saliva extracts
282 are surprising in some extent when increasing contact time leads to increased
283 bioluminescence inhibition, it suggests that composition of simulation media used
284 enhances extractability of xenobiotics present in chocolate material making the solution more toxic
285 (impact of time is also noticeable). There are studies being performed aimed at identification
286 of substances plausibly responsible for observed elevated toxicity levels.

287

288 3.2. TVOC results

289 For 6 samples (2 Capsule B, 6 Capsule B, 12 Capsule B, 0.5 Capsule NB, 1 Capsule NB, 2
290 Capsule NB), TVOC analyses were performed. The results are interpreted using the
291 method described for Microtox effects. Polynomial models proved to be most
292 satisfactory for the results obtained. In Table 1c, the calculated parameters of the
293 polynomial model are presented. The values of TVOC emissions for all objects studied
294 and all time periods are listed in the Table 3. An attempt was made to compare both the
295 models and the experimental values for Microtox toxic effects (in water and artificial saliva)
296 and TVOC concentrations for the same objects. As presented in Table 3, there is a significant
297 difference between the emitted TVOCs for objects 2 Capsule B and 0.5 Capsule NB when
298 compared to 1 Capsule NB and 2 Capsule NB. The polynomial model satisfactorily describes
299 all samples except for 12 Capsule B, which is characterized by high TVOC concentrations
300 for very short and very long times and minima for intermediate times. Specific to these
301 models is the low value for a_1 and very high values for a_2 . Thus, the a_2 value is
302 considered a better descriptor of the time trend. Therefore, for 2 Capsule B and 6
303 Capsule B samples, TVOC concentration generally decreases with time, while 12
304 Capsule B, 1 Capsule NB and 2 Capsule NB do not show significant time trends since
305 0.5 Capsule NB reveals a concentration increase with time.

306 Taking into account the information listed in the Table 3 it was noticed a clear difference in
307 assessed TVOC parameters between original brand products and non-brand products. It was
308 observed that in both cases (small toys and containers) the original brand products were
309 characterized by much higher TVOCs emission values than studied non-brand products. To
310 explain the occurrence of this type of phenomenon, it was necessary to conduct the FT-IR
311 analysis of the brand and non-brand products. This solution gives a possibility to define the
312 main polymer material from which studied products were made. The results of FT-IT analysis
313 of brand and non-brand products were present on the Supplementary Figures from 3 to 5.
314 Considering the obtained results of FT-IR analysis of small toys and containers, both original
315 brand and non-brand products, and comparing them to the FT-IT software database, it was
316 conclude that the TVOCs emissions might be indicated by two main factors: (i) the pigment
317 application (sample color) and (ii) the type of the main polymer material which was used at
318 the studied product manufacturing process. In a first case, the polymer containers defined as
319 Capsule NB and Capsule B were mainly made of a mixture polypropylene + polyethylene-p
320 (according to performed FT-IR analysis) – in this situation, the main polymer material was the
321 same, but the color of containers was different. Due to this fact it might be pointed out that the
322 application of defined pigment might influence on the TVOCs emission values. As for the
323 second case (small toys), the higher TVOCs emissions observed for original brand products
324 might be caused by the type of polymer that was used. The original brand products (Toy 1B
325 and Toy 2B) were mainly made of acrylonitrile-butadiene-styrene (ABS) copolymer. As for
326 the non-brand products (Toy 1NB and Toy 2NB), the small toys were mainly made of
327 polyethylene (Mn 1800). The ABS copolymer is characterized by much higher TVOCs
328 emissions due to the fact, that during a manufacturing process, tree types of polymers/reagents
329 must be used to obtain the desired copolymer. Taking into consideration this information, the
330 presence of the mentioned phenomena of TVOCs emissions, might be caused by the type of
331 the polymer which was used at the manufacturing process of the brand and non-brand
332 products.

334 **3.3. XenoScreen YES/YAS results**

335 Results of endocrine potential studies are presented in Table 4. In most cases, only androgenic
336 antagonistic behavior of extracts was observed, proving the objects studied to be a
337 major threat in relation to this endpoint. As other endpoints (oestrogenic agonistic,
338 oestrogenic



339 antagonistic, and androgenic agonistic) showed no significant values or variations, no models
340 could be calculated.

