Population and biomarker responses of *Daphnia magna* towards anticholinesterase exposures

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*Cogent Biology* (2019), 5: 1616363
Population and biomarker responses of *Daphnia magna* towards anticholinesterase exposures

Qianping Lang¹ and Shaonan Li¹*

**Abstract:** **Context:** Cholinesterase (ChE) had long been employed for revealing environmental existence of anticholinesterases, and β-N-acetylglucosaminidase (NAGase) is a newly developed biomarker of aquatic arthropods. Still, population consequences of ChE inhibition and the consequences in terms of NAGase remained unclear. **Objective:** To quantify relationship between level of ChE and that of NAGase deliberated from chemical suppressed population of *Daphnia magna*. **Methods:** A set of macrophyte-dominated systems were established indoor to test insecticide chlorpyrifos. Antibodies were developed for quantifying content of ChE in bodies and content of NAGase in media, which was achieved by indirect-competitive and indirect-noncompetitive enzyme-linked immunosorbent assay (ELISA), respectively. **Results:** Lowest observed effect concentration (LOEC) of chlorpyrifos, as it was counted by actual concentrations, was 0.128, <0.011, 0.092, and 0.092 µg/L for population density, inherent activity of ChE, apparent activity of ChE, and content of NAGase, respectively. Corresponding to 0.90–0.48 U/µg declination in inherent activity of ChE, atrophy of −1.65 to 23% in population and that of −4.1 to 24.89% in NAGase was detected, respectively. **Conclusion:** Population impact of an anticholinesterase could be predicted, with adequate accuracy, by either ChE or NAGase. **Subjects:** Environmental & Ecological Toxicology; Pesticides; Environment & Health **Keywords:** *Daphnia magna*; chlorpyrifos; free-living NAGase; somatic ChE; polyclonal antibodies; ELISA

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**PUBLIC INTEREST STATEMENT**

Is it possible to announce dynamic of a population and impact of a chemical on that by status of a biomarker? To find an answer for that, a species of crustacean *Daphnia magna* was employed to test its responses in terms of population, somatic cholinesterase, and deliberated NAGase toward organophosphorus insecticide chlorpyrifos. Result indicated that population impact of an anticholinesterase could be predicted, with adequate accuracy, by either ChE or NAGase. Corresponding to 0.90–0.48 U/µg declination in inherent activity of ChE, atrophy of −1.65 to 23% in population and that of −4.1 to 24.89% in NAGase was detected, respectively, in this study.
1. Introduction
A biomarker is employed, in the original intention, for revealing existence of a chemical or state of an organism under the chemical exposure. To be a biomarker of eco-toxicology, it is expected to have the capacity to reveal state of an organism at the level of population (Jemec, Drobné, Tišler, & Sepčić, 2010; Lam, 2009).

Cholinesterase (ChE) had long been used for indicating existence of anticholinesterases (e.g. organophosphorus and carbamate insecticides) in environmental components (Choi et al., 2011; Denoyelle, Rault, Mazzio, Mascle, & Capowiez, 2007; Gad, 2009; Kanno et al., 2010; Kawakami et al., 2008; Kirby et al., 2000; Mena et al., 2014). In spite of that, consequences of ChE inhibition had not yet been quantified at the level of population. One of the difficulties to do so is the demand for eliminating the interference caused by accelerated synthesis of ChE in animals being exposed to anticholinesterases (Xing et al., 2013) and the need for keeping the systems steady. To solve problem of the first, antibodies should be developed and then employed for determining inherent activity of somatic ChE being exposed to the chemicals.

β-N-acetylglucosaminidase (EC 3.2.1.52), also named chitobiase, NAGase, or β-N-Acetylhexosaminidase, is one of the chitinolytic enzymes that are responsible for breaking down of chitin in process of moult of arthropods (Cohen, 2010; Yebra, Kobari, Sastri, Gusmão, & Hernández-León, 2017). With respect to the aquatic arthropods, the NAGase is released, together with other digestive enzymes, into body of water at end of each moult, and the more in number and size of the animal, the greater in amount of the NAGase to be released (Sastri & Dower, 2006). This character makes it possible for man to link the free-living NAGase with biomass of aquatic arthropods (Avila, de Souza Machado, & Bianchini, 2012; Hanson & Lagadic, 2005; Sastri & Dower, 2009, 2006; Sastri, Juneau, & Beisner, 2013; Sastri et al., 2012; Suchy, Avila, Dower, Bianchini, & Figueiredo, 2016; Yebra et al., 2017).

Although the free-living NAGase had widely been used for assessing biomass of aquatic arthropods, the enzyme had seldom been used for determining population impact of a chemical. Qi et al. (2013) and Duchet et al. (2011) studied impact of some insecticides (i.e. spinosad, diflubenzuron, and guadipyr) on daphnids, and showed declinations in terms of activity of free-living NAGase along with declinations in terms of the population. In spite of that knowledge about patterns of NAGase in announcing population impact of a chemical was still lacking.

Different from the ChE, which responses to anticholinesterases only, the NAGase responses to all kinds of chemical provided the latter have the capacity to change biomass of a population. A shortcoming of the NAGase, however, is its multi in sources. NAGases from groups such as bacteria (Vrba, Šimek, Nedoma, & Hartman, 1993), protozoan (Baldwin and Bowers, 1995; Zubkov & Sleigh, 1998), and diatoms (Vrba et al., 2004, 1997) might disturb the biomass determination. Moreover, the NAGase, as it is expressed by catalytic activity, may over- or underestimatated the content of NAGase in case the chemical involved acted as a direct inhibitor (or stimulator) of the enzyme. In view of these, antibodies of NAGase should be developed and then employed for quantifying content of the enzyme in media.

