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Review

Daphnia swimming behaviour as a biomarker in toxicity assessment: A review

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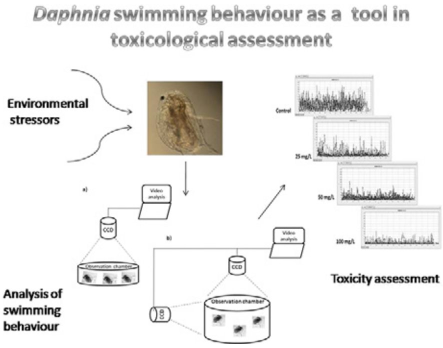
HIGHLIGHTS

- Swimming behaviour of *Daphnia* is affected by various toxicants.
- Parameters of daphnid swimming behavior may useful tool for toxicity assessment.
- Automated systems based on *Daphnia* swimming behavior allow quick evaluation of toxicity in water samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Daphnia is a motile common model organism widely used in ecotoxicological testing. Although mortality and immobilisation are the main endpoints used for determination of toxicity, detection of subtle alterations induced by some chemicals particularly at lower levels may require more sensitive biomarkers. As a number of studies indicated that swimming behaviour may be altered by pesticides, nanoparticles, bacterial products or other chemicals, analysis of its various parameters is considered as a novel methodological approach for toxicity assessment and monitoring of water quality. This paper presents the current state of knowledge on the effects induced by various chemical compounds on the parameters of swimming behaviour of *Daphnia* and systems developed for its analysis. Advantages and limitations of swimming behaviour as a tool in toxicological studies are also discussed.

Contents

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1. Introduction

Daphnia also called "water flea" is a very common planktonic invertebrate organism inhabiting freshwater ecosystems such as lakes and ponds (Mergeay et al., 2006). These microcrustaceans have developed specific organs that facilitate them to move in water. Regular beating with the second set of antennae enables these crustaceans to swim with characteristic hops (Dees et al., 2008). *Daphnia* swimming is dependent on body size (Dodson and Ramcharan, 1991) and may also be affected by various factors such as light, water temperature, presence of food and predators (Baylor and Smith, 1953; O'Keefe et al., 1998; Hamza and Ruggiu, 2000; Ziarek et al., 2011).

Daphnia are sensitive to various substances and can be easily cultured in laboratory conditions, therefore they are found to be very useful model organisms in toxicology. Most of toxicological research with these crustaceans are based only on acute toxicity data for evaluation of lethal concentration (LC50) for mortality or effective concentration (EC50) for immobilisation. However, in order to provide more detailed information on toxicity, particularly sublethal effects induced by lower concentrations of toxicants, more sensitive biomarkers are required. Although life history traits, grazing rate, reproductive effects and a number of physiological and biochemical parameters are reliable endpoints (Dodson et al., 1995), more sensitive biomarkers such as swimming activity has recently drawn more attention of scientists. Mobility of daphnids may be affected by various substances, therefore swimming endpoints have been widely used in toxicology. For example, Restani and Fonseca (2014) tested the acute effect on *Daphnia laevis* mobility fed with a saxitoxin- and neosaxitoxin-producing cyanobacteria *Cylindrospermopsis raciborskii* strain, CYRF-01, and compared the effects with those induced by the non-toxic strain. It was found that the animals exposed to the STX-positive strain were immobilized. A similar approach was made in a study by Ferrão-Filho et al. (2008) in which mobility of daphnids exposed to *Cylindrospermopsis raciborskii* was determined. Although these two experiments showed the influence of the toxic factors on mobility, no movement parameters were determined. Another study showed abnormal swimming of *Daphnia magna* induced by silver nanoparticles (Asghari et al., 2012). The animals treated with the nanoparticles were categorized to the following groups according to their swimming type: normal swimming, erratic swimming, *Daphnia* mainly at the bottom, and *Daphnia* mainly at the surface. The method used in this study enabled to determine the influence of toxicants on daphnid swimming however, was ambiguous and not quantitative. Since changes of swimming behaviour induced by some toxicants may be very subtle and differences of animal reactivity between experimental groups may not be noticed by ambiguous observations with a naked eye, an approach providing more detailed toxicological information with a wide range of quantitative and very sensitive parameters has been developed. This paper is a review of the current knowledge on the use of *Daphnia* swimming behaviour as a biomarker in ecotoxicological studies. In the first section of this work various swimming parameters and their alterations induced by different agents will be presented. Further, available systems for motion analysis will be discussed in terms of their specificity, limitations and applicability for the analysis of *Daphnia* swimming endpoints.

2. Parameters of *Daphnia* swimming behaviour

Daphnia swimming behaviour is complex, multiparametric and considered as one of the most sensitive biomarkers of toxicity (Duquesne and Küster, 2010). It may be characterized by several parameters reflecting changes induced by various compounds on sensitive (i.e. nervous and endocrine) systems. Most of behavioural parameters are determined with the use of digital analysis of video recording (Dodson et al., 1995). This section presents major *Daphnia* swimming parameters that may potentially be used in ecotoxicological testing and reviews the literature on the effects of various compounds on swimming behaviour of these microcrustaceans.

2.1. Swimming time

Swimming time is a parameter indicating the period of time (seconds, hours or days depending on a type of testing and toxicity of a tested agent) in which *Daphnia* exhibit the ability to move. Observation of mobility is easy even without video recording, however noticing the moment of immobilisation requires permanent observation which may be rather difficult and time-consuming. A few reports indicated that some chemical compounds may alter this parameter. For example, daphnids subjected to saxitoxin-producing *Cylindrospermopsis raciborskii* showed a decreased swimming time (Ferrão-Filho et al., 2014). Another study indicated that copper shortened average duration of swimming in *Daphnia magna* in a concentration-dependent manner (Untersteiner et al., 2003).

2.2. Swimming speed

Swimming mobility expressed by scalar quantity- speed and its vector quantity-velocity (usually expressed in millimetres per second or s^{-1}) is one of the most reliable and widely used parameter of *Daphnia* behavioural activity. As these two quantities seem to be used by authors interchangeably, standardization is required. It is to note that cladoceran movement is not constant since acceleration occurs after each hop generated by a single beat with the second antennae and subsequently the animal slows down when the second antennae return to the position to begin the next beating cycle. Therefore, average swimming speed refers to movement characterized by accelerations followed by slowdowns. This parameter depends on daphnid size. It was demonstrated that larger individuals tend to swim faster than the small-sized ones (Hylander et al., 2014). A number of results indicate that various substances may alter swimming speed. *Daphnia magna* exposed to titanium dioxide, carbon-based nanomaterials such as multi-wall carbon nanotubes, graphene and graphene oxide, carbon fullerene C(60) nanoparticles show the reduced swimming speed in a concentration-dependent manner (Lovern et al., 2007; Brausch et al., 2011; Noss et al., 2013; Cano et al., 2017). Although higher concentrations of multiwalled carbon nanotubes were found to decrease animal mobility, lower levels did not affect it (Stanley et al., 2016). Another nanoparticle, cerium dioxide which is used as an oxidation catalyst, gas sensor, or UV absorber and oxidative stress inducer was noted to reduce swimming speed of *Daphnia pulex* and *Daphnia similis* (Artells et al., 2013). A few authors suggested that the parameter may be also altered by some metals (Noss et al., 2013). Daphnids exposed to cadmium or copper tend to manifest inhibited speed when compared to the unexposed, similarly-sized control organisms (Baillieul and Blust, 1999; Untersteiner et al., 2003). The mechanisms by which heavy metals or metal nanoparticles decrease *Daphnia* swimming speed have not been elucidated, however it could be hypothesized that oxidative stress known to be induced by these substances in other animal species may also be responsible for the toxic effects on the behaviour of crustaceans (Fan et al., 2012; Manke et al., 2013; Cano et al., 2017). Another potential mechanism underlying the swimming speed decrease may be physical interferences of the nanoparticles with the daphnids' carapace (Gaiser et al., 2011).