341

342 *3.4. Comparison between Microtox[®] ecotoxic effects and TVOC concentrations*

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344 The comparison between Microtox[®] ecotoxic effects and TVOC concentrations was
345 performed by correlation analysis. In the first run, correlation between all water medium
346 effects with all artificial saliva effects resulted in a correlation coefficient of 0.63. This result
347 indicates that both series of effects are relatively well correlated, probably due to the very
348 high correlation between samples 6 Capsule NB and 12 Capsule NB in both series. In Table 5,
349 the correlation coefficients for the series MT water/TVOC and MT saliva/TVOC are
350 presented. Assuming that correlation coefficients (as absolute value) below 0.3 are
351 insignificant and between 0.31 – 0.60 are of relatively low significance, one might conclude
352 that reasonable correlation (negative) is detected for cases 2 Capsule B and 12 Capsule B (MT
353 water/TVOC) and for case 6 Capsule B (positive for MT saliva/TVOC).

354

355 **4. Conclusions**

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357 The results from studies of both toxicological and instrumental analyses of TVOC
358 demonstrate that there is a significant problem with assigning the health impact of products
359 intended for children using purely instrumental methods. Although significant emissions of
360 toxicants from toys were not observed, the toxicity of chocolate extracts was significant,
361 demonstrating either its low quality or high capacity as a sink for volatile organic compounds
362 emitted from materials in contact with it. The obtained results on acute toxicity of polymer
363 material extracts coincide with data presented by other researchers. In studies where *D. magna*
364 was used as biologically active element, no acute toxicity was detected in water extract of PE
365 such as ABS (Lither et al., 2009, 2012). On this basis, it can be assumed that the compounds
366 released from the surface of these materials are so in low concentration that they do not cause
367 visible adverse effects in test organisms, or the exposure time was too short. It therefore
368 seems necessary to check whether this toxicity occurs after prolonged exposure (chronic
369 toxicity). As for the general law regulations about the organic compounds emission from
370 plastic materials directly into the food products, in the EU there is document defined as non-
371 legislative acts - the Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic
372 materials and articles intended to come into contact with food. In the mentioned regulation,



373 the union list of authorised monomers, other starting substances, macromolecules obtained
374 from microbial fermentation, additives and polymer production aids was attached. Moreover,
375 mentioned document defines only a numerical values of SML [mg/kg] factor, which is the
376 specific migration limit applicable for the substance, expressed in mg substance per kg food.
377 There is a lack of general information about the limits of TVOCs emissions form plastic
378 materials which have direct contact with food products.

379 More alarming conclusions can be withdrawn from results on endocrine potential studies, as
380 the androgenic impact of extracts is clearly observable on samples extracted from plastic toys
381 and chocolate. **These results may indicate that compounds such as phthalates, bisphenol A or**
382 **compounds from the group of brominated flame retardants are released from the surface of**
383 **the materials. In *in vitro* conditions, all of these compounds have been found to exhibit anti-**
384 **androgenic activity (Wooten et al., 2013, Kharlyngdoh et al., 2015). Moreover, many studies**
385 **can be found in literature confirming that it is these groups of analytes that are mainly leached**
386 **from the surface of children's toys (Nerev et al., 2018, Ionas et al., 2014). The presence of**
387 **weak anti-estrogenic responses in some samples may indicate that other xenobiotics are also**
388 **released from the surface of the materials. Lack of significant correlations between**
389 **instrumental and biological assay data may also be associated with the occurrence of synergy**
390 **or antagonism effect. To date, few studies have been carried out to assess the toxicity and**
391 **endocrine nature of compounds migrating from children's toys. Further research is needed to**
392 **prove the applicability of bioassay studies in assessing the impact of xenobiotics present in**
393 **materials intended for kids or as a validated tool in product quality assessment. Clear**
394 **discrepancies between different commercially available products show that such an**
395 **assessment is plausible, while more environmentally benign than many instrumental methods.**
396 In TVOC emission profile studies, a similar characteristic of the emission profile typical of
397 the new type of plastic indoor materials was observed - a decrease in the TVOC emission rate
398 along with an increase in the storage/seasoning time inside the environmental chamber. This
399 type of relationship was observed for all studied samples of children's toys and plastic
400 containers **(Marć et. al., 2015, 2017, Tirendi et. al., 2009).** This may indicate that the
401 chemicals released from polymeric materials can be present in the gaseous phase between the
402 small polymeric material and the chocolate food product and might be adsorbed or dissolved
403 in the chocolate, directly affecting the human body. Additionally, significant amounts of
404 harmful organic compounds might be released directly to the gaseous phase from the surface
405 of plastic packaging, which has direct contact with the chocolate food product. However, it
406 should be clarified that further, more detailed studies are needed to define precisely the

407 individual chemicals that are emitted from the surface of plastic samples directly into the
408 gaseous phase – indoor environment. Defining the TVOC parameter or TVOC emissions
409 profile allows researchers to obtain screening information that provides the opportunity to
410 highlight potential problems. The future research, associated with the described
411 interdisciplinary studies, ought to be focused on the assessing the emission rates of selected
412 carcinogenic compounds or endocrine-disrupting chemicals that might be released from
413 plastic materials and have direct contact and interaction with chocolate food products.

