Using a new high-throughput video-tracking platform to assess behavioural changes in *Daphnia magna* exposed to neuro-active drugs.

Fatima Simao[1,2], Fernando Martínez-Jerónimo[3], Victor Blasco[4], Francesc Moreno[4], Josep Maria Porta[4], Joao Pestana[1], Amadeu M.V.M. Soares[1], Demetrio Raldúa[2] and Carlos Barata[2*]

[1] Centre for Environmental and Marine studies (CESAM), Department of Biology, University of Aveiro, Portugal

[2]Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA), Spanish Research Council (IDAEA, CSIC), Jordi Girona 18, 08034 Barcelona, Spain

[3] Instituto Politecnico Nacional, Laboratorio de Hidrobiología Experimental, Mexico City, Mexico

[4] Institut de Robòtica i Informàtica Industrial (CSIC-UPC), Barcelona,Spain

[*]E-mail contact: cbmqam@cid.csic.es

Abstract

One of the major challenges that faces today regulatory risk assessment is to speed up the way of assessing threshold sublethal detrimental effects of existing and new chemical products. Recently advances in imaging allows to monitor in real time the behaviour of individuals under a given stress. Light is a common stress for many different organisms. Fish larvae and many invertebrate species respond to light altering their behaviour. The water flea *Daphnia magna* as many other zooplanktonic species has a marked diel vertical phototactic swimming behaviour against light due to fish predation. The aim of this study was to develop a high-throughput image analysis to study changes in the vertical swimming behaviour to light of *D. magna* first reproductive adult females exposed to 0.1 and 1 μg/L of four psychiatric drugs: diazepam, fluoxetine, propranolol and carbamazepine during their entire life. Experiments were conducted using a new custom designed vertical oriented four 50 mL chamber device controlled by the Noldus software (Netherlands). Changes in speed, preferred area (bottom vs upper areas) and animal aggregation were analysed using groups of animals under consecutive periods of dark and apical light stimulus of different intensities. Obtained results indicated that light intensity increased the speed but low light intensities allowed to better discriminate individual responses to the studied drugs. The four tested drugs decreased the response of exposed organisms to light: individuals move less, were closer to the bottom and at low light intensities were closer each other. At high light intensities, however, exposed individuals were less aggregated. Propranolol, carbamazepine and fluoxetine were the compounds effecting most the behaviour. Our results indicated that psychiatric drugs at environmental relevant concentrations alter the vertical phototactic behaviour of *D. magna* individuals and that it is possible to develop appropriate high-throughput image analysis devices to measure those responses.

Introduction

One of the major challenges that faces today regulatory risk assessment is to speed up the way of assessing threshold sublethal detrimental effects of existing and new chemical products. Recently advances in automated video/ imaging allows to monitor in real time locomotor trajectories of individuals under a given stress and hence assessing multiple behavioural parameters in a relatively short time (Bownik, 2017). Behavioral responses are at the core of the adverse outcome pathway (AOP) concept that relates chemical exposure to subsequent molecular, cellular, physiological and behavioural changes that result in illness or injury to individuals (Ankley et al., 2010). The central nervous system (CNS) is the most complex organ that senses, processes and transmits information. Therefore, locomotor-based behavioural outputs of the CNS are highly sensitive measures of toxicant impact particularly for compounds with a neurodevelopmental or neurofunctional mode of action (Mora-Zamorano et al., 2018). Fong and Ford (2014) and Ford and Fong (2015) reported that antidepressant drugs induced phototaxis in amphipods, altered mobility of snails, memory, cognitive function and the ability to camouflage in cattlefish at environmental relevant doses as low as pg-ng/L. More recently Rivetti et al. (2016) reported that psychiatric drugs such as the antidepressant fluoxetine, the anxiolytic diazepam and the neuropatic carbamazepine altered phototaxis in the crustacean *Daphnia magna* at environmental relevant concentrations ranging from 1-1000 ng/L.

The ecotoxicological model crustacean species *D. magna* is a good candidate to study altered phototactic behaviour upon exposure to neuro-active drugs. *D. magna* share with vertebrates several of the neurotransmitters that are targeted by antidepressant and other neuro-active drugs. These include the presence of serotonin, dopamine, epinephrine and GABA receptor signaling pathways (Campbell et al., 2004; Campos et al., 2013; Ehrenström and Berglind,

1988; McCoole et al., 2012a; McCoole et al., 2012b; Weiss et al., 2012). *Daphnia* swimming behaviour is complex and hence precise of several measurement parameters. *Daphnia* move with a characteristic hops generated by rhythmic beating of the second antennae (Dodson and Ramcharan, 1991). This means that cladoceran movement is not constant, it accelerates after the beat of the second antennae and subsequently the animal sinks when the second antennae return to the position to begin the next beating cycle. Therefore swimming speed depends on the movement characterized by accelerations followed by slowdowns. This parameter depends on *Daphnia* size (Hylander et al., 2014) and thus it is not always a reliable parameter to measure in ecotoxicological studies. Instead the distance moved (expressed in millimetres) by daphnids measured 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 pesticides and neuroactive compounds (Bownik et al., 2018; Cooke, 1966; Chevalier et al., 2014; Hansen and Roslev, 2016; Zein et al., 2015). Additional parameters associated with the hop type movement that have been assessed in ecotoxicological studies are hopping frequency, swimming time or alternatively resting time between normal swimming (Bownik, 2017). Daphnia also have a collective behaviour termed warming, characterized by the aggregation of animals upon sensing light change, food presence or a predator pressure (Vollmer et al., 2006), that have been reported as a response to titanium oxide nanoparticles (Noss et al., 2013).