Daphnia magna is a type of species that belongs to Phylum Arthropoda, Class Crustacea, Order Cladocera, and Family Daphniidae. The short life-span and high rate of reproduction makes D. magna a potential species for exploring population sequences of chemical exposures. Chlorpyrifos was taken in this study as test substance. To be one of the most commonly used insecticides, population impacts of chlorpyrifos had been studied both indoor and outdoor (Daam, Crum, Van Den Brink, & Nogueira, 2008; Daam & Van Den Brink, 2007; Liu, Liu, Zhao, Sen, & Zhu, 2013; López-Mancisidor, Carbonell, Marina, Fernández, & Tarazona, 2008; Van Wijngaarden, Brock, & Douglas, 2005; Xiao, Liu, Yao, & Zhu, 2017). In this study, population impact of chlorpyrifos was tested by indexes, such as population density, inherent and apparent activity of ChE, and immuno-reactive content of NAGase. Aims of the study were: (1) To quantify sensitivity of indexes such as population density, inherent activity of ChE, apparent activity of ChE, and immuno-reactive content of
NA\text{G}ase in determining population impact of an anticholinesterase; (2) To determine relationships between state of the biomarkers and that of the population.

2. Materials and methods

2.1. Chemicals

2.1.1. Test substance
Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate): technical grade material from XinNong Chemical Engineering Company Limited with purity of 97% (w/w).

2.1.2. Reagents
Chlorpyrifos standard: Merck Life Science (Shanghai) Co. Ltd, China (Sigma-Aldrich-31,553).
Bradford Protein Assay Kit: Sangon Biotech (Shanghai) Co. Ltd. (CS03031-1001). Amplex\textsuperscript{\textregistered} Red Acetylcholine/Acetylcholinesterase Assay Kit: Invitrogen\textsuperscript{TM}, USA (A12217). Propionylthiocholine iodide: Merck Life Science (Shanghai) Co. Ltd., China (Aldrich-P2880). Bradford Protein Assay Kit: Sangon Biotech (Shanghai) Co., Ltd. (CS03031-1001). Goat anti rabbit Immunoglobulin G linked with horse radish peroxidase (IgG-HRP): HuaBio\textsuperscript{®} Co. Ltd., Hangzhou, China (HA1001). Tetramethyl benzidine (TMB): Merck Life Science (Shanghai) Co. Ltd. (Sigma-T5513). 4-Methylumbelliferyl N-acetyl-\beta-D-glucosaminide (4-MUF-NAG): Merck Life Science (Shanghai) Co. Ltd (Sigma-69585). Hydrolyzate 4-methylumbelliferyl (4-MUF): Merck Life Science (Shanghai) Co. Ltd. (Aldrich-M1381).

2.2. Test species
\emph{D. magna} (hereinafter was addressed as daphnid) was provided by the Chinese Center for Disease Control and Prevention, Beijing, China. The group was raised in fully defined Elendt M\textsubscript{4} medium (OECD, 2012) and fed with planktonic algae \emph{Chorella vulgaris} (hereinafter was addressed as algae). The population was kept in temperature of (22 ± 1)\textdegree C and light intensity of 1,500–2,500 lx. A cycle of 16 h light to 8 h dark was provided.

2.3. Test systems
The test systems were established in glass vessels of 30 cm high and 12.5 cm in diameter that could accommodate dilutions of 10 L. Shoots of hornwort \emph{Ceratophyllum demersum} of about 30 cm long were introduced at quantity of 35 per system. The hornwort was introduced to prevent overgrowth of algae (Hilt & Gross, 2008), which might have happened latter of the test. To take the date of insecticide administration as the day of 0, the hornworts were introduced at day of −22 together with diluent (i.e. M\textsubscript{4} medium) and the algae. For each system, inoculum of the algae of $7 \times 10^6$ cells/mL was introduced at volume of 5 mL. The daphnids were introduced at day of −20. Individuals of 60, with 20 of them longer than 1.5 mm (adults) and 40 of them shorter than 1.5 mm (neonates), were introduced for each system. For maintaining growth of the daphnids, the algae were supplemented at 3-d interval. The systems were kept in temperature of (25 ± 1)\textdegree C and light intensity of 2,000–3,000 lx, with a light cycle of 16 h light to 8 h dark. The diluent was replenished, whenever necessary, to compensate the losses due to evaporation and samplings.

2.4. Insecticide administration
The technical grade insecticide was prepared into a stock solution of 212 mg/L by acetone. The stock solution was further diluted by acetone to make working solution of 21.2 and 2.12 mg/L. All of them were spiked into systems to make dilutions with nominal concentration of 0.02, 0.10, 0.50, 2.50, and 12.50 μg/L, respectively. Three parallels were set for each of the five concentrations and for CK (i.e. the treatment free from insecticide administration). Totally, 18 systems were therefore employed in this study. Steps were taken to insure each of the systems (with diluent of 10 L) received the same volume of acetone, i.e. 588 μL.
2.5. Sampling
Dilution samples were collected with a sampler recommended by American Society for Testing and Materials (ASTM, 2016). Schedules of the samplings are listed in Table 1. For purpose of compensating dilution losses, systems were replenished immediately after each sampling.

2.6. Sample measurements

2.6.1. Population densities
The daphnids in sample were counted directly if they were in proper density. If not, they were filtrated before being counted. The adults and neonates were counted separately. After counting, adults of at least 7 were collected for measuring activity and immune-reactive content of ChE and content of total protein. Rest of the daphnids were returned.

2.6.2. Insecticide residues
Animal-free dilutions were filtered by 0.22 μm cellulose membrane (Bonna-Agela Technologies, TianJing, China) for removing of bacteria, algae, diatoms, protozoan, and small particles of other sources (Vrba & Machácek, 1994). Solid phase extraction (SPE) columns of Supelclean ENVI-18 of 500 mg (Sigma-Aldrich) were employed to extract residues of the insecticide in dilutions (Dong & Dong, 2008). Prior to the extraction, either NaOH or HCl of 0.1 mol/L was added to adjust the pH into 7, and the column was preconditioned in turn with 5 mL ethyl acetate, 5 mL acetone, and 10 mL de-ionized water. Dilution was loaded and let go at speed of 1 mL min⁻¹ or so. The moisture in column was removed thoroughly under reduced pressure. The column was then washed with ethyl acetate. All above operations were performed with AP-01D SPE vacuum manifold (Automatic Science Instrument Co., LTD, Tianjing). The effluent was collected for insecticide residue analysis.