414

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416

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422

423 **6. Conflict of interest**

424

425 The authors declare that they have no conflict of interest.

426

427 **7. References**

428

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542



543 **Figure Captions**

544

545 **Figure 1.** Figure 1. Schematic representation of samples studied (legend for results
546 description: foil B, foil NB – aluminum foil package for brand and non-brand product,
547 respectively; capsule B, capsule NB – plastic capsule/package inside chocolate to cover toy,
548 for brand and non-brand product, respectively; toy 1B, toy 2B, – toys inside plastic package of
549 brand original product; toy 1NB, toy 2NB – toys inside plastic package of non-brand (fake)
550 product; chocolate B, chocolate NB – brand (original) and non-brand (fake), respectively,
551 chocolate sample being in contact with aluminum foil)

552

553 **Supplementary Figure and Tables:**

554

555 **Supplementary Figure 1.** Analytical procedure of Microtox® acute toxicity determination.

556 **Supplementary Figure 2.** Diagram of the procedure used in order to assess the oestrogenic
557 and androgenic activity.

558 **Supplementary Figure 3.** The general view of FT-IR spectra of plastic container/capsules.

559 **Supplementary Figure 4.** The general view of FT-IR spectra of small polymeric toys -
560 original brand products.

561 **Supplementary Figure 5.** The general view of FT-IR spectra of small polymeric toys – non-
562 brand products.

563 **Supplementary Table 1.** General information about the working conditions of applied
564 analytical equipment and techniques during the conducted TVOCs research

Table 1. Values of the regression coefficients and correlations.

Extraction time [h] / object name	a_1	a_2	R^2
a)			
0.5/ Foil B	-0.14	1.55	0.1
1/ Foil B	-4.71	31.38	0.91
2/ Foil B	-4.86	33.84	0.78
6/ Foil B	-3.11	27.21	0.44
12/ Foil B	-4.72	30.18	0.52
0.5/ Foil NB	0.58	6.17	0.57
1/ Foil NB	-3.79	25.41	0.40
2/ Foil NB	-3.30	20.30	0.29
6/ Foil NB	-1.43	23.27	0.91
12/ Foil NB	-0.20	15.49	0.85
b)			
0.5/ Foil B	-4.33	29.16	0.28
1/ Foil B	1.65	-6.19	0.17
2/ Foil B	4.21	-24.63	0.48
6/ Foil B	-1.53	5.02	0.21
12/ Foil B	-0.07	-1.89	0.1 <i>ns</i>
0.5/ Foil NB	3.27	-22.34	0.68
1/ Foil NB	-0.87	1.27	0.61
2/ Foil NB	0.63	-4.49	0.1 <i>ns</i>
6/ Foil NB	-1.94	21.07	1.00
12/ Foil NB	-1.63	19.32	0.83
c)			
2/ Capsule B	499.9	-3400	0.58
6/ Capsule B	103.3	-1373	0.84
12/ Capsule B	-463.8	8415	0.1 <i>ns</i>
0.5/ Capsule NB	-679.1	5700	0.66
1/ Capsule NB	44.9	-214	0.95
2/ Capsule NB	63.9	291	0.65

Table 2. Results of Microtox® toxicity effect in water and artificial saliva extracts

