JOURNAL OF ENTOMOLOGICAL SOCIETY OF IRAN
2014, 33(4): 43-54

Cellular energy allocation of *Glyphodes pyloalis* (Lep.: Pyralidae): changes related to exposure to TiO_2 nanoparticles

N. Memarizadeh[1&*], M. Ghadamyari[1], M. Adeli[2] and Kh. Talebi-Jahromi[3]
1. Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran, 2. Department of Chemistry, Faculty of Sciences, University of Lorestan, Khoramabad, Iran, 3. Department of Plant Protection, College of Agricultural and Natural Resources, University of Tehran, Karaj, Iran.
*Corresponding author, E-mail: nmemarizadeh@yahoo.com

Abstract

In order to study the pollution potential of TiO_2 nanoparticles (TiO_2-NPs) on ecological health, this research was carried out on the cellular energy allocation (CEA) of *Glyphodes pyloalis* Walker exposed to TiO_2-NPs. The newly ecdysed fifth instar larvae of *G. pyloalis* were treated with LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50} concentrations of TiO_2-NPs and the amount of energy available (E_a), energy consumption (E_c) and cellular energy allocation were compared. The resulting calculated energy reserves (lipid, carbohydrate, glycogen and protein) showed that increasing the time of exposure, the total lipid and carbohydrate amounts significantly decreased, when the LC_{30}, LC_{40} and LC_{50} concentrations were applied. The amounts of glycogen in the larvae treated with LC_{10}, LC_{20} and LC_{30} concentrations of TiO_2-NPs were increased, whereas the LC_{40} and LC_{50} concentrations led to decrease in the amount of glycogen. The significant reduction in the amount of total protein compared to the control and over all three days of treatment was observed for LC_{50} concentration of TiO_2-NPs; however, the LC_{10} concentration lead to a significant increase of the total protein after three days. E_a decreased in a dose-response related manner and over all time points, but it significantly increased in treated larvae by LC_{10} and LC_{20} concentrations after two days. E_c increased as concentrations grew to LC_{30} and then started to decrease. The results showed that CEA was not affected by LC_{10} concentration, but significantly decreased when the concentration increased and at all time points probably as a cost to deal with TiO_2-NPs detoxification. Therefore, it will be possible to use the CEA as an appropriate early biomarker for the impacts of TiO_2-NPs.
Key words: TiO_2-NPs, cellular energy allocation, *Glyphodes pyloalis*, lipid, glycogen, protein

چکیده

تخصیص انرژی سلولی (Lep.: Pyralidae) *Glyphodes pyloalis*: تغییرات مربوط به مواجهه با نانوذرات TiO_2