The insecticide residue analysis was conducted with a Shimadzu GC-2010 Plus equipped with an electron capture detector (ECD) and a capillary column of HP-50 (30 m × 0.32 mm × 0.25 μm). The temperature of the injection port and that of the detector was set to be 240°C and 250°C, respectively. The temperature of the column was set to be 150°C, 0-2 min after sample injection and it was made to rise to the point of 190°C at the rate of 15°C/min. One minute after that the temperature of the column was made to rise to the point of 240°C at the rate of 5°C/min. Sample was injected at splitless mode. Injection volume was 1 μL. Nitrogen (in purity of 99.999%) was given as carrier with constant pressure of 15 psi. Detection limit and retention time of chlorpyrifos was found to be 1 pg and 8.05 min, respectively. Recovery test showed that at the rate of 10~1,000 μg/L the fortified chlorpyrifos recovered from 98.1% to 107.6% with standard deviation from 0.4% to 7.2%.

2.6.3. Somatic ChE
In presence of 0.7 mL ice cold 50 mmol/L Tris-HCl (pH 7.50) that contained 0.25% (W/V) Triton X-100 and 1 mmol/L EDTA, adults of at least 7 were homogenized in a 2-mL centrifuge tube using a PRO 200 homogenizer (PRO Scientific, USA). The homogenates were let to go through 30 min centrifugation at 10,000 r/min. The supernatant was taken as source of enzyme for measuring total activity and immuno-reactive content of ChE, as well as content of total protein.

| Purpose                                      | Volume (mL/system) | Day after insecticide administration |
|----------------------------------------------|--------------------|--------------------------------------|
| For insecticide residue analysis             | 100-500            | 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39 |
| For measuring content of NAGase              | 20                 | −12, −9, −6, −3, 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39 d |
| For animal counting and ChE activity measures | 100-500            | −12, −9, −6, −3, 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39 |

Table 1. Sampling schedules
2.6.3.1. Total activity. In view of the low content of ChE in daphnids, instead of using the method innovated by Ellman et al. (1961), total activity of ChE was measured by a sensitive method recommended by Zhou, Zhang, Richard, and Haugland (2000) in which the substrate acetylcholine was converted by ChE to choline, which was in turn oxidized by choline oxidase to betaine and H$_2$O$_2$, the latter of which, in the presence of horseradish peroxidase (HRP), reacted with Amplex Red reagent 10-acetyl-3,7-dihydroxyphenoxazime (ADHP) in a 1:1 stoichiometry to generate the highly fluorescent product resorufin. Measurements were performed with Amplex® Red Acetylcholine/Acetylcholinesterase Assay Kit excepting that PTCh was used instead of acetylcholine chloride (Component G) (Diamantino, Almeida, Soares, & Guilhermino, 2003; Liu, Yuan, & Li, 2016). Samples and negative control of 100 μL was added into separate wells of a flat-bottomed high binding black polystyrene microplate (Corning Inc., USA). Reactions were started by adding working solution of 100 μL that contained 400 μM Amplex Red reagent, 2 U/mL HRP, 0.2 U/mL choline oxidase, and 100 μmol/L PTCh. After 30 min incubation at room temperature (i.e. 25°C) in dark, the fluorescence was measured by a GEMINI XPS fluorescence microplate reader (Molecular Devices, USA) at excitation wavelength of 530 nm and emission wavelength of 595 nm, respectively. Background fluorescence for each of the points was correct by negative control in which 50 mmol/L Tris-HCl of pH 8.0 was added instead of samples. Activity of ChE in samples was quantified by acetylcholinesterase enclosed in Kit (Component H).

2.6.3.2. Total protein content. Total protein content in supernatant was determined by the method innovated by Bradford (1976). Measurements were performed by Bradford Protein Assay Kit: To each well of a microtiter plate (JET BIOFIL®, Guangzhou), supernatant of 20 μL and working solution of Bradford of 200 μL were added. After 5-min incubation at 25°C, OD$_{595}$ was read from a VERSA max microplate reader (Molecular Devices, USA). The OD$_{595}$ for samples was corrected by that for negative control in which the phosphate buffer (1×PBS) was added instead of the supernatant. The standard curve was established by seriously diluting bovine serum albumin (BSA) of 5 mg/mL from 150 to 10 μg/mL (n = 7) with 1×PBS. Plot OD$_{595}$ against concentration of BSA, an equation of $y = 0.0021x + 0.5664$ was set with $R^2 = 0.9985$.

2.6.3.3. Immuno-reactive content of ChE. Polyclonal antibodies were employed for measuring immuno-reactive content of ChE in rude enzyme. The antibodies were developed against a type of ChE of about 84 kDa (Lang, 2016). The enzyme was purified from whole body of D. magna by a 3-step procedure, i.e. crude enzyme extraction, ammonium sulphate precipitation, and DEAE-Sepharose™-Fast-Flow chromatography (Yang, Niu, & Li, 2013). It was then delivered to Huabio® Co. Ltd., Hangzhou, China for producing of antibodies. A type of rabbit immunoglobulin G (IgG) of 3.35 mg/mL was finally gained. It was then employed for measuring content of ChE in supernatant by a protocol of indirect and competitive enzyme linked immunosorbent assay (ELISA).