Cyanobacterial and bacterial products were reported to induce alteration of swimming mobility of *Daphnia*. Inhibition of the mean velocity was noted in daphnids exposed to *Cylindrospermopsis raciborskii* producing neurotoxic saxitoxin (Ferrão-Filho et al., 2014). The inhibition was an effect of the toxin-induced blockade of sodium channels in neurons. Interestingly, swimming speed of *Daphnia* exposed to some bacterial products may be initially altered with subsequent return to normal values suggesting that some mechanisms of adaptation exist. Similar adaptation was observed in daphnids exposed to β-cyclocitral, a molecule produced by freshwater cyanobacteria, *Microcystis aeruginosa* NRC-1. The molecule increased the swimming speed of *Daphnia magna,* however after a few minutes of the exposure the parameter returned to normal values (Jüttner et al., 2010). Acclimation to normal swimming values was also observed in daphnids exposed to Volatile Organic Compounds (VOC). The animals manifested a temporal increase of the swimming speed, however subsequent acclimation to normal swimming was observed (Watson et al., 2007). Alteration of swimming speed was noted in crustaceans exposed to pesticides. An organophosphate, praoxym-methyl and a carbamate, carbaryl were reported to increase the parameter (Duquesne and Küster, 2010). On the other hand, daphnids exposed to dichlorophenol and cypermethrin showed the decreased mobility (Christensen et al., 2005; Bahrndorff et al., 2016). Binary mixtures of glyphosate and copper or both chemicals alone induced reduction of this parameter in juvenile *Daphnia magna* (Hansen and Roslev, 2016). It is noteworthy that effects induced by a single compound on swimming speed be opposite to those when the compound is mixed with other molecules. For example, a study by Bownik et al. (2015) showed that a bacterial osmoprotectant, ectoine alone induced reduction of swimming speed of *Daphnia magna*, however when daphnids were treated with combinations of ectoine and hydrogen peroxide they showed less reduced speed than the animals treated with the oxidant alone (Bownik and Stępniewska, 2015a). Ectoine turned out to be a potent protective agent alleviating the formaldehyde-induced decrease of swimming speed (Bownik and Stępniewska, 2015b). The results showed less reduced mobility of daphnids exposed to the combination of ectoine and formaldehyde in comparison to the animals treated with formaldehyde alone.

Effects of chemicals used in human medicine, 17alpha-ethynylestradiol (EE2) and norethindrone (NOR) a constituent of oral contraceptive showing high stability in water environment were assessed with the use of daphnid swimming speed. It was noted that although NOR found to decrease this parameter, EE2 had no modulatory effect (Goto and Hiromi, 2003). Swimming speed was also found to be a reliable endpoint of toxicity induced by food additives. It was found that sucralose, a non-calorie sweetener may increase mobility of *Daphnia magna* (Wiklund et al., 2012) suggesting excitatory effects on the nervous system.

Results obtained by Barrozo et al. (2015) who found reduction of *Daphnia magna* swimming speed by a dopamine agonist bromocriptine, provided some insight into the role of dopamine in swimming behaviour of *Daphnia*. The results indicate that a dopamine receptor signalling pathway, mediated by putative D2-like receptors, is likely to be involved in the control of *Daphnia* swimming behaviour.

Swimming speed of *Daphnia magna* is affected by physical stressors. High salinity water was found to induce initial size-dependent reduction of this parameter in *Daphnia* (Baillieul et al., 1998). However, subsequent acclimation of the animals that survived the exposure was noted. Heat stress may also alter swimming speed of these crustaceans. It was reported that a gradual increase of temperature in 0.1 °C showed increase of mobility until immobilisation at a critical value of 42 °C (Bownik et al., 2014). (See Table 1.)

Analysis of swimming speed may also be performed with the use of some biomarkers related to this parameter. Horizontal swimming velocity is a parameter indicating the velocity of daphnids swimming at a given height in the water column (Pan et al., 2015). However no application of this parameter in toxicological studies was found. Acceleration is another parameter related to swimming speed expressed as mm/s^2 determined by Hansen and Roslev, 2016. The authors found that *Daphnia magna* exposed to the mixture of glyphosate and copper showed a concentration-dependent inhibition of the parameter.

2.3. Behavioural strength

Behavioural strength (Ren et al., 2009a, 2009b; Zhang et al., 2012) or mobility strength, (Chen et al., 2012) have been used for determination of environmental factors on the behaviour of some organisms. However it is not a precise approach and it represents intensity of several behaviour parameters referring to instantaneous motion. Scaling factor that changes from 0 (Lose the ability of movement) to 1 (Full behaviour) illustrates the behavioural responses of *Daphnia* (Ren et al., 2009a, 2009b; Chen et al., 2012). The parameter can be measured in optical systems and those based on changes of alternating current (described below) (Ren et al., 2015). Behavioural strength was determined in *Daphnia magna* exposed to pesticides: an insecticide deltamethin, a fungicide chlorothalonil and nitrofen (Ren et al., 2009a, 2009b). Higher concentrations of these compounds induced more significant behavioural responses, as well as a shorter response time for the first phase, which was regarded as avoidance behaviour. Organophosphorus pesticides such as parathion, malathion and dichlorvos were also found to alter behavioural strength (Ren et al., 2009a, 2009b). The reactions of the exposed animals were classified into the categories of no effect, stimulation, acclimation, adjustment, toxic effect and readjustment. The results indicated that animals exposed to the pesticides showed avoidance response.

2.4. Hopping frequency

Daphnia move with a characteristic hops generated by rhythmic beating of the second antennae (Dodson and Ramcharan, 1991). The number of hops (per minute) may serve as a reliable behavioural

Table 1
Effects of various stressors on various parameters of *Daphnia* swimming behaviour.