One of the most ecological relevant swimming behavioural in *Daphnia,* however, is its negative phototaxis, which is directly linked to diel vertical migration along the water column, which prevents *Daphnia* to be preyed upon fish during daylight (Cousyn et al., 2001; De Meester, 1993). Behavioural reactions during diel vertical migrations associated with phototactic behaviour are light-dependent. Therefore, phototaxis may be altered not only by toxicants but it can be also a natural response of *Daphnia* to changing light conditions.

Experimental systems for determination of the vertical position of daphnids across light and dark periods required special vertical containers, an apical and intensity regulated visible light source, an additional light source for video recording the animals in darkness or under visible light not detected by the animals (i.e. infrared light) and software calibration. Despite the increasing number of studies that have used automated video recording system to monitor *Daphnia* swimming behaviour (Bownik, 2017), few used infrared light-based monitors (Bahrndorff et al., 2016; Chevalier et al., 2014) and none combined both visible and infrared light to allow the simultaneous measurement of behavioural responses under dark and light. Indeed studies that have monitored phototactic behaviour in *Daphnia* across dark and light periods are mostly based on manual monitoring of the relative position of animals without video recording (Cousyn et al., 2001; De Meester, 1993; Rivetti et al., 2016).

The aim of this study was to develop a high-throughput image analysis to study changes in the vertical swimming behaviour to light of *D. magna* individuals exposed to 0.1 and 1 µg/L of four psychiatric drugs: diazepam, fluoxetine, propranolol and carbamazepine during their entire life. Previously we found that these four drugs altered reproductive behaviour at low environmental relevant doses but only three of them, diazepam, fluoxetine and carbamazepine also altered phototaxis behaviour (Rivetti et al., 2016). In the previous studies (Cousyn et al., 2001; De Meester, 1993; Rivetti et al., 2016) phototaxis was measured as the proportion of animals swimming close to the light source in vertical cylindrical (i.e. 125 mL) glass column (i.e. 25 cm height, 5 cm internal cross-section), placed in a darkened box, and illuminated from above. To mimic the above mentioned device, experiments were conducted using a new custom designed vertical oriented four 50 mL chamber device controlled by the Noldus software (Netherlands). Changes in locomotor activity, preferred area (bottom vs upper areas) and animal aggregation were analyzed using groups of animals under consecutive periods of dark and apical light stimulus of different intensities.

1. Methods

2.1 Chemicals

Fluoxetine hydrochloride (CAS-No 56296-78-7; analytical standard, purity 100%), diazepam (CAS-No 439-14-5; analytical standard, purity 99%), carbamazepine (CAS-No 298-46-4; analytical standard, purity 99%) and propranolol hydrochloride (CAS-No 318-98-9; analytical standard, purity 99%) were purchased from Sigma-Aldrich (USA/Netherlands). All other chemicals were analytical grade and were obtained from Merck (Germany).