For purpose of determining optimal dilutions of antigen and antibodies, the purified ChE was serially diluted from 13.19 to 0.50 μg/mL (n = 8) with dilution buffer, i.e. phosphate buffer of 10 mmol/L (pH 7.4). To each well of a microtiter plate (JET BIOFIL®, Guangzhou), dilution of the purified ChE of 100 μL was added. After 2-h incubation at 37°C, the plate was washed three times with washing buffer, i.e. phosphate buffer of 10 mmol/L that spiked with Tween-20 of 0.1%. The plates were then blocked by skimmed milk of 2.0% at quantity of 200 μL/well. After 0.5 h incubation at 37°C, the plate was washed with the washing buffer. The IgG was serially diluted from 1.675 to 0.21 μg/mL (n = 8) with skimmed milk of 2.0%. To each well of the plate, the purified ChE of 50 μL was added together with the antibodies of 50 μL. After 1 h incubation at 37°C, the plate was washed with the washing buffer. IgG-HRP was diluted 5,000 times with skimmed milk of 2.0%. To each well of the plate, it was added at quantity of 100 μL. After 1 h incubation at 37°C, the plate was washed with the washing buffer. To each well of the plate, TMB was added at quantity of 100 μL. After 15 min incubation at 37°C, sulphuric acid of 2 mol/L was added at quantity of 50 μL. OD$_{450}$ was read from a VERSA max microplate reader (Molecular Devices, USA). At IgG concentration of 0.41875 μg/mL and ChE concentration of 5.277 μg/mL, the OD$_{450}$ was determined to be 0.998.
For standard curve establishment, the purified ChE was diluted from 2.19 to 0.03 μg/mL (n = 7) with the dilution buffer. The OD$_{450}$ was tested at IgG concentration of 0.41875 μg/mL. Plot percentage inhibition in OD$_{450}$ against concentration of ChE, an equation was established as \( y = 94.2641 - 29.9902x \) with \( R^2 = 0.9936 \).

For testing of samples, the IgG was employed at concentration of 0.42 μg/mL. Result of the test was corrected by negative control in which the dilution buffer was added instead of the supernatant.

2.6.3.4. Specific activity. Apparent activity of ChE was obtained by dividing total activity of ChE with total protein content. Inherent activity of ChE was obtained by dividing total activity of ChE with immuno-reactive content of ChE (Liu, Yuan, & Li, 2012).

2.6.4. Free-living NAGase
Water samples were filtered with cellulose membrane (Bonna-Agela Technologies, TianJing, China) of 0.22 μm according to Vrba and Macháček (1994). The filtrate was taken as source of the enzyme for testing immuno-reactive content of NAGase. The filtrate was concentrated with 3 kD millipore ultrafiltration tubes (Shanghai Root Biological Technology Company) in case the NAGase content was too low to be tested.

2.6.4.1. Activity. A fluorospectrophotometric method was employed to test the total activity of NAGase in filtrates (Hanson & Lagadic, 2005). To start the procedure, the filtrate was added to a well of a 96-well polystyrene plate (JET BIOFIL®, Guangzhou, China) at quantity of 150 μL. Citrate-phosphate buffer of 0.15 mol/L (pH = 5.5) containing 0.31 mmol/L 4-MUF-NAG and ethylene glycol monomethyl ether of 6.2% (w/w) was then added at quantity of 50 μL. The mixture was incubated at 25°C for 1 h. The reaction was terminated by adding 0.25 mol/L NaOH of 50 μL. The NaOH were added synchronously the substrate and in negative control. Fluorescence from enzymatic decomposition of the substrate was measured using a GEMINI xps fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm, respectively.

For establishing of standard curve, stock solution of 4-MUF of 5 mmol/L in ethylene glycol monomethyl ether was seriously diluted from 0.5 to 0.001 mmol/L (n = 10) with citrate-phosphate buffer of 0.15 mol/L (pH = 5.5). Plot response of the microplate reader against concentration of the 4-MUF, an equation of \( y = 221196x + 680.98 \) was obtained with \( R^2 = 0.9981 \).

2.6.4.2. Immuno-reactive content of NAGase. Polyclonal antibodies were produced for measuring immuno-reactive content of NAGase in filtrate. The antibodies were developed against a type of NAGase of electrophoretic purity. The enzyme was purified from whole body of \( D. \) magna by a 4-step procedure, i.e. crud enzyme extraction, ammonium sulfate precipitation, DEAE Sephadex A-25 chromatography, and Sephadex G-150 chromatography (Zeng & Li, 2013), and it was then delivered to HuaBio® Co. Ltd., Hangzhou, China for producing of antibodies. A type of rabbit antiserum was finally gained. It was then employed for measuring content of NAGase in filtrated media by a protocol of indirect and non-competitive ELISA.

For purpose of determining the optimal dilutions of antigen and antibodies, the purified NAGase was serially diluted from 25.2 to 0.375 μg/mL (n = 8) with dilution buffer, i.e. phosphate buffer of 0.01 mmol/L (pH 7.4). To each well of a microtiter plate (JET BIOFIL®, Guangzhou), dilution of the purified NAGase of 100 μL was added. After 2-h incubation at 37°C, the plate was washed three times with washing buffer, i.e. phosphate buffer of 10 mmol/L spiked with Tween-20 of 0.1%. The plates were then blocked by skimmed milk of 2.0% at quantity of 200 μL/well. After 0.5-h incubation at 37°C, the plate was washed with the washing buffer. The antiserum was serially diluted from 1:1,000 1:14,000 (n = 8) with skimmed milk of 2.0%. To each well of the plate, the
diluted antiserum was added at quantity of 100 μL. After 1 h incubation at 37°C, the plate was washed with the washing buffer. IgG-HRP was diluted 5,000 times with skimmed milk of 2.0%. To each well of the plate, it was added at quantity of 100 μL. After 1-h incubation at 37°C, the plate was washed with the washing buffer. To each well of the plate, TMB was added at quantity of 100 μL. After 15 min incubation at 37°C, sulphuric acid of 2 mol/L was added at quantity of 50 μL. OD_{450} was read from a VERSA max microplate reader (Molecular Devices, USA). At antiserum dilution of 1:6,000 and NAGase concentration of 8.961 μg/mL, the OD_{450} was found to be 0.998.