Daphnia species	Stressful agent	Parameter of swimming behaviour	Effect	References
Nanoparticles and heavy metals				
Daphnia magna	Multi-wall carbon nanotubes, graphene graphene oxide	Swimming speed	Decrease	Cano et al., 2017
Daphnia magna	Water-stirred C(60) carboxylic acid functionalized fullerenes (fC(60))	Vertical position	Alteration	Brausch et al., 2011
		Swimming speed	Decrease	
Daphnia magna	Nanosized titanium dioxide	Swimming speed	Decrease	Noss et al., 2013
		Swarming	Increase	
Daphnia magna	Multiwalled carbon nanotubes	Swimming speed	Decrease	Stanley et al., 2016
Daphnia magna	Fullerenes: nano-C60 and C60HxC70Hx	Hopping frequency	Increase	Lovern et al., 2007
Daphnia magna	Potassium dichromate	Vertical migration	Altered	Gutierrez et al., 2012
Daphnia magna	Copper	Swimming time	Decrease	Untersteiner et al., 2003
		Swimming speed	Decrease	
Cyanobacterial and bacterial products				
Daphnia magna	Saxitoxin-producing *Cylindrospermopsis raciborskii*	Swimming speed	Decrease	Ferrão-Filho et al., 2014
		Swimming time	Decrease	
		Resting time	Increase	
		Distance travelled	Decrease	
Daphnia magna	β-Cyclocitral	Swimming speed	Initial increase followed by acclimation	Jüttner et al., 2010
Daphnia magna	Ectoine	Swimming speed	Decrease	Bownik et al., 2015
Daphnia magna	Ectoine + hydrogen peroxide	Swimming speed	Alleviation of hydrogen peroxide-induced decrease	Bownik and Stępniewska, 2015a
Daphnia magna	Ectoine + formaldehyde	Swimming speed	Alleviation of formaldehyde-induced decrease	Bownik and Stępniewska, 2015b
Daphnia magna	Ectoine + heat stress	Swimming speed	Alleviation of Decrease of swimming velocity induced by heat Stress	Bownik et al., 2014
Daphnia pulex	Algal extracts	Horizontal distribution	No effect	Lauren-Määttä et al., 1997
Pesticides				
Daphnia pulex	Chlorpyrifos	Cumulative distance	Decrease	Zein et al., 2014
		Change in angle	Increase	
Daphnia magna	Binary mixtures of glyphosate and copper [Cu(II)]	Swimming speed	Decrease	Hansen and Roslev, 2016
		Acceleration	Decrease	
		Distance moved	Decrease	
		Inactive time	Increase	
Daphnia pulex	Imidacloprid	Cumulative distance	Increase	Zein et al., 2014
		Change in angle	Decrease	
Daphnia magna	Dichlorvos	Behavioural strength	Decrease	Ren et al., 2015
Daphnia magna	4-Nonylphenol	Change in direction	Increase	Zein et al., 2015
Daphnia magna	Dichlorophenol	Swimming activity	Decrease	Bahrndorff et al., 2016
Daphnia magna	Bromocriptine	Swimming velocity	Decrease	Barrozo et al., 2015
Daphnia pulex	Diazinon	Cumulative distance	Increase (lower concentrations)	Zein et al., 2015
			Decrease (higher concentrations)	
		Change of direction angle	Increase	
Daphnia magna	Cypermethrin	Swimming speed	Decrease	Christensen et al., 2005
Daphnia magna	Carbaryl	Swimming speed	Increase	Dodson et al., 1995
		Number of hops	Decrease	
		Upward angle	Increase	
		Downward angle	Decrease	
Daphnia magna	Paraoxon-methyl	Swimming speed	Increase	Duquesne and Küster, 2010
Daphnia magna	Endosulphan	Vertical migration	Altered	Gutierrez et al., 2012
Other agents				
Daphnia pulex	Nicotine	Cumulative distance	Increase	Zein et al., 2014
		Change in angle	Increase	
Daphnia magna	Clove essential oil (eugenol)	swimming speed	Decrease	Bownik et al., 2015
Daphnia magna	Norfloxacin	Time ratio of vertical to horizontal swimming	Increase	Pan et al., 2017
		Duration of quiescence	Increase	
		Swimming activity	Increase	

(continued on next page)

Table 1 (*continued*)

Daphnia species	Stressful agent	Parameter of swimming behaviour	Effect	References
Daphnia magna	VOC (Volatile Organic Compounds)	Swimming speed	temporal increase followed by the acclimation	Watson et al., 2007
Daphnia pulex	Physostigmine	Cumulative distance Change in angle	Increase Increase	Zein et al., 2014
Daphnia similis	Cerium dioxide	Swimming speed	Decrease	Artells et al., 2013
Daphnia pulex	Cerium dioxide	Swimming speed	Decrease	Artells et al., 2013
Daphnia magna	17alpha-Ethynylestradiol	Swimming speed	No effect	Goto and Hiromi, 2003
Daphnia magna	Norethindrone	Swimming speed	Decrease	Goto and Hiromi, 2003
Daphnia magna	Sucralose	Swimming speed Swimming height	Increase Increase	Wiklund et al., 2012
Physical factors				
Daphnia magna	Heat stress	Swimming speed	Increase	Bownik et al., 2014
Daphnia magna	Increased salinity	Swimming speed	Decrease	Baillieul et al., 1998.
Daphnia magna	Solar radiation	Gravitaxis	Increase	Gonçalves et al., 2007
Daphnia magna	Ultraviolet radiation	Swimming speed Distance travelled	Decrease Decrease	Hylander et al., 2014

parameter of microcrustaceans. Counting of single hops with a naked eye may be difficult, therefore a frame-by-frame analysis with the use of software is recommended for simple and precise determination of this parameter. It is to note that the number of hops is independent of body size (Dodson and Ramcharan, 1991), however it may also be species-dependent. Length of separate hops may also affect average swimming velocity. For example, two individuals belonging to the same species with the same mean swimming speed may have different hopping frequency, time between hops and a distance of a single hop (Fig. 1). Some compounds were found to alter daphnid hopping frequency. Depressants of the nervous system may increase time period between each hop which in turn may alter overall swimming velocity. Conversely, some neurostimulatory agents may decrease time gaps between hops or increase distance of single hops. Various toxic agents may alter hopping frequency in an opposite way. Nanoparticles such as fullerenes nano-C60 and C60HxC70Hx were found to increase this parameter in *Daphnia magna* (Lovern et al., 2007) which may be explained as avoidance and escaping response. On the other hand, *Daphnia* subjected to carbaryl a potent inhibitor of acetylcholinesterase showed a decreased number of hops per second (Dodson et al., 1995). Hopping frequency is a valuable swimming parameter and determination of this parameter together with swimming velocity may provide detailed information on potential neurotoxicity of a compound and its effects on the regulation and level of muscle contractions in daphnids.