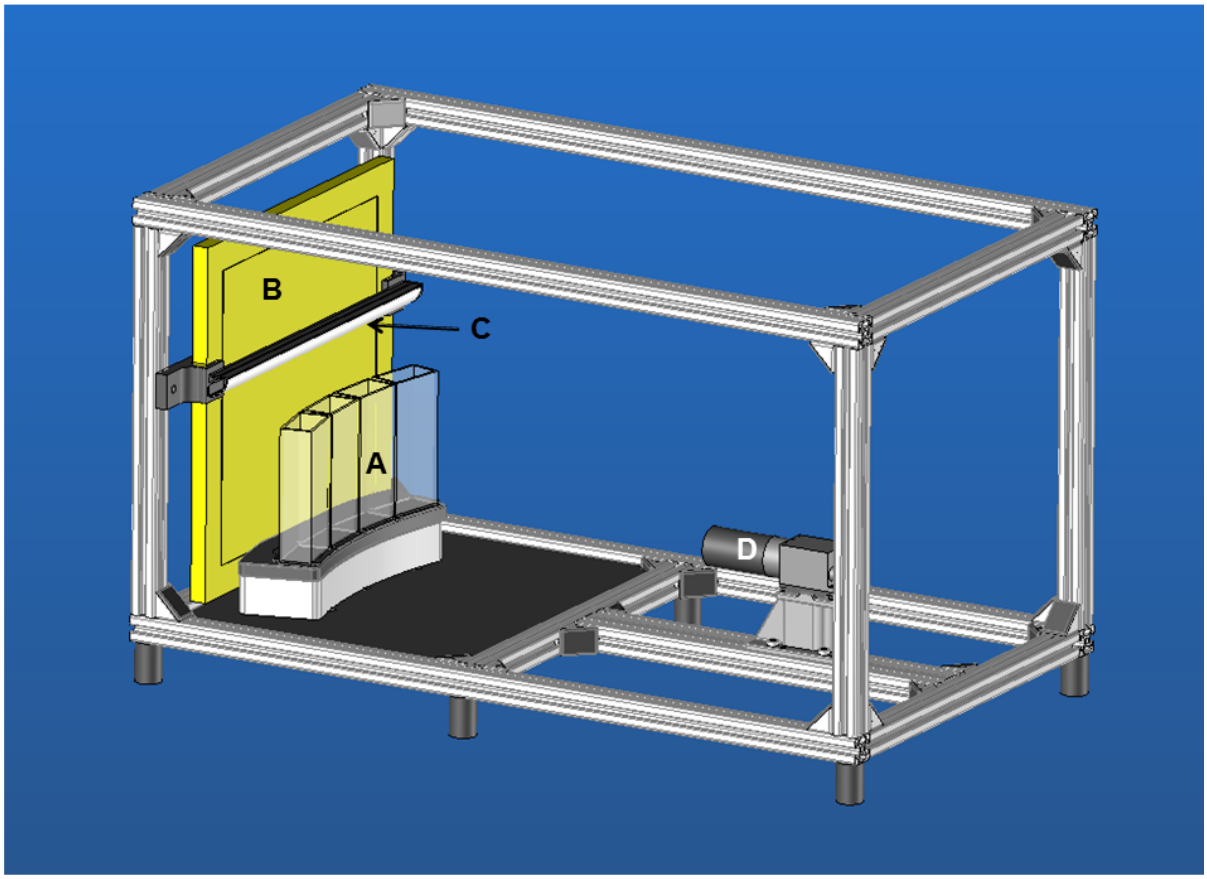
2.2 Experimental animals

A single *D. magna* clone F, extensively characterized in previous studies (Barata and Baird, 2000) was used for all assays. Bulk cultures of 10 animals/l were maintained in ASTM hard synthetic water (ASTM, 1994) as it has been described previously (Barata and Baird, 2000). Bulk cultures were fed daily with *Chorella vulgaris* Beijerinck ($5x10^5$ cells/ml, corresponding to 1.8 µg C/ml; (Barata and Baird, 2000). The culture medium was changed every other day, and neonates were removed within 24 h. Photoperiod was set to 14h light: 10h dark cycle and temperature at 20 ± 1 ºC.

2.3 Behavioral exposure and video tracking system

Changes in swimming behaviour were quantified by determining the response of groups of first egg bearing females in the presence and absence of the tested chemical concentration. Experiments were initiated with neonates (< 24 h old) exposed until adulthood (when females carried the first clutch of eggs into their brood pouch, approx. 8 days at 20ºC) to 0.1 and 1µg/L of fluoxetine, diazepam, carbamazepine and propranolol. Previous studies indicated that the tested chemical concentrations altered reproductive and/or phototaxis (Rivetti et al.,

143 2016). Animals were exposed in groups of five individuals to the tested chemicals in 150 mL
144 of ASTM hard water at the food ration of 5 x 10^5 cells/mL of *C. vulgaris*. The same
145 concentration of ethanol 20 µL/L was used in all treatments as a carrier solvent and a solvent
146 treatment was also included. Each treatment was replicated twice. The test medium was
147 changed every other day.



148
149 Figure 1. Schematic representation of the vertical oriented four chamber behavioural device
150 showing the four optical 70 mL glass cells (A), the infrared backlight diode infrared (LED)
151 panel placed behind the cells (B), the visible LED strip on the top of the cells (C) and the
152 uEye 5246-CP-Gl-Mono-CMOS-GigE near infrared camera positioned squarely 35 cm from
153 the rack containing the experimental cells (D). Further details are described in the text.

154

155

156 An experimental setup for monitoring and recording groups of *Daphnia* individuals
157 simultaneously was designed (Fig. 1). Four optical 70 mL glass chambers (45 mm height x
158 12.5mm width x 22.5 mm depth) that were supplied by Hellma were used as exposure

chambers and assembled in an horizontal rack. An infrared backlight Elit 220 x 220 mm-IR 850-24V-5mm-emitting diode (LED) panel with a wavelength of 850 nm was placed behind the chambers to ensure homogeneous cell illumination. An anti-flicker visible LED strip (4000K) of 25 cm mounted on the top of the chambers provided uniform illumination for the video-recording changes to light stimuli. Video-tracking was recorded by an uEye 5246-CP-Gl-Mono-CMOS-GigE near infrared camera (IDS Imaging) with an optical 12 mm HR 2·2" F1.45 lend and a resolution of 1280 × 1024 pixels that was operating at 20 fps and positioned squarely 35 cm from the rack containing the experimental chambers. An IBP850 filter mounted to the camera only allowed to monitor infrared light. The visible LED strip and GigE camera were connected to a portable computer by a Mini USB-IO box and a USB 2.0 and controlled by by Ethovision XT 11.5 sofware (Noldus Information Technology, Leesburg, VA). After inserting the exposure chambers, the rack was covered with an opaque polymer mask to block external light sources and cover the exposure cell walls to limit diffusive light and reflections.

Several trails were performed consecutively. In each trail groups of five adult *Daphnia* from the experimental treatments were distributed among the four cgambers (two chambers per treatment) filled with 50 mL of ASTM. Replicated treatments were randomized across chambers. Animals were then acclimated in the dark for 5 min before video recording. The recording area of each chamber was divided by half to allow recording the relative position of animals in the vertical axis. The video tracking conditions used consisted on five 5 min cycles including a dark period followed by low light intensity (water surface: cell bottom, 84.5: 48.7 lux), dark period, high intensity (water surface: cell bottom, 2270: 1330 lux) and a final dark period. The position of each individual daphnia and the time spend on the top and bottom of the chamber was recorded using EthoVision XT 11.5 video tracking system. In each chamber, individual tracks of the five experimental animals were analysed separately

using the social module of Ethovision XT 11.5 for total distance moved (mm) and time spend in the bottom half part of each experimental chamber calculated for each dark or light period. For each of the five individuals the average distance among the remaining ones was used as a measurement of aggregation. Responses were calculated per min.