Extraction time [h] / object name	Water extraction		Artificial saliva extraction	
	Bioluminescence inhibition [%]	SD	Bioluminescence inhibition [%]	SD
0.5/ Foil B	5.862	0.023	-25.427	0.035
1/ Foil B	23.620	0.043	7.881	0.039
2/ Foil B	23.596	0.025	-20.376	0.039
6/ Foil B	15.508	0.039	15.669	0.015
12/ Foil B	14.445	0.019	-13.512	0.030
0.5/ Foil NB	13.409	0.001	-13.219	0.033
1/ Foil NB	37.354	0.010	17.876	0.018
2/ Foil NB	40.539	0.015	-11.181	0.040
6/ Foil NB	35.199	0.016	-8.220	0.013
12/ Foil NB	30.547	0.003	18.427	0.039
0.5/ Capsule B	-0.531	0.036	-22.855	0.027
1/ Capsule B	13.689	0.004	-28.576	0.014
2/ Capsule B	20.499	0.019	-49.231	0.085
6/ Capsule B	35.744	0.006	-22.879	0.112
12/ Capsule B	11.693	0.022	-22.661	0.028
0.5/ Capsule NB	2.412	0.019	-20.924	0.015
1/ Capsule NB	36.889	0.036	9.142	0.029
2/ Capsule NB	13.770	0.025	-19.689	0.029
6/ Capsule NB	40.105	0.018	-32.164	0.042
12/ Capsule NB	22.618	0.028	-20.954	0.047
0.5/ Toy 1B	-0.531	0.036	-22.855	0.027
1/ Toy 1B	2.932	0.006	-22.932	0.050
2/ Toy 1B	32.879	0.007	3.896	0.020
6/ Toy 1B	15.819	0.030	-24.171	0.042
12/ Toy 1B	32.296	0.003	-32.720	0.038
0.5/ Toy 2B	12.407	0.001	-16.125	0.136
1/ Toy 2B	7.419	0.002	-7.737	0.032
2/ Toy 2B	23.828	0.034	-14.305	0.032
6/ Toy 2B	6.921	0.021	-20.661	0.065
12/ Toy 2B	52.023	0.033	-31.369	0.041
0.5/ Toy 1NB	6.298	0.031	-18.750	0.008
1/ Toy 1NB	29.836	0.048	-15.454	0.034
2/ Toy 1NB	13.347	0.026	-16.947	0.026
6/ Toy 1NB	39.166	0.016	-34.117	0.006
12/ Toy 1NB	15.036	0.018	-29.046	0.029
0.5/ Toy 2NB	-9.460	0.026	-21.009	0.024
1/ Toy 2NB	24.147	0.013	-12.907	0.028
2/ Toy 2NB	3.728	0.031	-22.862	0.018
6/ Toy 2NB	- ¹	-	-26.985	0.100
12/ Toy 2NB	3.669	0.058	-17.358	0.036
0.5/ Chocolate B	15.912	0.056	26.540	0.056
1/ Chocolate B	19.891	0.157	42.277	0.054
2/ Chocolate B	47.140	0.012	52.922	0.011
6/ Chocolate B	68.144	0.010	61.003	0.019
12/ Chocolate B	65.233	0.022	64.473	0.019
0.5/ Chocolate NB	25.556	0.031	41.463	0.019
1/ Chocolate NB	20.311	0.096	51.217	0.011
2/ Chocolate NB	51.280	0.017	58.282	0.009
6/ Chocolate NB	72.615	0.005	83.284	0.038
12/ Chocolate NB	70.762	0.008	72.516	0.006

¹ Unrepeatable results, omitted in data treatment

Table 3. TVOCs emission values for all cases and times studied.

object	Sample mass [g]	Sample seasoning time inside a chamber [h]	TVOC emission [$\text{ng}\cdot\text{g}^{-1}$]
Capsule B	3.9902	0.5	3273.24
		1	2610.25
		2	2886.47
		6	2280.37
		10	2935.57
Capsule NB	3.8232	0.5	1114.40
		1	1123.72
		2	620.06
		6	564.16
Toy 1B	6.8347	10	538.73
		0.5	1420.44
		1	384.33
		2	440.33
Toy 2B	7.3696	6	343.14
		10	1109.06
		0.5	210.72
		1	1252.19
Toy 1NB	1.8211	2	1211.94
		6	986.86
		10	1484.63
		0.5	96.91
Toy 2NB	2.9535	1	88.05
		2	79.62
		6	100.55
		10	183.46
Toy 2NB	2.9535	0.5	415.57
		1	310.08
		2	280.00
		6	560.19
		10	545.37

Table 4. Results of endocrine potential determinations for extracts.