2.5. Horizontal distribution

This parameter of microcrustacean swimming behaviour indicates the level of collective response to different environmental factors such as predation pressure or food concentration and has been used mainly in ecological studies (Porter et al., 1982; Adamczuk and Mieczan, 2013; Nihongi et al., 2016). This parameter may be expressed as a number of daphnids per square centimeters or meters, or by the percentage of time spent in a part of the water column. Determination of horizontal distribution may be possible mainly in natural reservoirs, mesocosm studies but it may be difficult to study toxic effects on daphnids in

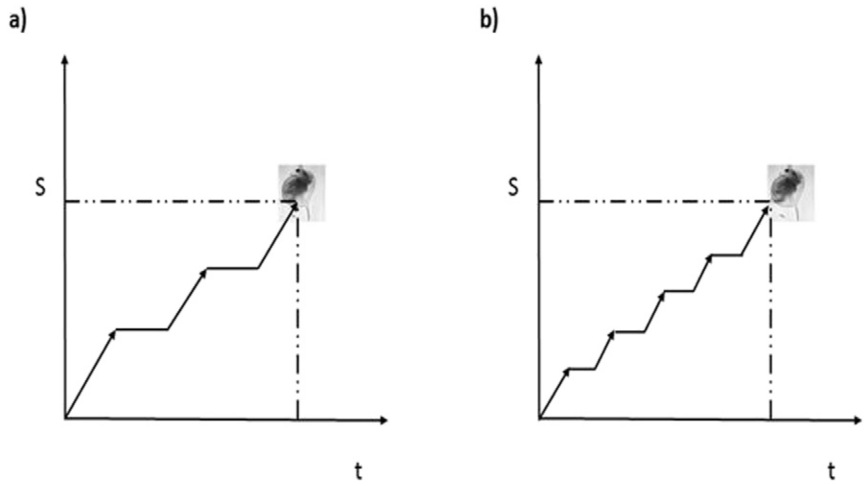

Fig. 1. Exemplary movement of two individual daphnids swimming with the same velocity, but with different numbers of hops and time between each hop. Panel a) shows a daphnid moving with 3 long hops and 3 long periods of time between jumps. The same velocity value may be observed in daphnids with more frequent hops of shorter distance and shorter periods of time between hops.

laboratories since it requires large tanks with enough space for aggregation of these animals into groups. However this behavioural parameter may be useful in ecotoxicological studies since it would enable observation of possible *Daphnia* avoidance to toxic substances. Horizontal distribution was determined for *Daphnia pulex* exposed to toxic algal extracts containing cyanotoxin microcystins-LR, however the results indicated this toxin induced no evident alteration of this parameter (Lauren-Määttä et al., 1997).

2.6. Vertical distribution and migration

Measurement of vertical distribution allows the assessment of the depth of the water column at which animals are aggregated. Distribution of organisms in the water column is a consequence of the ascents and descents through different time periods. Typically, alterations of vertical migration may be a behavioural response to light (phototactic migration) (Ringelberg, 1995), predation (Rinke and Petzold, 2008) and temperature (Geritsen, 1982). Some authors determined vertical distribution in ecotoxicological studies and suggested it as a sensitive parameter for the use in standard toxicity assays. It was found that diel vertical migration may be altered by chromium and sublethal concentrations of a persistent insecticide, endosulphan. It is hypothesized that such changes are a consequence of neurotoxic effects leading to disorientation of an organism (Gutierrez et al., 2012). Vertical migration of cladocerans may be also affected by some cyanobacterial species (Forsyth et al., 1990; Berthon and Brousse, 1995). Nanoparticles, such as water-stirred fullerenes (C60) and sonicated carboxylic acid functionalized fullerenes (FC60) were also found to alter the vertical position of *Daphnia magna* in response to the addition of food (Brausch et al., 2011). It is important to note that behavioural reactions during diel vertical migrations associated with phototactic behaviour are light-dependent. Therefore, the parameter may be altered not only by possible toxicants but it can be also a natural response of *Daphnia* to changing light conditions. An interesting methodological approach for analysis of horizontal distribution was proposed by Jeong et al. (2014). A rectangular chamber containing daphnids was divided into parts marked for the location of the crustaceans. When daphnids migrate through the sections a camera transfers the data to a computer. The frequencies of appearance in each section are obtained at 1 min interval and the distribution level is calculated and expressed as distribution index. Behaviour of daphnids swimming at a given height in the water column may be determined by horizontal swimming velocity (Pan et al., 2015). Although this parameter has not been used in ecotoxicological studies so far, some related endpoints such as swimming height and time ratio between vertical to horizontal swimming were measured (Pan et al., 2017).

Vertical position may be altered by various environmental conditions such as light, predator or food presence (Young and Watt, 1993). It is noteworthy that experimental systems for determination of these parameters require special containers with depth gauge or software calibration. Container width or diameter also affect this parameter since animals kept in higher containers with small width or diameter may be forced to swim vertically (Dodson et al., 1995). Some chemical substances were reported to affect this parameter. Sucralose induced the increased swimming height of *Daphnia magna* (Wiklund et al., 2012). Swimming altitude is a basis to evaluate the altitude index in an automated system called as Toximeter® (Lechelt et al., 2000) (described in the next section) and it was one of the parameters proposed for a biological early warning system for water quality (Jeong et al., 2014).

2.7. Time ratio of vertical to horizontal swimming

Time ratio between vertical and horizontal swimming indicates which type of swimming dominates in the mobility profile. Measurement of this parameter requires 3-dimentional systems monitoring simultaneously vertical and horizontal movements. Although the ratio may be a reliable parameter in ecotoxicological studies it should be noted that it may also be affected by the day-night cycle. The results indicate that the parameter may be increased in daphnids exposed to an antibiotic, norfloxacin (Pan et al., 2017). This parameter may be very useful for indirect evaluation of the animal energetic potential since vertical swimming of crustaceans requires more energy than horizontal swimming and the animals swimming upward must overcome water viscosity in addition to gravitional force (Boudrias, 1991). Therefore, the decreased ratio between vertical and horizontal swimming of the intoxicated daphnids may suggest energy depletion and/or some metabolic disorders.

2.8. Distance travelled

Distance moved (expressed in millimetres) by daphnids measured ~~for~~ repeatedly or continuously for a period of time may be a valuable swimming parameter indicating the locomotor activity. Some authors reported that this parameter may be altered by some pesticides. Fig. 2 presents exemplary trajectories of 10 swimming daphnids exposed to various concentrations of a neonicotinoid and analysed by a frame-by-frame method. The shortest tracks are evident at the highest concentration of the compound, 50 mg/L and 100 mg/L in comparison to the non-treated daphnids exhibiting the longest pathways. It was found that distance moved by the juvenile *Daphnia magna* was decreased by the binary mixture of glyphosate and copper [Cu(II)] (Hansen and Roslev, 2016). Daphnids exposed to the mixture showed shorter tracks when compared to those treated with glyphosate alone, however they were longer than those left by the animals exposed to the metal alone. Concentration-dependent effects on the cumulative distance were reported in daphnids exposed to an organophosphorous pesticide, diazinon (Zein et al., 2015). The parameter increased at lower concentrations of this insecticide, on the other hand, shorter distances were travelled by daphnids exposed to its higher levels. Shorter distances were also travelled by daphnids exposed chlorpyrifos, an organophosphorous insecticide (Zein et al., 2014). On the other hand, stimulatory effects on cumulative distance were observed in crustaceans treated with nicotine, physostigmine, a reversible cholinesterase inhibitor and imidacloprid, a neonicotinoid insecticide. These ambivalent responses to the pesticides may be explained by the fact that neurotoxic substances possess various mechanisms of action resulting in excitation or depression of the nervous system which in turn may induce opposite effects on cumulative distance.