2.5 Chemical analyses

Stability of each compound during the tests was confirmed using solid-phase extraction and liquid chromatography-tandem mass spectrometry following (Rivetti et al., 2016). Duplicated water samples of freshly made and old (48 hours) test solutions were collected and pre-concentrated using Oasis HLB SPE cartridges (200 mg), conditioned with 10 mL of methanol followed by 10 mL of water. Five hundred mL of ASTM water were pre-concentrated at a flow rate of 10 ml/min and eluted with 2 x 5 ml of methanol. The eluate was then reduced under nitrogen to almost dryness and reconstituted in 500 µL of methanol. All compounds were measured using LC-ESI-MS/MS (TqDetector, Acquity Waters, USA) following a previous study reporting an analytical method for simultaneous identification of a wide range of pharmaceuticals with minor changes (López-Serna et al., 2011). Separation was performed by using a Luna C18 (150 mm×2 mm ID, particle size 5 µm, Phenomenex, Torrance, USA) equipped with a SecurityGuard pre-column. The mobile phase composition consisted of binary mixtures with 0.1% formic acid in ACN (A) and 0.1% formic acid in water (B). The gradient of elution started at 5% A , then increased to 40% A in 5 min, 60% A in 10 min, reaching 100% A in 20 min and then return to initial conditions within 5 min. The system was operated at room temperature, the flow rate was set at 200 µL min−1 and 10 µL were injected. Fluoxetine, carbamazepine, diazepam and propranolol were analysed under positive electrospray ionization mode (ESI+). Acquisition was performed in SRM mode using two transitions from [M+H]+ precursor ion to daughter ions to identify each compound. The transitions used as well as the cone voltages and collision energies were in accordance with

209 the above mentioned work (López-Serna et al., 2011). Quantification was based on external
210 calibration standard 8 point curves (range between 0.5-1000 μg/L). Limits of detection and
211 quantification (LD,LQ) defined as the minimum detectable amount of analyte with a signal to
212 noise ratio of 3:1 and 10:1, respectively, were 1.35, 4.52 ng/l for fluoxetine; 0.15,0.52 ng/l for
213 diazepam; 0.07,0.021 ng/l for carbamazepine and 0.02,0.06 for propranolol. The data were
214 acquired and processed using the MassLynx v4.1 software package.

215 **2.6 Data analyses**

216 Effects of the studied chemical treatments on measured behavioural parameters across and
217 within experimental photoperiods (dark, low light and high light intensity) were compared by
218 two way ANOVA. Further treatment differences against control treatments were assessed by
219 Dunnet's post hoc tests. Prior to analyses we ensured that the measured variables meet the
220 ANOVA assumptions of normality and/or variance homoscedasticity (Zar, 1996).
221

222 **Results**

223 **3.1 Chemical analyses**

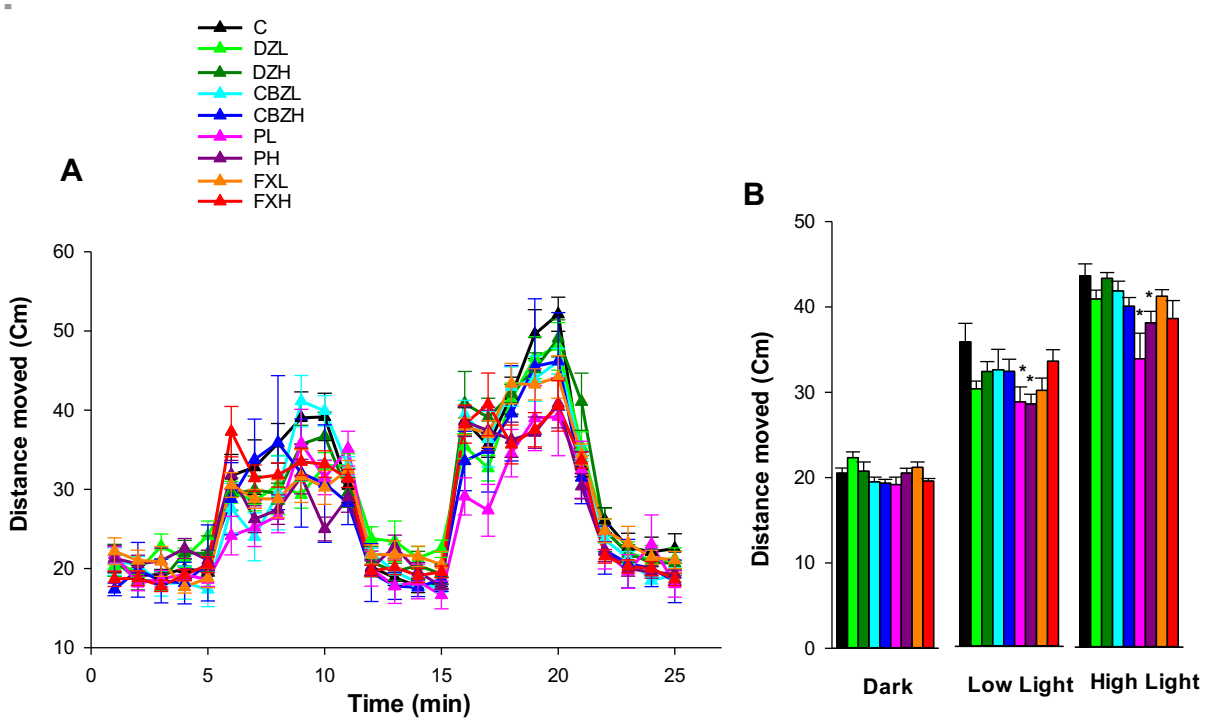
224 Measured residue levels of the tested concentrations in freshly prepared solutions (Table 1, 0
225 h) were pretty close to nominal values being in 6 out of 8 cases within 10% of nominal ones
226 and having the max deviation of 29 %. In all treatments measured concentrations of old test
227 solutions were within 14 % of freshly prepared ones (Table 1, 48 h). For the sake of clarity
228 hereafter we will refer to nominal values.