For standard curve establishment, the purified NAGase was diluted from 34.7 to 0.2 μg/mL (n = 7) with the dilution buffer. The OD_{450} was tested at antiserum dilution of 1:6,000. Plot OD_{450} against concentration of the ChE, an equation was established as $Y = 1.2864 \times (1 - e^{-2.1191x})$ with $R^2 = 0.9914$.

For testing of samples, the antiserum was employed at dilution of 0.1:6,000. Result of the test was corrected by negative control in which the dilution buffer was added instead of the filtrated medium.

2.7. Data processions
Analysis of variation (ANOVA) and the related multiple comparisons (Tukey’s test) and regression analysis were performed using DPS® (Tang, 2013). Constrained ordination and partial redundancy analysis (RDA) were performed using CANOCO 5 (Ter Braak & Šmilauer, 2012). The numeric data used for the ordinations had been log transformed to down weight high abundance values and to approximate log-normal distribution of data (Choung et al., 2013; Daam & Van Den Brink, 2007; Zafar, van Wijngaarden, Roessink, & van Den Brink, 2011).

3. Results
3.1. Insecticide exposures
Samples were collected at pre-determined interval as shown in Table 1 for measuring actual concentration of chlorpyrifos in dilutions. Result of the measurement is listed in Table 2. One can see from Table 2 that measured concentrations of chlorpyrifos dissipated quickly in dilutions from 0 to 39 d. The quick dissipations of the insecticide may due to processes, such as evaporation, adsorption, degradation, and samplings. One can see from Table 2 that the measured concentrations were 23–30% lower than the nominal ones at Day 0. This might be the outcome of absorption by periphytons and hornwort.

3.2. Responses
3.2.1. Population
Samples were collected every 3 days for counting number of daphnids in each of the systems. The results were diagrammed in Figures 1 and 2. One can see from Tables 1 and 2 that both the neonates and adults amplified gradually from −12 to 39 d in media free from insecticide administration. The neonates amplified from the minimal of 61.0 number/L at −12 d to the maximal of 141.3 number/L at 33 d, while the adults amplified from the minimal of 12.0 number/L at −12 d to the maximal of 70.7 number/L at 15 d. The change was far from violent. Generally speaking, the systems involved were in steady.

To take density of both adults and neonates as dependent variables and concentration and time as explanatory variables to perform constrained ordination and then to perform partial RDA. Result showed that the explanatory variables could account for variation of the dependent variables of 99.9%. The principal response curves (PRC) was established indicated that the effects of “Dose” (including its interaction with “Day”) were significant, according to Monte Carlo permutation test ($F = 158,380, P = 0.002$). Judged by the value of species score (i.e. dependent variable scores), which was −1.0747 for neonates and −0.9192 for adults, respectively, the neonates were more sensitive than the adults.
Table 2. Measured concentration of chlorpyrifos in dilutions

| Days after dose administration | 0.02  | 0.10  | 0.50  | 2.50  | 12.50 |
|-------------------------------|-------|-------|-------|-------|-------|
| 0                             | 0.014 | 0.077 | 0.371 | 1.908 | 9.631 |
| 3                             | 0.011 | 0.040 | 0.206 | 1.123 | 5.590 |
| 6                             | 0.009 | 0.020 | 0.098 | 0.641 | 2.127 |
| 9                             | 0.007 | 0.010 | 0.059 | 0.251 | 1.053 |
| 12                            | ND    | 0.007 | 0.027 | 0.090 | 0.594 |
| 15                            | ND    | ND    | 0.016 | 0.061 | 0.423 |
| 18                            | ND    | ND    | 0.011 | 0.038 | 0.262 |
| 21                            | ND    | ND    | 0.008 | 0.013 | 0.107 |
| 24                            | ND    | ND    | ND    | 0.010 | 0.053 |
| 27                            | ND    | ND    | ND    | 0.008 | 0.024 |
| 30                            | ND    | ND    | ND    | 0.008 | 0.024 |
| 33                            | ND    | ND    | ND    | ND    | 0.010 |
| 36                            | ND    | ND    | ND    | ND    | ND    |
| 39                            | ND    | ND    | ND    | ND    | ND    |

*Data listed in table are result of measurements from pooled samples. ND: Not detectable, i.e. lower than 0.01 μg/L.
3.2.2. Somatic ChE

Adults were collected from samples for measuring activity and immuno-reactive content of ChE and content of total protein. The activity was divided by immuno-reactive content and by total protein to obtain inherent and apparent activity of ChE, respectively. The outcomes were diagrammed in Figures 3 and 4, respectively. One can see from the two figures that the chlorpyrifos had no induction on inherent activity of ChE but apparent activity of ChE was induced by the insecticide at lower concentrations.

To take both inherent and apparent activity of ChE as dependent variables, time and concentration as explanatory variables to perform constrained ordination and then to perform partial RDA. Result indicated that the explanatory variables could account for variation of the dependent variables of 99.1%. The PRC thus established indicated that the effects of “Dose” (including its interaction with “Day”) were significant ($F = 19.689, P = 0.002$). Judged by the value of dependent
variable score, which was $-1.4140$ for inherent activity and $-0.0266$ for apparent activity of ChE, respectively, the inherent activity was much more sensitive than the apparent activity of ChE.

3.2.3. Free-living NAGase
Dilution samples were collected every 3 days for measuring immuno-reactive content of NAGase in them. Results of the measurement were diagrammed in Figure 5. One can see that the content of NAGase in dilutions decreased at higher concentrations and in early stage of exposure. The content of NAGase increased from the minimal of $0.656\, \mu g/mL$ at $-12\, d$ to the maximal of $1.92\, \mu g/mL$ at $36\, d$ in media free from insecticide administration. The change was not violent. Generally speaking, the systems employed were in steady.