2.9. Swimming trajectory

Swimming trajectory is a pathway left by a moving organism and may be characterized by length (usually expressed in millimetres) and shape. This parameter may be analysed in 2-or 3-dimensional systems. 3-dimensional trajectories of *Daphnia pulex* in different light conditions were determined by Uttieri et al. (2004). Some results indicate that trajectories of the intoxicated daphnids are different from those of the non-treated ones (Shimizu et al., 2002; Noss et al., 2013). Alterations of this parameter may suggest disorders in the crustacean nervous system manifested by various symptoms such as loss of orientation. Exemplary swimming trajectories of daphnids swimming in different concentrations of neurotoxic neonicotinoid acetamiprid recorded for 1 min shown in Fig. 2 indicate less curved trajectories of the control crustaceans covering almost the whole area of the observation dish while the tacks of the animals treated with the pesticide swim only around small areas close the walls. 3-dimensional analysis was used to measure this parameter in daphnids exposed to nanosized titanium oxide (Noss et al., 2013). The increased tendency for aggregation of the daphnids in the centre of the test vessel, was observed as a response to increasing particle concentrations. Some mathematical approaches were also introduced to analyse *Daphnia* swimming trajectories. Lagragian description allows to classify and compare trajectories, providing knowledge

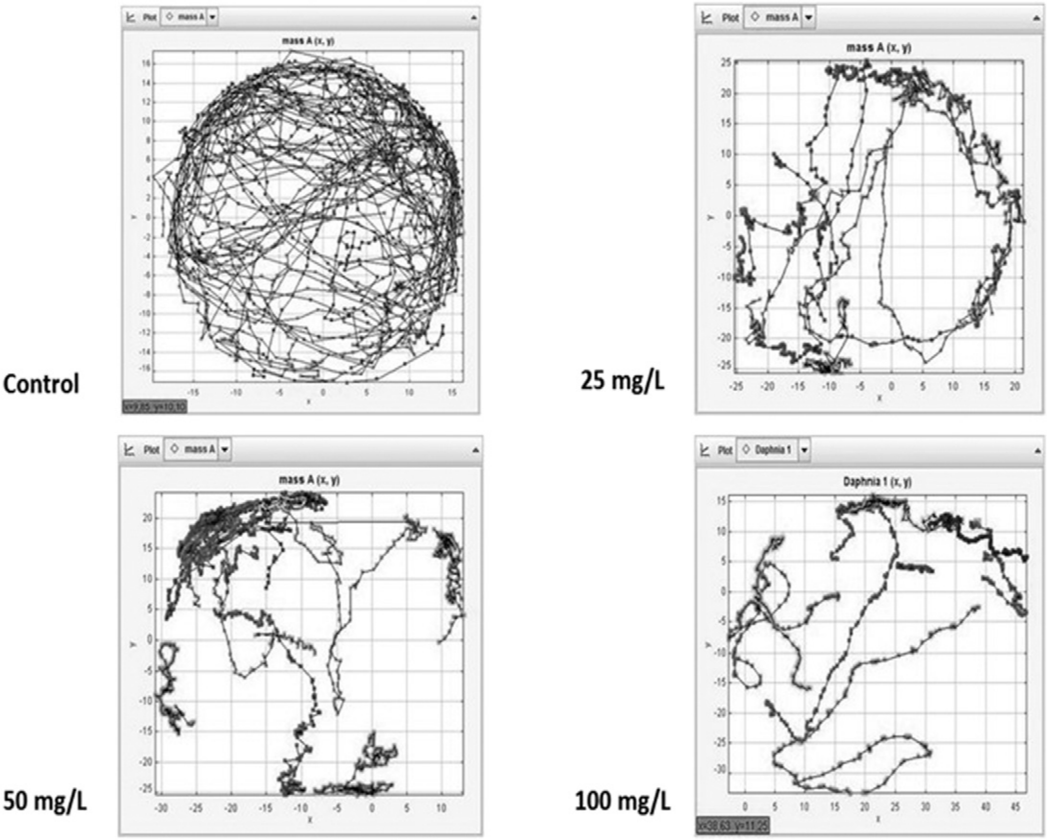

Fig. 2. Swimming trajectories of 10 individual *Daphnia* exposed to various concentrations of neonicotinoid pesticide acetamiprid (mospilan 20 SP). Images generated in Tracker® software.

about the complex behaviour of zooplanktonic organisms (Uttieri et al., 2004). Another approach to characterize swimming trajectory is using fractal dimensions (Nikitin et al., 2015; Shimizu et al., 2002) with box counting approach. This method was used in a 2-dimensional system where x and y coordinates were established to estimate the fractal dimension according to a mathematical equation (Katz and George, 1985).

2.10. Number of turnings, turning angle

Although *Daphnia* change swimming direction in response to different factors such as food, light or predator pressure, this type of a behaviour may also occur in response to toxic agents. The literature data indicates that determination of directional change was mostly done with the use of two parameters: number of turnings (per second or

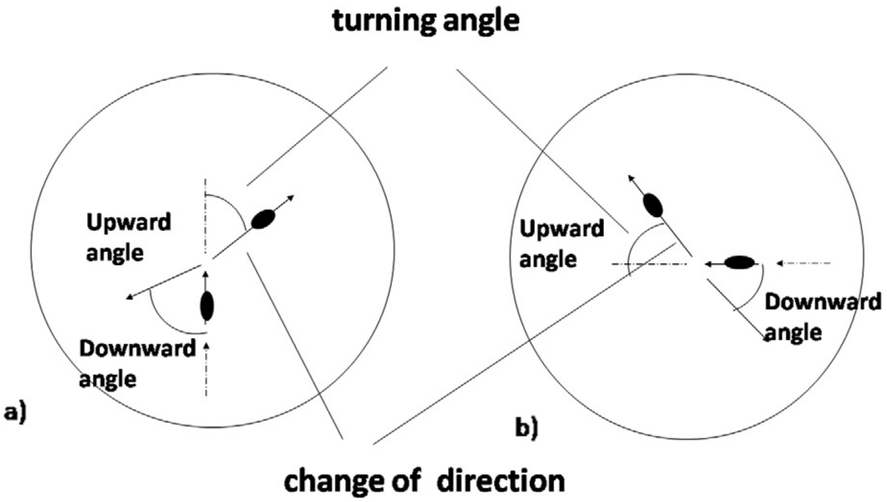

Fig. 3. Changes of swimming direction may be measured by downward angle and upward angle or a number of turnings. The parameters may be determined in daphnids moving vertically (panel a) and horizontally (panel b).

minute) and change of angle (expressed in radians). Moreover, both vertical and horizontal movement was measured with two parameters: upward and downward angles (Fig. 3). Swimming direction of daphnids may be determined in 2-dimensional or 3-dimensonal systems. Results obtained in ecotoxicological studies revealed that swimming direction may be altered by various neurotoxic compounds. The increased change of angle was found in daphnids exposed to diazinon, physostigmine, nicotine and chlorpyrifos. Oppositely, imidacloprid reduced the change of angle direction (Zein et al., 2014, 2015). Fullerenes (C60) were also found to alter average turning angle and modal angle downward of *Daphnia magna* (Brausch et al., 2011). Daphnids treated with carbaryl exhibited the increased upward angles when compared to downward angles (Dodson et al., 1995). Monitoring of swimming direction was proposed as a method of assessing spatial orientation of cladocerans exposed to xenobiotics (Goodrich and Lech, 1990).