Extraction time [h] / object name	YES +		YES -		YAS +		YAS -	
	water	artificial saliva	water	artificial saliva	water	artificial saliva	water	artificial saliva
0.5/ Foil B	0	0	0	1	0	0	2	2
1/ Foil B	0	0	0	0	0	0	3	0
2/ Foil B	0	0	0	0	0	0	2	5
6/ Foil B	0	0	0	2	0	0	5	4
12/ Foil B	0	0	0	0	0	0	6	3
0.5/ Foil NB	0	0	0	1	0	0	2	2
1/ Foil NB	0	0	0	0	0	0	3	1
2/ Foil NB	0	0	0	2	0	0	2	5
6/ Foil NB	0	0	0	2	0	0	5	5
12/ Foil NB	0	0	0	1	0	0	3	3
0.5/ Capsule B	0	0	1	0	0	0	2	1
1/ Capsule B	0	0	0	0	0	0	0	1
2/ Capsule B	0	0	0	0	0	0	2	3
6/ Capsule B	0	0	0	2	0	0	4	5
12/ Capsule B	0	0	1	0	2	0	6	3
0.5/ Capsule NB	0	0	0	1	2	0	1	2
1/ Capsule NB	0	0	0	0	0	0	0	1
2/ Capsule NB	0	0	0	0	0	0	2	0
6/ Capsule NB	0	0	0	0	0	0	4	3
12/ Capsule NB	0	0	0	0	2	0	6	2
0.5/ Toy 1B	0	0	1	1	0	0	2	0
1/ Toy 1B	0	0	0	0	0	0	3	2
2/ Toy 1B	0	0	0	0	0	0	2	2
6/ Toy 1B	0	0	0	0	0	0	4	5
12/ Toy 1B	0	0	0	0	0	0	3	0
0.5/ Toy 2B	0	0	1	1	0	0	2	1
1/ Toy 2B	0	0	0	0	0	0	3	2
2/ Toy 2B	0	0	0	0	0	0	2	4
6/ Toy 2B	0	0	0	0	0	0	4	5
12/ Toy 2B	0	0	0	0	0	0	2	3
0.5/ Toy 1NB	0	0	2	0	0	0	2	3
1/ Toy 1NB	0	0	0	0	0	0	3	1
2/ Toy 1NB	0	0	0	0	0	0	2	5
6/ Toy 1NB	0	0	0	0	0	0	4	0
12/ Toy 1NB	0	0	1	0	2	0	6	3
0.5/ Toy 2NB	0	0	1	0	0	0	2	2
1/ Toy 2NB	0	0	0	0	0	0	2	2
2/ Toy 2NB	0	0	0	0	0	0	2	5
6/ Toy 2NB	0	0	0	0	0	0	2	2
12/ Toy 2NB	0	0	0	0	0	0	2	2
0.5/ Chocolate B	0	0	0	0	0	0	2	3
1/ Chocolate B	0	0	0	0	0	0	2	2
2/ Chocolate B	0	0	0	0	0	0	4	5
6/ Chocolate B	0	0	0	0	0	0	5	3
12/ Chocolate B	0	0	0	0	0	0	4	3
0.5/ Chocolate NB	0	0	0	0	0	0	2	3
1/ Chocolate NB	0	0	0	0	0	0	2	2
2/ Chocolate NB	0	0	0	2	0	0	3	5
6/ Chocolate NB	0	0	0	0	0	0	5	4
12/ Chocolate NB	0	0	0	0	0	0	4	3