4. Data analysis and discussions

4.1. NOEC, LOEC, and MATC
For determining the values of no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), and maximal allowable toxicant concentration (MATC) of the insecticide, ANOVA and the followed multiple comparisons were performed time by time for each of the test indexes. Result of the comparisons is listed in Table 3. One can see from Table 3 that among 78 samples collected from 3 to 39 d, 28, 40, 28, and 24 of them were different from control as they were counted by population density, inherent activity of ChE, apparent activity of ChE, and immuno-reactive content of NAGase, respectively. One can also see from Table 3 that the LOEC was $0.50$, $<0.02$, $0.50$, and $0.50\, \mu g/L$, respectively.

To take data enclosed in Table 2 into calculation, one could figure out time-weighted average (TWA) up to the date the impact lasted. TWA-based NOEC, LOEC, and MATC could then be determined for each of the test indexes. One can see from Table 4 that TWA-based LOEC of chlorpyrifos was $0.128$, $<0.011$, $0.092$, and $0.092\, \mu g/L$, respectively, for population density, inherent activity of ChE, apparent activity of ChE, and immuno-reactive content of NAGase.
4.2. Sensitivity of the biomarkers

As shown in Table 4, LOEC for inherent activity of ChE, apparent activity of ChE, and immuno-reactive content of NAGase was <0.04, 1, and 1 time as much as that for population density in case it was counted by nominal concentrations, and value was <0.086, 0.719, and 0.719 time as much as that for population density in case it was counted by actual concentrations. This indicated that the inherent activity of ChE was most sensitive among the three in announcing population impact of chlorpyrifos. Previous study conducted by Liu et al. (2012) proofed that inherent activity of ChE was more sensitive than apparent one in detecting chemical existence of anticholinesterases. Result of our study proofed that the inherent activity was more sensitive in detecting population level impact of an anticholinesterase.

Judged by the NOEC and LOEC as shown in Table 4, immuno-reactive content of NAGase was not so sensitive as inherent activity of ChE, whereas it was sensitive enough for announcing population level impact of the chemical.

4.3. Population impact of the insecticide

Population impacts of chlorpyrifos had been studied either indoor or outdoor in past few years. Enclosed in Table 5 are some of the results. One can see from Table 5 that studies conducted by Liu et al. (2013) and Xiao et al. (2017) gave nominal LOEC of <5 and <2.3 µg/L, respectively, and the other four studies gave the LOECs from 0.05 to 10 µg/L. The nominal LOEC of 0.5 µg/L determined in this study was just in range of above.
Although the chemical analysis was performed in all of the six studies listed in Table 5, only those conducted by López-Mancisidor et al. (2008), Daam and Van Den Brink (2007), and Van Wijngaarden et al. (2005) gave information of initial concentration and half-life that enabled us to calculate the concentration at a time (i.e. $C_t$) and TWA-based LOEC. The latter was determined to be 0.121, <0.033, 0.092 µg/L, respectively, as shown in Table 5. These values are comparable with the TWA-based LOEC of 0.128 µg/L determined in this study. In addition to that, outdoor studies conducted by Zafar et al. (2011) revealed TWA-based LOECs of <0.098, <0.116, and <0.126 µg/L. All these suggested that the systems we employed was sensitive enough for testing population impacts of an insecticide.

### 4.4. Risk assessment

For purpose of determining risk quotient (RQ) (Sumon, Rashid, Peeters, Bosma, & Van Den Brink, 2018) of chlorpyrifos, GENEEC2 was employed for determining surface water contamination. The GENEEC2 was employed because it was found to give outcomes comparable to those obtained from field investigations (Wu et al., 2011). Result of the simulation gave peak value of 5.70 and 4-d, 21-d, 60-d, and 90-d average of 5.48, 4.22, 2.55, and 1.88 µg/L, respectively. All these values are much higher than the value of NOEC, LOEC, and MATC obtained in this study (i.e. 0.026, 0.128, or 0.058 µg/L). This suggested that the chlorpyrifos would be Liu harm to the population of daphnid if it was applied for rice pests (e.g. planthopper) control at recommended rate of 720 g/ha.

### 4.5. Correlations between population and biomarkers

#### 4.5.1. Somatic ChE

To plot a population against activity or rate of change in activity of ChE, a set of scatter diagrams are obtained as Figure 6. Equations corresponding to them are listed in Table 6.

| Test indexes                        | Population density | Inherent activity of ChE | Apparent activity of ChE | Immuno-reactive content of NAGase |
|-------------------------------------|--------------------|--------------------------|--------------------------|-----------------------------------|
| NOEC (µg/L)                         | Nominal            | 0.10                     | ?                        | 0.10                              |
|                                     | TWA               | 0.026                    | ?                        | 0.019                             | 0.019 |
| LOEC (µg/L)                         | Nominal            | 0.50                     | <0.02<sup>b</sup>        | 0.50                              |
|                                     | TWA               | 0.128                    | <0.011<sup>a</sup>       | 0.092                             | 0.092 |
| MATC<sup>c</sup> (µg/L)             | Nominal            | 0.224                    | ?                        | 0.224                             |
|                                     | TWA               | 0.058                    | ?                        | 0.042                             | 0.042 |
| Time lasting for the effect<sup>d</sup> (d) | Up to 12         | Up to 6                  | Up to 18                  | Up to 18 |

<sup>a</sup>TWA is equal to the sum of the portion of each time period multiplied by the level of chemical during the time period divided by the period up to which the effect lasts. The level of chemical during the time period is counted as geometric average in that period.

<sup>b</sup>This value is indefinite because no test had been performed with nominal concentration lower than 0.02 µg/L.

<sup>c</sup>MATC is the geometric average of NOEC and LOEC.