229 **Behavioural responses**

230 Results are depicted in Fig 2-4, which include temporal tracking responses of the studied
231 individuals (graphs A) and overall ones across periods of dark and light (graphs B). The

232 distance moved of experimental animals per min, which is a measure of locomotor activity,

233 increased from dark to low and high intensity lights (Fig 2A,B).



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235 Figure 2. Locomotor activity measured as the distance moved (Mean ± SE, N=10) of exposed
236 and unexposed D. magna individuals across consecutive 5 min periods of dark, low light
237 intensity, dark, high light intensity and dark. Graphs A and B depict, respectively, the
238 tracking responses each min or across periods of dark and light.*indicated significant
239 ($p<0.05$) differences from control treatments following ANOVA and Dunnetts post hoc tests.
240 C, DZP, CBZ, P, FX, L and H are respectively control, diazepam, carbamazepine,
241 propranolol, fluoxetine, 0.1 and 1 µg/L treatments.

242

243 Under exposure to light propranolol decreased the locomotion of exposed organism (Fig 2B).

244 Differences across photoperiods and of propranolol accounted for significant ($P<0.05$) effects

245 of photoperiod ($F_{2,243} = 494.1$), treatment ($F_{8,243} = 5.01$) and its interaction ($F_{16,243} = 1.88$)

246 in two way ANOVAs.

247 To analyse phototaxis we determined the cumulative time that animals remained at the bottom

248 of the chambers relative to the total (%), which showed significant effects of photoperiod ($F_{2,243} = 24.8$)

249 and treatment $F_{8,243} = 4.24$) and no interaction ($P>0.05$; $F_{16,243} = 1.24$).

Unexposed daphnids of the tested clone in darkness showed moderate levels of positive geotaxis, as 70% of time animals swam close to the bottom of the cells (Fig 3 A, B). Carbamazepine and the highest concentration of fluoxetine increased positive geotaxis. Light induced a strong negative phototaxis in all animals as the time remaining in the bottom increased, being greater in those individuals exposed to low concentrations of carbamazepine, propranolol and high concentrations of fluoxetine within the low light intensity period.