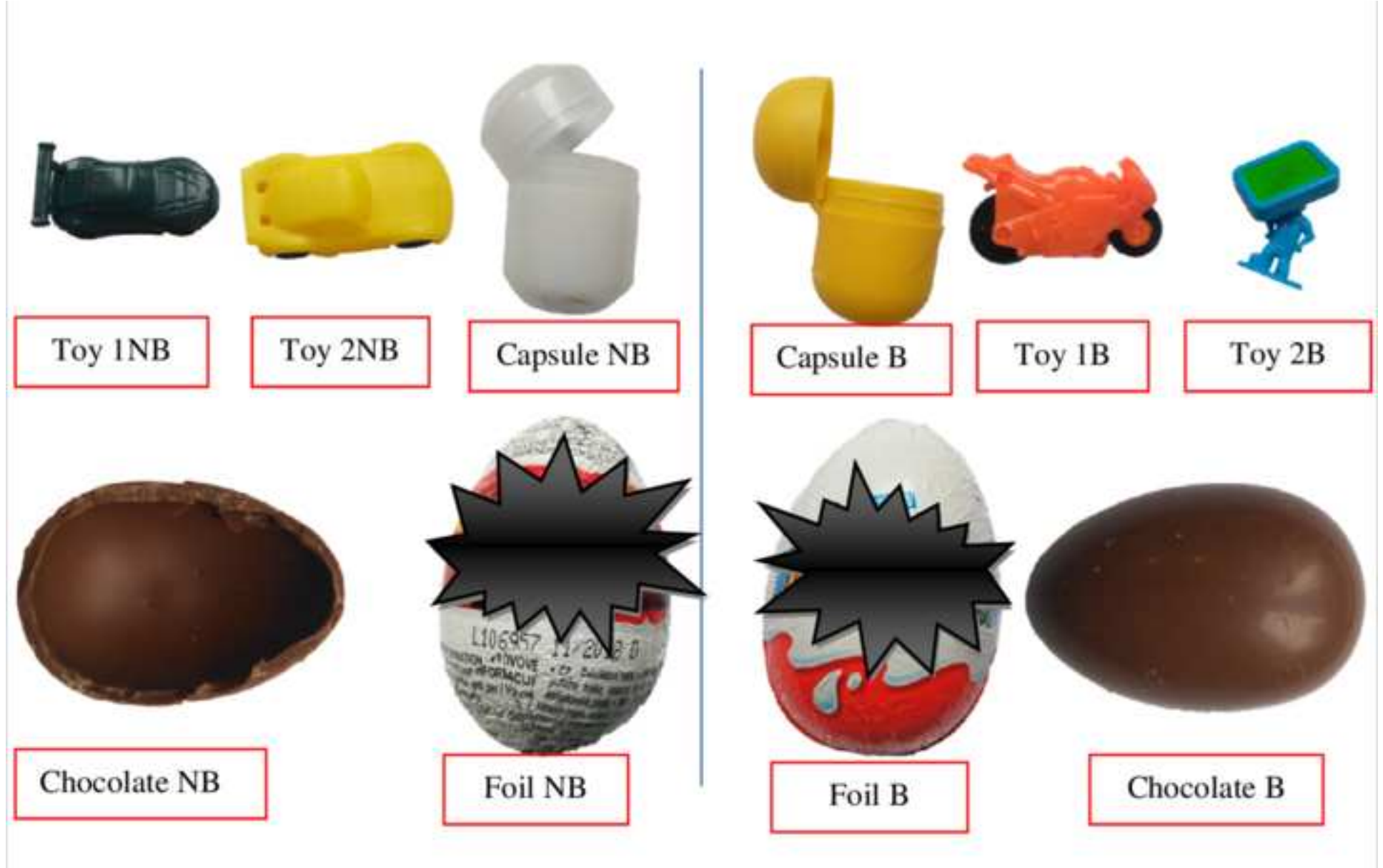
Explanation of numbers

0	1	2	3	4	5	6
Weak effect			→	Strong effect		

Table 5. Correlation coefficients between bioluminescence effects for different times in relation to TVOC emissions for the same times

Objects	Water media	Artificial saliva
Capsule B	-0.90	-0.07
Capsule NB	-0.22	0.69
Toy 1B	-0.88	-0.10
Toy 2B	0.43	-0.29
Toy 1NB	-0.17	-0.54
Toy 2 2NB	-0.37	-0.37