<sup>d</sup>According to Van Den Brink et al. (2009), the impact could not be recognized unless it lasted for a period of two successive times or more.
| No. | Condition | Population or community in concern | Nominal NOEC/LOEC (μg/L) | Time lasting for the effect | TWA-based NOEC/LOEC (μg/L) | Reference |
|-----|-----------|-----------------------------------|--------------------------|---------------------------|----------------------------|------------|
| 1   | Outdoor   | Moina micrura                     | 0.1/1                    | 9 weeks                   |                            | Daam et al., 2008 |
| 2   | Outdoor   | Daphnia group galeata             | 0.1/1                    | 43 d                      | 0.0144/0.121               | López-Mancisidor et al., 2008 |
| 3   | Indoor    | Cladocera community\(^{a}\)       | ?/<5                     | >35 d                     |                            | Liu et al., 2013   |
| 4   | Indoor    | Simocephalus sp.                  | ?/<2.3                   | >56 d                     |                            | Xiao et al., 2017 |
|     |           | Scapholeberis sp.                 | ?/<2.3                   |                           |                            |             |
| 5   | Indoor    | Simocephalus vetulus              | 0.005/0.05               | >14 d                     | /<0.0326                  | Daam & Van Den Brink, 2007 |
|     |           | Chydorus sphaericus               | 0.005/0.05               |                           |                            |             |
| 6   | Indoor    | Daphnia group galeata             | 1/10                     | >35 d                     | 0.0551/0.551              | Van Wijngaarden et al., 2005 |
|     |           | Simocephalus vetulus              | 0.1/1                    | 21 d                      | 0.00919/0.0919            |             |
|     |           | Daphnia group galeata             | 0.1/1                    | >28 d                     | 0.00735/0.0735            |             |

\(^{a}\)A community that consisted of Simocephalus sp., Moina sp., Chydorus sp., and Alona sp.
To plot a population against inherent activity instead of apparent activity of ChE, the equations obtained were improved as well (to compare Equation A with B, Equation C with D, Equation E with F, and Equation G with H in Table 6).

From practical point of view, both Equation G and H could be used for population dynamic prediction, and judged by the value of $R^2$ and $P$, Equation G was superior to Equation H. Look into the scatterplot as shown in Figure 6(g), one can see atrophy of $-1.65$ to $23\%$ in terms of the population along with declination of $0.90-0.48$ U/$\mu$g in terms of the inherent activity of ChE. This gave the limit of $23\%$ beyond which declination in terms of the rate in production in population could not be predicted with adequate accuracy by ChE.

An amplification of $1.65\%$ was sighted in population being exposed to the lowest concentration of $0.02$ $\mu$g/L (Figure 6(e)). We would rather regard it as a true amplification than regard it as a testing error because the value of $1.65\%$ was an average of $3 \times 7 = 21$ measurements. Amplification with respect to the population can be regard as a type of hormesis in presence of outside stimuli (Calabrese, 2015). This study revealed weak hormesis of anticholinesterase at the level of population.
Table 6. Regressive equations established between population and ChE (3 to 21 d after dose administration)

|   | y                      | x                      | n   | Logistic equation                                                                 | $R^2$  | $P$   |
|---|------------------------|------------------------|-----|-----------------------------------------------------------------------------------|--------|-------|
| A | Population density from 3 to 21 d (number/L) | Inherent activity from 3 to 21 d (U/µg) | 105 $^a$ | $y = \frac{213.939372}{1 + \exp(1.199015 - 3.303077x)}$ | 0.5568 | 0.0000 |
| B | Population density from 3 to 21 d (number/L) | Apparent activity from 3 to 21 d (U/µg) | 105 $^a$ | $y = \frac{180.945731}{1 + \exp(1.289695 - 410.040519x)}$ | 0.4792 | 0.0000 |
| C | Population density of 18-d average from 3 to 21 d (number/L) | Inherent activity of 18-d average from 3 to 21 d (U/µg) | 5 $^b$ | $y = \frac{194.181822}{1 + \exp(0.946662 - 3.666915x)}$ | 0.9897 | 0.0103 |
| D | Population density of 18-d average from 3 to 21 d (number/L) | Apparent activity of 18-d average from 3 to 21 d (U/µg) | 5 $^b$ | $y = \frac{648.734214}{1 + \exp(1.934723 - 80.564603x)}$ | 0.9097 | 0.0903 |
| E | Decline of 18-d average in population density (%) from 3 to 21 d (%) | Decline of 18-d average in inherent activity from 3 to 21 d (%) | 5 $^b$ | $y = \frac{25.046257}{1 + \exp(4.124150 - 0.142111x)}$ | 0.9810 | 0.0190 |
| F | Decline of 18-d average in population density (%) from 3 to 21 d (%) | Decline of 18-d average in apparent activity from 3 to 21 d (%) | 5 $^b$ | $y = \frac{23.160000}{1 + \exp(11.710990 - 2.593855x)}$ | 0.9701 | 0.0299 |
| G | Decline of 18-d average in population density (%) from 3 to 21 d (%) | Inherent activity of 18-d average from 3 to 21 d (U/µg) | 5 $^b$ | $y = \frac{25.045463}{1 + \exp(-10.088550 + 15.733024x)}$ | 0.9810 | 0.0190 |
| H | Decline of 18-d average in population density (%) from 3 to 21 d (%) | Apparent activity of 18-d average from 3 to 21 d (U/µg) | 5 $^b$ | $y = \frac{23.160000}{1 + \exp(-255.751281 + 24202.098357x)}$ | 0.9638 | 0.0362 |

$^a$Constituting 5 levels × 7 dates × 3 replications. $^b$Each is an average of 7 dates × 3 replications.
4.5.2. Free-living NAGase

To plot population density against content or change in content of NAGase, a set of scatter diagrams are obtained as shown in Figure 7. The equations corresponding to them are listed in Table 7.