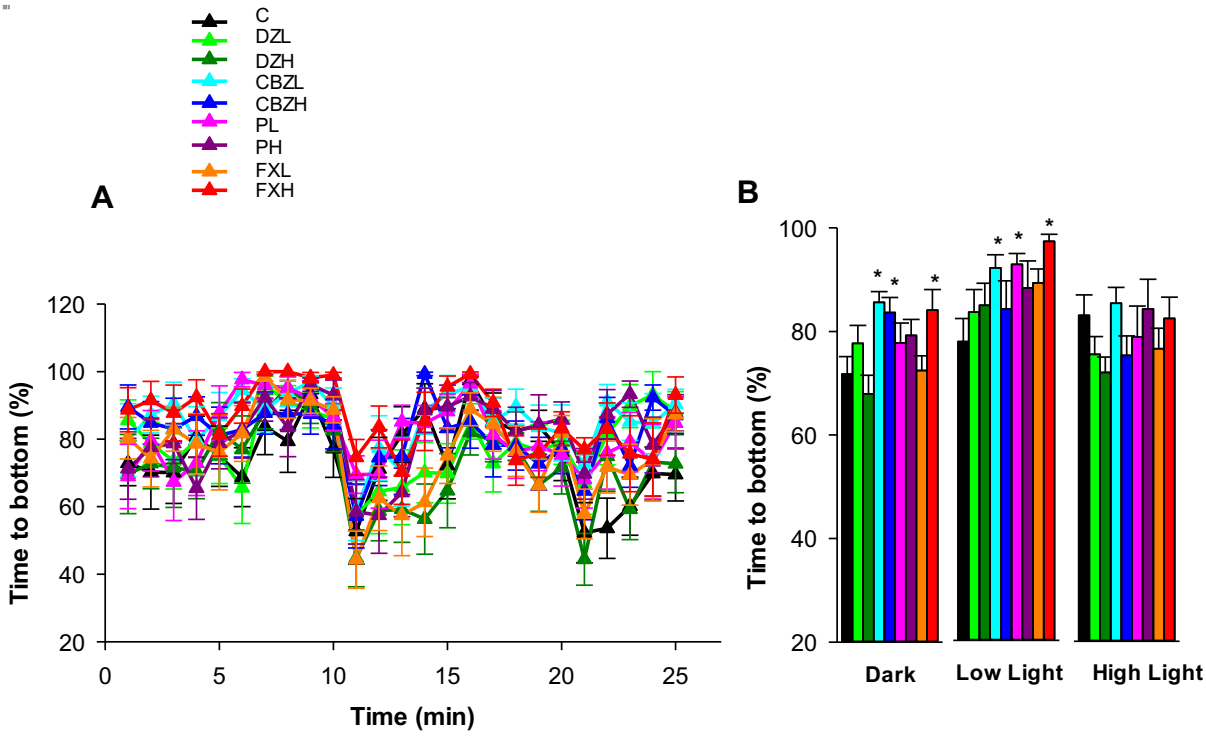

Figure 3. Phototaxis measured as the cumulative time that animals remained at the bottom of the cells relative to the total (%) (Mean ± SE, N=10) of exposed and unexposed D. magna individuals across consecutive 5 min periods of dark, low light intensity, dark, high light intensity and dark. Graphs A and B depict, respectively, the tracking responses each min or across periods of dark and light.*indicated significant ($p<0.05$) differences from control treatments following ANOVA and Dunnetts post hoc tests. Abbreviations are described in Fig 2.

The averaged distance among individuals was used as a measurement of aggregation, which decreased in unexposed daphnids from dark to high light intensity, which means that light intensity increased animal aggregation. Effect of the tested chemical concentrations on

aggregation varied across photoperiod periods. Under darkness the highest concentrations of carbamazepine, propranolol and fluoxetine increased aggregation; at low light intensity low levels of propranolol and both concentrations of fluoxetine increased aggregation; at high light intensities diazepam, high concentrations of carbamazepine and low concentrations of fluoxetine decreased aggregation.

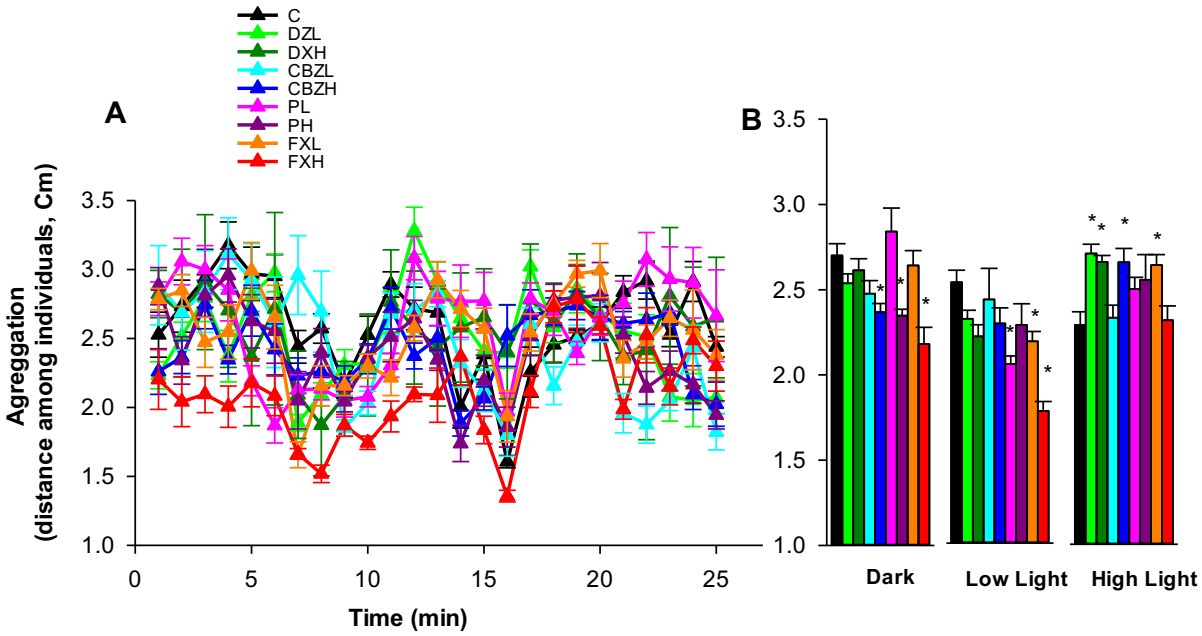

Figure 4. Aggregation behaviour defined as averaged distance among individuals (Mean ± SE, N=10) of exposed and unexposed D. magna individuals across consecutive 5 min periods of dark, low light intensity, dark, high light intensity and dark. Graphs A and B depict, respectively, the tracking responses each min or across periods of dark and light.*indicated significant ($p<0.05$) differences from control treatments following ANOVA and Dunnetts post hoc tests. Abbreviations are described in Fig 2

The previous results accounted for significant ($P<0.05$) photoperiod ($F_{2,243} = 34.29$), treatment ($F_{8,243} = 7.17$) and interaction ($F_{16,243} = 4.43$) effects.