Figure 1
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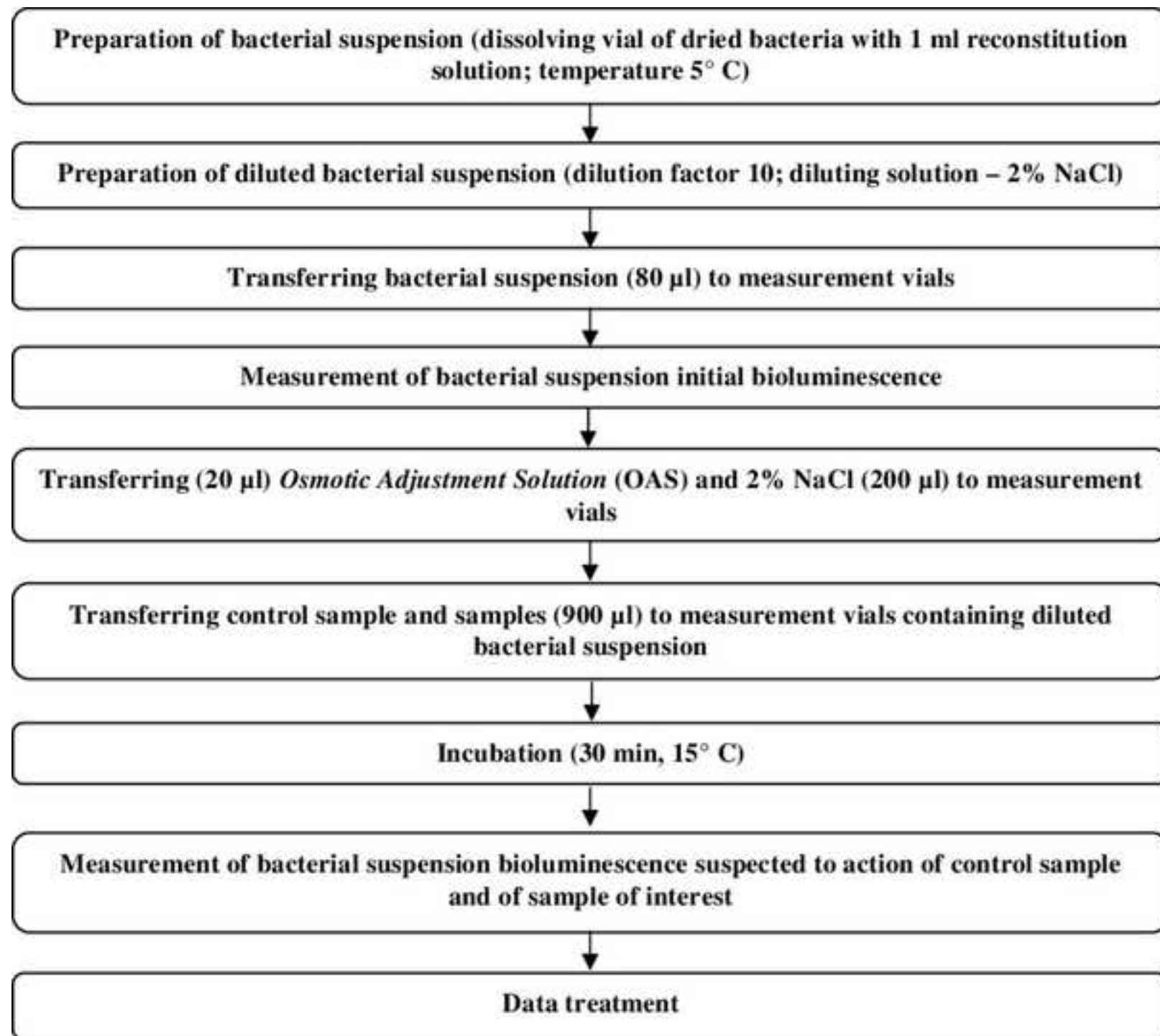
S.1. Materials and methods

S.1.1. Chemicals and reagents

Chemicals used in the research were: sodium chloride (Sigma Aldrich, Germany), dipotassium phosphate (Ciech S.A., Poland), calcium chloride (Eurochem BGD, Poland), magnesium chloride, potassium chloride, potassium carbonate, lactic acid, urea (POCH S.A., Poland), acetic acid (35-38% w/w), ammonium hydroxide (25 % w/w), (Chempur, Poland), distilled water. Microtox[®] 500 kit (Microtox Diluent, 2 % NaCl, lyophilized *Vibrio fischeri*, Osmotic Adjusting Solution (OAS), Microtox Acute Reagent, Reconstitution Solution (RS) was purchased from ModernWater Ltd. (GB). Ethanol (EtOH, CAS no. 64-17-5), acetic acid (CAS no. 64-19-7), dimethyl sulfoxide (DMSO, CAS no. 67-68-5), and Parafilm[®] were purchased from Sigma-Aldrich (Germany). All reagents were of analytical grade or higher. XenoScreen YES/YAS reagents (CPRG (chlorophenol red- β -D-galactopyranoside), basal medium, copper sulfate, L-aspartic acid, L-threonine, hAR and hER yeast cells stabilized on the filtration paper, vitamin, vials with calibrators: 5 α -dihydrotestosterone, 17 β -estradiol, flutamide, 4-hydroxytamoxifen) were purchased from Xenometrics G. A. (Switzerland). culture flasks with gas-permeable filter caps, gas-permeable plate sealers, 96-well plates, were purchased from GenoPlast Biochemicals (Poland). The equipment and instruments used were: Microtox[®] 500 (Modern Water Ltd. (GB)), electronic multi- and single-channel pipettes (Eppendorf (Germany)), CP411 pH-meter (Metron (Poland)), shaker type water bath 357 (Elpan Laboratory Instruments (Poland)).