2.11. Resting time, duration of quiescence

A few studies revealed that daphnids may react to some toxicants by temporal immobilisation followed by the return to normal swimming. This transient lack of movement that may last a few seconds or several hours is named as resting time or quiescence. Resting time was reported to be found in daphnids exposed to saxitoxin-producing cyanobacteria, *Cylindrospermopsis raciborskii* (Ferrão-Filho et al., 2014). Although swimming of the exposed animals was ceased, after some period of time they recovered with the mobility as for the control animals. In another study inability of animals to move (quiescence) was observed in daphnids exposed to norfloxacin (Pan et al., 2017). The mechanism of swimming cessation by the antibiotic has not been elucidated so far, however neurotoxicity should not be excluded.

2.12. Sinking rate

Short moments of sinking lasting fractions of a second may be observed in normally swimming daphnids. This phenomenon occurs between each hop as a result of gravitational force. Sinking rate is a parameter depending on several factors such as the size, shape of the falling and water viscosity (Brooks and Hitchinson, 1950; Dodson and Ramcharan, 1991), presence of food and light intensity (Gorski and Dodson, 1996). Little is known of the effects of toxicants on daphnid sinking rate. This parameter named as ‘sinking velocity’ was determined in *Daphnia magna* exposed to titanium dioxide nanoparticles (Noss et al., 2013). Although the authors found that sinking velocity was higher in the animals with increased body length no effects of the nanoparticles on this parameter were observed. However it may be hypothesized that elevation of sinking rate may be concomitant with the reduction of swimming velocity in the intoxicated daphnids.

2.13. Gravitaxis

This parameter is useful in measuring downward movement initiated by daphnids in the presence of stressful factors. Gravitaxis was measured in *Daphnia spinulata* exposed to solar radiation. Most of the animals swam downwards, regardless of the radiation treatment imposed to the samples (Gonçalves et al., 2007). Determination of this parameter would be recommended in studies on stressful factors dispersed from above water surface such as various types or radiation. Although this parameters has not been used in ecotoxicological studies it may be a promising endpoint providing data on how toxicants may alter animal reactions to external stimuli such as light.

2.14. Swarming

Swarming is a collective behavioural response leading to vortex formation (Vollmer et al., 2006). This type of reaction occurs normally in daphnids responding to light change, food presence or predator pressure (Øien, 2004; Mach and Schweitzer, 2007). Characteristic manifestation of swarming is attraction of the animals to the centre in the water column. This behaviour of massive migration has not been frequently used in toxicological assessment, however it was observed in daphnids exposed to titanium dioxide nanoparticles (Noss et al., 2013). Intensive swarming was observed in daphnids treated with higher concentrations of the nanoparticles. The animals aggregated in the centre of the test chambers during the initial phase of the exposure. As this parameter is complex, for more detailed characterization of alterations it may be supplemented by additional data such as direction of swarming, speed, animal distribution and change of swarm shape.

2.15. Spinning

Spinning, a type of moving in small circles characteristic to daphnids exposed to stressful agents is not typical behaviour for free-swimming *Daphnia* (Fig. 4). This type of reaction was observed in daphnids exposed to carbaryl and *Chaoborus* kairomone (Dodson et al., 1995). The authors interpreted this phenomenon as continuous escape response. However, in daphnids treated with high concentration of toxicants, this kind of a behaviour may also suggest loss of the body balance control as a result of neurotoxic changes. Unfortunately, no detailed analysis of spinning is available in the literature. This parameter might be additionally characterized by its frequency, speed and direction.

3. Systems for the analysis of swimming behaviour

First studies on *Daphnia* swimming behaviour were performed with the use of VHS video cameras and tape recorders which did not offer such flexibility and simplicity of use in comparison to the modern digital solutions. Current standard experimental equipment typically consists of one observation container or flat vessel (observation dish) or a set of containers in which the exposed daphnids are kept and monitored by one (for 2-dimesional systems) or two (for 3-dimensional systems) video cameras connected to a computer with software for movement analysis (Fig. 5a and b) (Shimizu et al., 2002; Noss et al., 2013). The movement of animals may be recorded constantly or periodically and transferred by a video camera to a computer for the analysis by a dedicated software. These systems may include special chambers enabling constant water flow or temperature control. Although modern video cameras allow transferring the signal to a computer by high speed USB (Universal Serial Bus) or FireWire connection at high resolution. 2-dimensional systems are the simplest sets that allow to monitor speed, swimming tracks, turning angles and the other parameters, however low water/medium level in the observation chambers in such systems will not allow measurement of vertical swimming parameters such as vertical velocity or gravitaxis (Fig. 5a). More detailed analysis

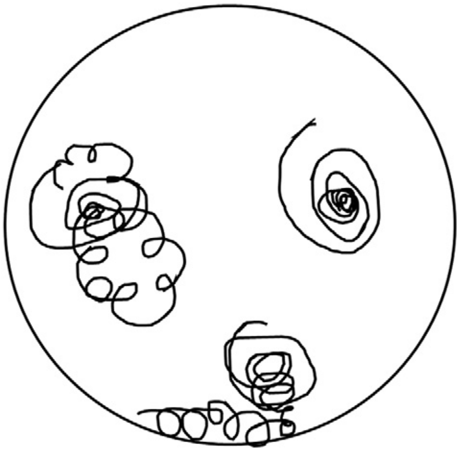

Fig. 4. Exemplary spinning movement of *Daphnia*.

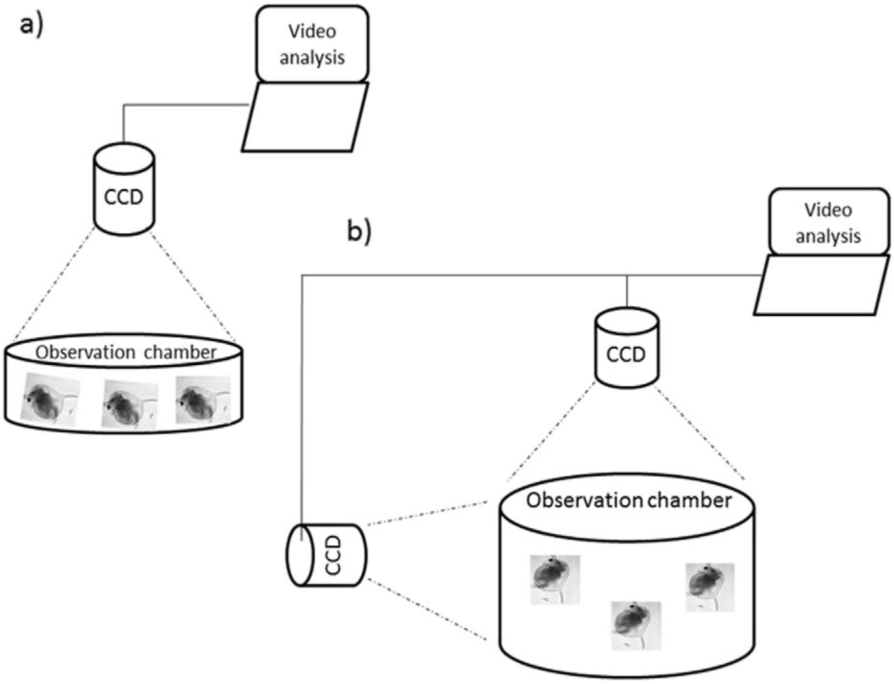

Fig. 5. Typical 2- (panel a) and 3-dimensional (panel b) systems for analysis of *Daphnia* swimming behaviour. Daphnids kept in the observation chamber is monitored by a single (a) or two CCD-video cameras (b). Image analysis is performed by the use of computer software.

of swimming parameters may be performed by 3-dimensional systems with 2 or more cameras (Fig. 5b). Such systems may facilitate analysis of all the behavioural parameters mentioned in this review.