Discussion

The results reported in this study indicated that it is possible to implement existing high-throughput video recording based behavioural platforms with vertical oriented exposure

285 chambers for continuous tracking groups of *Daphni*a behavioural responses across light and
286 dark periods. Our system was able to monitor changes in speed (distance moved), spatial
287 distribution and aggregation upon exposure to environmental relevant concentrations of the
288 four tested neuro-active chemicals. Light intensity increased speed, negative phototaxis and
289 aggregation of individuals from the tested *D. magna* clone, which is in line with previously
290 reported negative phototaxis of this and other *D. magna* clones (Cousyn et al., 2001; De
291 Meester, 1991; De Meester, 1993; Rivetti et al., 2016). Propranolol, carbamazepine and
292 fluoxetine increased geotaxis under darkness and negative phototaxis at low light intensities.
293 Propranolol and to a lesser extent the other tested drugs tent to reduce locomotor activity of
294 animals exposed to light. Increased geotaxis upon exposure to low concentrations of
295 fluoxetine (i.e. 0.1 µg/L) agrees with previous results reported in amphipods but observed
296 change in speed and phototaxis opposed (Bossus et al., 2014; Guler and Ford, 2010). In
297 amphipods fluoxetine and other selective serotonin reuptake inhibitors (SSRI) increased the
298 speed of animals under light and increased positive phototaxis (Bossus et al., 2014; Guler and
299 Ford, 2010). Rivetti et al. (2016) also found that except propranolol, the tested drugs
300 increased phototaxis in *D. magna*. Note, however, that in the previous study phototaxis was
301 calculated using 10 discrete point measurements of the position of individuals relative to a
302 higher intensity light source (500 Wm-2, which was equivalent to a 5350 lux at the surface
303 and 3220 lux at the bottom), whereas our measurements were based on a continuous 5 min
304 monitoring of the time spend in a relative position relative to a lower intensity light source
305 (48.7-84.5 lux). Indeed in our study, at the highest light intensity (1330-2270 lux), exposed
306 animals did not change their position to light relative to the control ones. Thus it is possible
307 that at even higher light intensities than those used in the present study the studied drugs
308 could act on phototaxis differently.

309 The studied light intensity ranges of the present work (40- 2270 lux) were similar to those
310 measured in an oligotrophic lake inhabited by *Daphnia* species in Central Europe from spring
311 to summer (Tilzer et al., 1995). It is well known that negative phototaxisis together with being
312 smaller at first reproduction are fish anti-predator defence *Daphnia* mechanisms, and that
313 these defences are more effective under low light intensities of 37-153 lux (Effertz and von
314 Elert, 2014; Effertz and von Elert, 2017). At higher light intensities, fish predation
315 efficiencies towards *Daphnia* preys are high and hence anti-predatory defences are less
316 effective (Tałanda et al., 2018). This means that in the present study the higher reported
317 effects at low than at high light intensity agree with reported cost-benefits of anti-predatory
318 defences.
319 Aggregation behaviour has also been shown to reduce the vulnerability to predation.
320 Predators dislike to attack aggregated prey (Allen, 1920; Neill and Cullen, 1974). Jensen et
321 al. (1999) found that light, when it was heterogeneously distributed from the surface,
322 enhanced aggregation. In our tested system light was attenuated by half from the top to the
323 bottom of the experimental cells and aggregation in control treatments increased from
324 darkness to low light intensity, thus our results agree with the previous study. On the
325 contrary, at high light intensities aggregation decreased. The observed greater dispersion of
326 individuals at high light intensity means that some animals could be situated close to the
327 surface having a positive phototaxis relative to the unexposed ones. These latter results agree
328 with the observation of Rivetti et al. (2016), which was scoring those animals not being at the
329 bottom of the water column upon exposures to high light intensities.
330 Observed behavioural responses across increasing concentrations (0.1, 1 μg/L) were not
331 always monotonic, which was the case for phototaxis of carbamazepine under low intensity
332 light and aggregation behaviour under low intensity light for propranolol and under high
333 intensity light for fluoxetine. Guler and Ford (2010) found that fluoxetine decreased negative

phototactic behaviour in the amphipod *E. marinus* in a non-monotonic manner having the greatest effects at 100 ng/l. In *Gammarus pulex* low concentrations (1-100 ng/l) of fluoxetine, carbamazepine and ibuprofen increase ventilation, whereas at high concentrations these drugs increase locomotion. (Boström and Berglund, 2015). Neurotransmitter receptors can suffer ligand-induced desensitization becoming unresponsive upon prolonged exposure to their neurotransmitter (Nicosia et al., 2003; Yamauchi et al., 2006). There is also reported information in *Drosophila* that many different receptor types are involved in the modulation behaviour of the serotonergic systems and that dysregulating the system with too match or too little serotonin influences similarly locomotor behaviour (Majeed et al., 2016). Thus, desensitisation or the innerent complexity of neurotransmitter systems could explain the reduced behaviour effect at higher concentrations.