Supplementary Table 1. General information about the working conditions of applied analytical equipment and techniques during the conducted TVOCs research

Sampling stage of analytes emitted from the surface of studied polymer products to the gaseous phase.	
Device applied to seasoning/conditioning samples of polymer products	Markes' Micro-Chamber/Thermal Extractor™ – μ-CTE™ 250 (<i>Markes International Inc., Gold River, California, USA</i>) containing four microscale chambers
Internal volume of a single chamber	114 cm ³
Seasoning/conditioning temperature	40° C
Conditions for conditioning/seasoning samples inside the chambers - procedure applied for collecting the organic compounds samples from gaseous phase	Seasoning/conditioning of polymer products (children's toys samples and plastic containers) under static conditions, without forced flow rate of inert gas through the chambers; Seasoning/conditioning at predefined time intervals: 0.5 h, 1.0 h, 2 h, 6 h, 10 h – starting from the moment when the studied samples were placed inside the chambers.
Nitrogen flow rate during the stage of collecting the organic compounds samples	50 ml/min
The elution time of the gaseous phase from the chamber to the sorption medium after a defined conditioning/seasoning time	25 min
Tool used to collect the organic compounds samples from gaseous phase	Cylindrical container filled with defined amount of sorption medium Tenax TA, 35/60 mesh (<i>Markes International Ltd.</i>) containing specify amount of internal standard solution (deuterated derivative of toluene - 40 ng per tube)
The liberation stage of analytes retained on a sorption medium (Tenax TA)	
Device employed to liberate the organic compounds from the sorption medium	Two-stage thermal desorber (<i>Omnisfera S.C., Gdansk, Poland</i>)
The 1 st stage of thermal desorption heating temperature	280° C Microtrap* temperature – 0° C
The heating period of a Tenax TA tube	15 min
Helium flow rate passing through the Tenax TA tube	40 ml/min
The 2 nd stage of thermal desorption heating temperature	Microtrap temperature - 300° C
Microtrap ballistic heating period	5 min
Helium flow rate passing through the microtrap in a straight line to the GC column.	2.2 mL/min
The conditions of final determination stage of analytes thermally extracted from the sorption medium	
Gas chromatography system	Hewlett-Packard 5890 GC Series II
Detector	Flame Ionisation Detector
Detector temperature	250° C
TD-GC transfer line temperature	150° C
Capillary column type	DB-1 (<i>J&W</i>) 30 m x 0.32 mm x 5 μm
Carrier gas type (flow rate)	Helium (2.2 mL/min)
Oven temperature program	40° C by 1 min; 5° C/min up to 125° C; 10° C/min up to 220° C hold 220° C by 5 min
* the microtrap tube is filled with two types of sorption medium - Carbotrap (approx. 27 mg) and Tenax TA (approx. 37 mg).	



PREPARATION OF YEAST CULTURE

- Transfer of specially prepared filtration discs with hER α and hAR yeast cells applied into the growth medium
- Incubation (2-3 days, temp 32°C, shaking)

PREPARATION OF PLATES FOR DILUTIONS

ACTUAL SAMPLES

Transfer of 73 μ l of the sample into a multi-well plate

CONTROL SAMPLES

Adding 100 μ l of the DMSO solution into vials containing control solutions to determine oestrogenic properties (17 β -estradiol, 4-hydroxyamoxyphe) and androgenic properties (flutamide, 5 α -dihydroxytestosterone)

Transfer of 73 μ l of the sample onto a multi-well plate

- Preparation of a series of 8 dilutions (DMSO diluting agent, dilution quotient of 3)
- Addition of 180 μ l of the growth medium solution and transfer of 20 μ l of prepared dilutions using a multi-channel pipette

PREPARATION OF PLATES FOR DETERMINATIONS

- Application of 20 μ L of growth medium onto plates
- Application of 20 μ L of selected dilutions of samples and control solutions onto plates
- Transferring 60 μ L of the CPRG solution onto samples intended for determination of agonistic YES/YAS properties and 60 μ L of the CPRG solution containing an appropriate amount of 17 β -estradiol in the case of determination of antagonistic YES properties and 5 α -dihydroxytestosterone for determination of antagonistic YAS properties.
- The addition of 100 μ L of YES/YAS fungi solution to appropriate plates.

INCUBATION OF PLATES FOR DETERMINATIONS

- Protection of samples using a sealing membrane.
- Transfer of plates into a plastic container with wet tissue paper.
- Incubation (48 hours, temp 32°C, shaking)

READING PLATES FOR DETERMINATIONS

- Thorough mixing of the content of the wells using a multi-channel pipette
- Reading plates using a spectrophotometer at wavelength of 690 nm in terms of the growth of cells and wavelength of 570 nm to determine the expression of β -galactosidase