3.1. Digital processing of video clip

Processing of video clips with software for motion analysis is the second step of determination of swimming behaviour with simultaneous measurement of several parameters. Although in some highly developed systems for behavioural analysis dedicated software such as Noldus EthoVision® may be preferred (Stanley et al., 2016), a typical digital analysis of a video clip with *Daphnia* swimming may be processed with freely available open- access software such as Ctrax®, Tracker®, ImageJ® (Barrozo et al., 2015; Bownik et al., 2015a). For example, Tracker® offers very simple technique of parametric analysis. The video files are analysed by a frame-by-frame method. By clicking with the cursor on one individual *Daphnia* (interpreted by the program as a mass point) the program records the position in separate frames

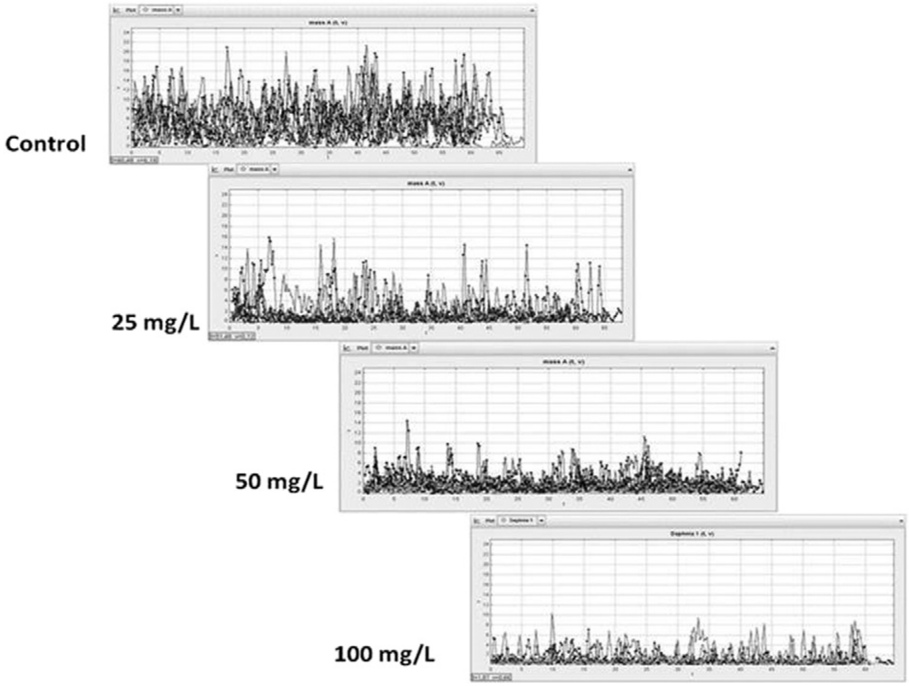

Fig. 6. Exemplary amplitudes of swimming speed of 10 Daphnia individuals. Individual tracks were superimposed. Graphs were generated in Tracker® software.

and generates the whole swimming trajectory measuring simultaneously several parameters such as its maximal, minimal and mean velocity, turning angles, etc. This program may be used in a 2-dimensional system (Bownik et al., 2015a) in which daphnids move in the observation dishes virtually only in two dimensions and trajectory is represented by x and y coordinates. The selected swimming parameters are calculated by the software and plotted in separate graphs. Amplitudes of average swimming velocity of individual daphnids may be superimposed and presented in one graph (Fig. 6).

3.1.1. Automated systems

Systems monitoring swimming activity of daphnids are very flexible and allow high level of automation for speeding up the analysis. Several automated approaches useful for rapid determination of toxicity and helpful tools for drinking water quality monitoring as biological early warning system (BEWS) have recently been developed (Jeong et al., 2014). This sensitive system is able to detect marked changes of swimming behaviour induced by toxicants present in a tested water sample. An alarm is triggered when the toxic level increases above the established threshold from the observation data (Jeon et al., 2008).

BehavioQuant (Quantitative analysis of Behaviour) is a computer-controlled image-processing data acquisition system used for analysis of behaviour for various aquatic species including *Daphnia* (Butterworth et al., 2001). This system allows automatic measurements of several parameters, such as mobility, number of turns, inconstancy of movements, swimming height, horizontal pace of preference.

Daphnia toximeter also known as Daphtox® is another type of an automated system for toxicity analysis in which 14 swimming parameters of *Daphnia* such as velocity, turns, circling movement, orientation with respect to light and gravity as well as cell form and size may be measured (Lechelt et al., 2000; Jüttner et al., 2010; Häder and Erzinger, 2017). Effects of sucralose and VOC on *Daphnia* swimming behaviour were conducted with the use of this system. *Daphnia* toximeter was recommended as a tool for a routine monitoring of VOC compounds in water (Watson et al., 2007; Wiklund et al., 2012). The improved version of Daphtox® is Daphtox II®.

Grid Counter device is another system which has been developed as a biomonitoring method to detect the abnormal swimming of *Daphnia magna*. The system includes six independent channels monitoring swimming activity of the cladocerans using a digital 'Grid Counter' triggering an alarm when detecting abnormal water quality (Jeon et al., 2008).

Systems based on visible light do not allow tracking of swimming daphnids in dark experimental conditions. Therefore, approaches which are not based on typical cameras could be very useful especially for continuous monitoring of swimming. Movement speed in addition to animal size may be measured with a photoelectrochemical array of individually-addressable electrodes in which photocurrent is generated. Mobility is detected by measuring the 'dark' transients of daphnid shadow passing over each electrode (Rees and Compton, 2009). Unfortunately, no wider application of such system in ecotoxicological research was found. Another system, the infrared light-based monitor for recording of *Daphnia* swimming behaviour was proposed by Bahrndorff et al. (2016). It allows determination of behavioural responses in darkness, which is advantageous to traditional visible light-based systems, however it provides less parameters. Another interesting approach for determination of behavioural strength is to use a system with electrodes sending and receiving alternating current (Fig. 7) in which animals are kept in a flow-through test chamber closed off on both sides with nylon nets (Ren et al., 2015). One pair of electrodes at the walls of the test chambers send a high frequency signal of current, which is received by a second pair of non-current-carrying electrodes. Behaviour strength of *Daphnia magna* is transformed by an A/D transformer and the signal changes formed by the A/D transformer are analysed by software. Values from 0 (no swimming behaviour) to 1 (Full Behaviour strength) illustrate differences in the swimming behaviour (Zhang et al., 2012). A novel approach is also tracking of daphnids labelled with fluorescent nanoparticles in UV light (Ekvall et al., 2013).