One can see from Table 7 that to plot a population at a date against the NAGase at the same date, the equations obtained were not ideal (see Equation A in Table 7), whereas to plot the population of 18-d average against the NAGase of the same average, the equations obtained were much improved (to compare Equation A with B in Table 7). This revealed advantage of period-estimation in utilization of NAGase.

To plot a population at a date against NAGase 0, 3, 6, 9, 12, 15, and 18 d after the date, the equation obtained was found to have $R^2$ of 0.7989, 0.9168, 0.9286, 0.9374, 0.9407, 0.9114, and 0.9176, respectively, indicating that a population could be better estimated by NAGase times after the date.

Equation B as shown in Table 7 could be used for predicting change in production rate of a population. Precondition is required, however, in doing so. It is known that instant level of NAGase is jointly determined by production and decay of the enzyme, and in case the population is in steady, decay of the NAGase is balanced by production of the enzyme, and that is the moment at which production rate of the population could be predicted with adequate accuracy by turnover rate of the enzyme in medium free from arthropods (Sastri & Dower, 2009, 2006; Sastri et al., 2013, 2012; Sastri & Roff, 2000; Yebra et al., 2017). So you can take measurement level of NAGase as a reflection of the level in production in condition that the system involved is in steady, and impact of a chemical on NAGase can be regard as a reflection of its impact on production.

Look into the scatterplot as shown in Figure 7(b), one can see declination from 179 to 135 number/L in terms of the population along with declination from 1.21 to 0.91 μg/mL in terms of the NAGase.

Figure 7. Status of population and that of NAGase 3–21 d after dose administration.

A: $y$ – Population density from 3 to 21 d (number/L); $x$ – NAGase content from 3 to 21 d (μg/mL). B: $y$ – Population density of 18-d average from 3 to 21 d (number/L); $x$ – NAGase content of 18-d average from 3 to 21 d (μg/mL). C: $y$ – Decline of 18-d average in population density from 3 to 21 d (%); $x$ – Decline of 18-d average in NAGase content from 3 to 21 d (%).
|   | y  | x                                      | n  | Logistic equation                                                                 | $R^2$ | $P$    |
|---|----|----------------------------------------|----|-----------------------------------------------------------------------------------|-------|--------|
| A | Population density from 3 to 21 d (number/L) | NAGase content from 3 to 21 d (µg/mL) | 126 $^a$ | $y = \frac{168.645199}{1 + \exp(6.647678 - 11.412781x)}$ | 0.7989 | 0.0000 |
| B | Population density of 18-d average from 3 to 21 d (number/L) | NAGase content of 18-d average from 3 to 21 d (µg/mL) | 6 $^b$ | $y = \frac{180.027737}{1 + \exp(5.306716 - 6.970247x)}$ | 0.9945 | 0.0004 |
| C | Decline of 18-d average in population density from 3 to 21 d (%) | Decline of 18-d average in NAGase content from 3 to 21 d (%) | 6 $^b$ | $y = \frac{100.636599}{1 + \exp(3.719743 - 0.101274x)}$ | 0.9940 | 0.0005 |

$^a$Constituting 6 levels × 7 dates × 3 replications. $^b$Each is an average of 7 dates × 3 replications.
One could use Equation C for predicting impact of a chemical on population. In utilization of Equation C, however, a group of CK need to be set. Look into the scatterplot as shown in Figure 7(c), one could see 4.81% increase in terms of the NAGase corresponding to 1.65% in terms of the population. This was a further proof for existence of anticholinesterase-derived hormesis at the level of population. Look into the scatterplot as shown in Figure 7(c), one can see declination from −1.65 to 23% in terms of the population along with declination from −4.81 to 24.89% in terms of the NAGase.

5. Conclusions
A set of macrophyte-dominated systems were established indoor to explore impact of anticholinesterase on *D. magna* at the level of population. The indexes observed including population density, inherent activity of ChE, apparent activity of ChE, and immuno-reactive content of NAGase. Result of the study showed that:

1. The systems established in this study were generally steady.
2. The LOEC of chlorpyrifos was determined to be 0.50, <0.02, 0.5, and 0.5 μg/L for population density, inherent activity of ChE, apparent activity of ChE, and immuno-reactive content of NAGase, respectively, in case it was counted by nominal concentrations, and it was determined to be 0.128, <0.011, 0.092, and 0.092 μg/L, respectively, in case it was counted by nominal concentrations. This suggested that the NAGase was sensitive enough for announcing population level impact of a chemical, and the inherent activity of ChE was most sensitive among the three in announcing population level impact of an anticholinesterase.
3. Based on the values of NOEC and/or LOEC determined in this study and the values of exposure exported by GENEEC2, which indicated the peak, 4-d, 21-d, 60-d, and 90-d average of 5.70, 5.48, 4.22, 2.55, and 1.88 μg/L, respectively, one could say that chlorpyrifos would impose harm on populations in case it was applied at the recommended rate.
4. The values of the NOEC and/or LOEC determined in this study were in range of those obtained from other studies. This suggested that systems involved were reliable in determining population impact of an insecticide.
5. Regressive analysis indicated that state of a population at a date could not be estimated with adequate accuracy by standing state of either ChE or NAGase ($R^2 = 0.5568$ and 0.7989, respectively). In spite of that, average state of a population over a period of time could be estimated with adequate accuracy by either ChE or NAGase of the same average ($R^2 = 0.9810$ and 0.9940, respectively). Moreover, state of a population at a date could be estimated with better accuracy by standing state of NAGase times after the date ($R^2 = 0.9407$ in maximal).
6. Along with declination from 0.90 to 0.48 U/μg in terms of inherent activity of ChE, one can see atrophy of −1.65 to 23% in terms of population and that of −4.1 to 24.89% in terms of NAGase.
7. Anticholinesterase-induced hormesis (1.65%) was identified at the level of population.
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