There is an increasing number of studies that have used video tracking devices to assess changes in *Daphnia* swimming behaviour upon exposure to chemicals (Artells et al., 2013; Bahrndorff et al., 2016; Barrozo et al., 2015; Bownik et al., 2018; Cano et al., 2017; Cruzeiro et al., 2017; Chevalier et al., 2015; Ferrario et al., 2018; Häder and Erzinger, 2017; Hansen and Roslev, 2016; Huang et al., 2017; Huang et al., 2015; Liu et al., 2018; Madeira et al., 2018; Nielsen and Roslev, 2018; Nikitin et al., 2018; Noss et al., 2013; Parolini et al., 2018; Ren et al., 2017; Ren et al., 2015; Stanley et al., 2016; Yang et al., 2018; Zein et al., 2014; Zhang et al., 2016). However, only few of them reported behavioural effects at environmental relevant concentrations far below those causing any sublethal effects on stress markers or life-history traits (Nielsen and Roslev, 2018). Thus many studies may have falsely concluded that the tested chemicals have behavioral disrupting modes of action when in fact a much simpler explanation was not previously ruled out (e.g., caused systemic toxicity). This means that there is an urgent need for developing sensitive behavioral assays able to detect neurofunctional effects, which should occur at concentrations far below those causing any

toxic response. Our results together with few other studies conducted in *Daphnia* (Nielsen and Roslev, 2018) provide an example that neuro-active drugs altered behavioral responses at environmental relevant concentrations. Concentrations of 12-540 ng/L of fluoxetine, the active ingredient of Prozac, in surface waters and effluents have been found in US (Kolpin et al., 2002).Concentrations of diazepam ranging from 4 to 40 ng/l have been found in Spanish urban rivers (Valcárcel et al., 2012). Carbamazepine is fairly persistent in water and hence can be found at concentrations ranging from 1 to up to 3000 ng/l in rivers receiving waste water treatment effluents (Muñoz et al., 2009; Tixier et al., 2003). Propranolol is also quite persistent in water and can be found at 10-60 ng/l in surface water (Bendz et al., 2005; Muñoz et al., 2009).

The observed behavioural effects of the studied drugs at ng/L are likely to be related to the disruption of neurofunctional processes of the central nervous system. The mechanisms of action of the SSRI fluoxetine on *D. magna* are better known than those of the remaining tested chemicals. Fluoxetine enhances brain serotonin activity in *Daphnia* (Campos et al., 2016), increases development and reproductive rates (Campos et al., 2012) and alters phototaxis behavior. Recent studies using knockout *Daphnia* individuals lacking serotonin showed that these animals had the opposite phenotype as those exposed to fluoxetine: animals matured latter, reproduced less and were more mobile than wild type animals (Rivetti et al., 2018). There is thus a neurofunctional link between fluoxetine, its pharmacological target serotonin and effects (life-history and behavioral changes).
The pharmacological target of carbamazepine is to block voltage dependant sodium channels (Ambrósio et al., 2002), however carbamazepine also increases extracellular serotonin levels (Lamichhane et al., 2014). Accordingly, carbamazepine may also act like fluoxetine increasing serotonin activity and hence altering similarly behavioural responses to light. Diazepam decreases anxiolytic behaviour in fish and increases locomotion activity in decapod crustaceans, probably acting on GABA receptors (Ford and Fong, 2015; Whitman and Miller,

1982). Diazepam ameliorates also expressed anti-predatory life-history behaviour in *Daphnia* interacting with GABA (Weiss et al., 2012). Phototactic behaviour is an adaptive anti-predatory behaviour (Cousyn et al., 2001) and hence could be also regulated by GABA and be affected by diazepam. In our study diazepam was the tested drug having less behavioural effects but at the highest light stimuli was the compound that decreased to a greater extent aggregation, which can be interpreted as an anti-predatory strategy (Jensen et al., 1999). Propranolol not only binds to β- adrenergic receptors but also to 5-HT1 receptors in humans acting as a serotonin receptor antagonist (Tierney, 2001). There is reported information that propranolol at low concentrations (0-1,1 μg/L) inhibits *Daphnia* swimming activity (Nielsen and Roslev, 2018), which is in line with our results.

In summary the four tested neuro-active drugs affected phototactic behaviour at environmental relevant concentrations and showed a response pattern that could be explained by reported neurofunctional mechanisms. Fluoxetine and carbamazepine acted on behaviour similarly probably since both drugs may affect serotonin activity. Propranolol was the only tested drug altering significantly ($P<0.05$) locomotor activity, which was probably linked with reported antagonistic effects on serotonin receptors. Effects of diazepam were restricted to aggregation behaviour, which may be linked with its reported neurofunctional effects with GABA (Weiss et al., 2012). Reported responses were not always monotonic, which means that environmental risk assessment of pharmaceuticals need to focus in determining specific physiological effects that for neuro-active pharmaceuticals may occur at the ng/l range. This is the case for anti-depressants and probably by β-blockers (Fong and Ford, 2014; Ford and Fong, 2015; Nielsen and Roslev, 2018).

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614 Table 1. Nominal and measured (Mean ±SD) concentrations (µg/l) of the tested chemicals in
615 freshly prepared (0 h) and old (48 h) test solutions.

Chemical	Nominal	Measured (0 h)			Measured (48 h)	
		N	Mean	SD	Mean	SD
Fluoxetine	0.1	4	0.108	0.004	0.094	0.01
	1	4	1.122	0.061	1.113	0.056
Carbamazepine	0.1	4	0.112	0.01	0.098	0.01
	1	4	1.011	0.072	0.846	0.080
Diazepam	0.1	4	0.114	0.04	0.106	0.009
	1	4	1.160	0.013	0.981	0.022
Propranolol	0.1	4	0.129	0.018	0.111	0.015
	1	4	1.222	0.089	1.124	0.101

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