4. Limitations and future perspectives of the analysis of *Daphnia* swimming behaviour

Although analysis of *Daphnia* swimming behaviour seems to be a reliable tool for evaluation of toxicity, it is not without limitations which may hopefully be reduced by future designs. Firstly, analysis of literature indicates that there exist some inconsistency in terminology. Some swimming parameters have different names. For example, vertical distribution (Geritsen, 1982), swimming height (Jeong et al., 2014) or swimming depth (Ringelberg, 1995) denominate the same parameter. Swimming velocity which is a vector quantity, seems to be used interchangeably with a scalar term, swimming speed. Therefore, there is a need for standardization of terminology in order to avoid confusion. Secondly, experimental systems used for determination of swimming parameters very poorly simulate natural conditions. Two dimensional approaches do not reflect natural swimming of *daphnia* since the cladocerans kept in observation dishes with low water level are forced to swim only horizontally. Such an experimental variant does not allow measurement of a number of swimming parameters such are gravitaxis, change of upward and downward swimming and downward or upward angular alteration. Moreover, two dimensional systems would rather

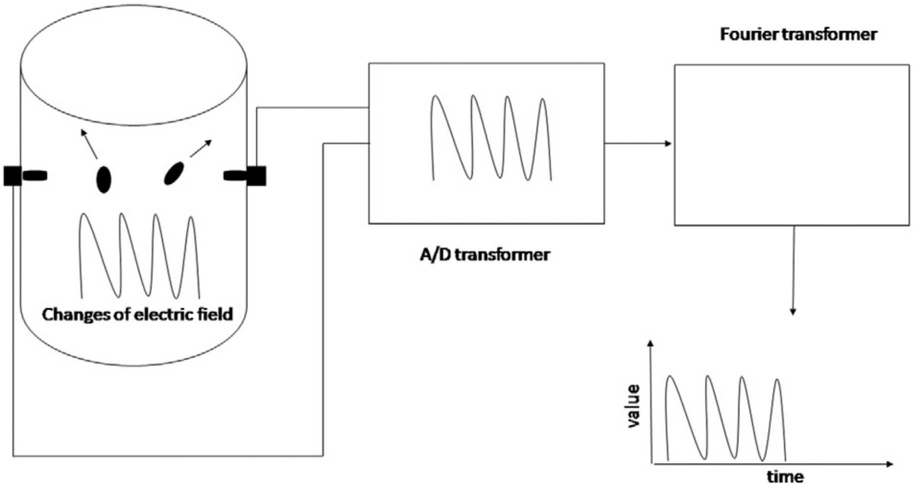

Fig. 7. System for determination of behavioural strength based on analysis of changes in current flow (Ren et al., 2015).

not be recommended for chronic toxicity experiments since prolonged swimming in not natural position may induce additional stress and thereby increase behavioural reaction to a tested compound. A better methodological approach would be provided by three dimensional sets in which natural movement may be measured with the use of more parameters. As many of three dimensional parameters have been used only in ecological studies (Uttieri et al., 2014), there is a methodological gap in ecotoxicology which will be hopefully filled in the near future. It is also to note that laboratory equipment may affect measurements of *Daphnia* swimming behaviour. Dimensions of experimental vessels such as shape, height, width or diameter should be carefully selected specifically for the type of the measured parameter. Some authors indicated that high beakers with small diameter may significantly affect velocity of horizontal swimming but decrease the velocity of vertical swimming (Pan et al., 2015). Vessels with too low volume containing high numbers of animals may result in inhibition of swimming velocity (Dodson et al., 1995) and even the non-treated daphnids moving along the walls may show escaping movement (Fischer and Moore, 1993). Physical parameters of water may affect swimming behaviour of *Daphnia*. Aquatic solutions of some compounds may possess high viscosity which in a consequence may decrease swimming speed and gravitaxis. Furthermore, organic solvents such as ethanol or dimethylsulfoxide used for many lypophilic compounds may also alter mobility of daphnids, inducing additive or synergistic effects with a tested compound. Therefore, it is recommended to introduce an additional control group of daphnids exposed to solvent alone. Another issue is that individual daphnids may show various behavioural reactions, therefore experimental groups should consist of appropriate number of animals in order to obtain statistically significant differences. It is also important that *Daphnia* that are transferred to test vessels may show some stress symptoms. Therefore, a proper time of acclimation to new conditions should be maintained, especially in two dimensional systems since low water level in observation dishes may induce initial escape movements. As daphnids alter their swimming behaviour in response to changes of light intensity, some period of time is also required to adapt to light especially when the animals are kept in darkness before taking measurements.

Tracking of swimming crustaceans with systems based on visible light cannot be used for determination of *Daphnia* swimming in dark experimental conditions. On the other hand, approaches with alternating current (Rees and Compton, 2009), infrared light (Bahrndorff et al., 2016) or labelling daphnids with fluorescent particles (Ekvall et al., 2013) seem to be a promising solution allowing assessment of toxicity in dark conditions.

Both two and three dimensional systems are simple laboratory sets not simulating environmental factors such as microbial activity, photodegradation, thermal stratification, water turbidity, wind conditions that may additionally affect toxicity of a tested compound and thereby change daphnid swimming reactions. Therefore microcosm and mesocosm systems allowing determination of interactions of multiple environmental factors with a tested compound could be an alternative. However, as they cover larger areas, tracking of individual daphnids swimming away of a monitoring camera or other sensors may not be possible. Moreover, application of some toxicants at higher concentrations to containers of that size would be very costly and very difficult to control its constant level due to bacterial metabolism and possible accumulation.

Concluding remarks

A number of scientific reports indicate that swimming behaviour of *Daphnia* is generally accepted as a reliable biomarker for determination of toxicity induced by various groups of chemicals. Thanks to high sensitivity of crustaceans simplicity and a high level of automation of the monitoring systems in which a number of parameters simultaneously can be measured as effective water-quality screening tools (Zein et al., 2014). Many of the presented parameters have been used in ecological studies but there are some still awaiting its application in ecotoxicology. Many authors of the reviewed studies also strongly recommend swimming behaviour of *Daphnia* as a biomarker indicating possible detrimental effects induced by toxic chemicals. However, it is noteworthy that this method has some limitations and methodological difficulties such as no possibility to simulate three dimensional swimming in two dimensional systems or problem of monitoring in dark experimental conditions that may be solved by using alternative method of their detection.

In summary, the reviewed literature shows that many various toxicants may induce disturbances of swimming behaviour of daphnids. As these dysfunctions may be analysed in automated systems it can be concluded that various parameters of swimming behaviour may be considered as a sensitive method recommended for assessment of chronic toxicity and also as a promising approach for monitoring of water quality.

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