نرگس معماری‌زاده، محمد قدمیاری، محسن عادلی و خلیل طالبی جهرمی

به‌منظور مطالعه پتانسیل آلودگی نانوذرات TiO_2 (TiO_2-NPs) بر سلامتی اکولوژیکی، این تحقیق برای بررسی تخصیص انرژی سلولی (CEA) حشره *Glyphodes pyloalis* Walker در مواجهه با نانوذرات TiO_2 انجام شد. لاروهای سن پنجم *G. pyloalis* که تازه پوست‌اندازی کرده بودند، با غلظت‌های LC_{10}، LC_{20}، LC_{30}، LC_{40} و LC_{50} نانوذرات TiO_2 تیمار شدند و میزان انرژی در دسترس (E_a)، انرژی مصرفی (E_c) و تخصیص انرژی سلولی آن‌ها مقایسه شد. نتایج محاسبه انرژی ذخیره‌ای (لیپید، کربوهیدرات، گلیکوژن و پروتئین) نشان داد زمانی‌که غلظت‌های LC_{30}، LC_{40} و LC_{50} به‌کار رفتند، با افزایش زمان مواجهه، مقدار لیپید و کربوهیدرات کل به‌طور معنی‌دار کاهش یافت. میزان گلیکوژن در تیمارهای انجام‌شده با غلظت‌های LC_{10}، LC_{20} و LC_{30} نانوذرات TiO_2 افزایش یافت، درحالی‌که غلظت‌های LC_{40} و LC_{50} منجر به کاهش میزان گلیکوژن شدند. کاهش معنی‌دار میزان پروتئین کل تیمار با غلظت LC_{50} در مقایسه با شاهد و در طول هر سه روز مشاهده شد، درحالی‌که غلظت LC_{10} منجر به افزایش معنی‌دار میزان پروتئین کل بعد از سه روز می‌شود. E_a، به‌صورت وابسته به دز و در طول تمام نقاط زمانی تیمار کاهش یافت، ولی در تیمارهای انجام‌شده با غلظت‌های LC_{10} و LC_{20} بعد از دو روز به‌طور معنی‌داری افزایش نشان داد. E_c، با افزایش غلظت تا LC_{30} افزایش و سپس شروع به کاهش کرد. نتایج مطالعه نشان داد که CEA با غلظت LC_{10} تحت تأثیر قرار نمی‌گیرد اما با افزایش غلظت نانوذرات TiO_2 و در تمام نقاط زمانی آزمایش، به‌طور معنی‌دار کاهش می‌یابد که احتمالاً به‌دلیل هزینه سم‌زدایی نانوذرات TiO_2 است. بنابراین، این امکان وجود دارد که CEA به‌عنوان نشانگر زیستی و پیش‌آگاهانه برای اثرات نانوذرات TiO_2 به‌کار رود.
واژگان کلیدی: TiO_2-NPs، تخصیص انرژی سلولی، *Glyphodes pyloalis*، لیپید، گلیکوژن، پروتئین

Introduction

Nanotechnology is the control of matter at dimensions between approximately 1 and 100 nanometers to produce special physical, chemical and biological properties in it at the nanoscale (Buzea *et al*., 2007). Since the potential uses and benefits of nanotechnology in agricultural productivity enhancement are enormous, the use of synthetic nanoparticles is growing rapidly and therefore the study on the safety of nanoparticles (NPs) in the environment seems to be very important (Kahru *et al*., 2008; Clemente *et al*., 2012). NPs by having the special surface properties may bind and transport toxic chemical pollutants, as well as possibly being toxic by

generating reactive radicals (Moore, 2006). Wide usage of titanium dioxide nanoparticles (TiO_2-NPs) in different field is due to its high stability, anticorrosion and photocatalytic property (Linhua *et al*., 2009). Cytotoxicity, phytotoxicity, lung inflammation and oxidative stress in mammals, plants and microorganisms have been reported as side effects of TiO_2-NPs (Ferin *et al.*, 1992; Wang *et al*., 2007, 2009; Warheit *et al*., 2007). Despite the fact that TiO_2 has been classified as innocuous to the organism through World Health Organization (WHO, 1996) but recently the International Agency for Research on Cancer (IARC, 2010) classified this material as "possibly carcinogenic for humans".

The vast majority of nanoecotoxicological studies with TiO_2-NPs have hitherto evaluated the toxicity of TiO_2-NPs to aquatic organisms (Lovern & Klaper, 2006; Federici *et al*., 2007; Zhu *et al*., 2008; Clemente *et al*., 2012). Furthermore, many studies in the aquatic environment have reported the influence of various toxicants on the cellular energy allocation (De Coen & Janssen, 1997, 2003; Smolders *et al*., 2004; Amorim *et al*., 2012)

Amorim *et al*. (2012) showed that the energy reserves (lipids, carbohydrates, and proteins) in the white worm, *Enchytraeus albidus* (Oligochaeta), was influenced by copper (Cu) salt and Cu-NPs. So, they showed that there was no apparent difference between the impacts of the two Cu exposure forms. Exposure to contaminants affected the physiological energetic and can disturb energy allocation as a cost of handling toxicants or due to the interaction of the toxicant with storage processes within the organisms (Ireland & Richards, 1977; Callow & Sibly, 1990; Amorim *et al*., 2012). Thus the effect of NPs, as probable and potential contaminant, can be elucidated by physiological response of an organism.

Cellular energy allocation (CEA) test is a fast method for measuring energy budget of an organism (Widdows & Donkin, 1992; De Coen & Janssen, 1997; Verslycke *et al*., 2004; Bagheri *et al*., 2010). CEA is calculated by divided amount of energy available (E_a) to energy consumption (E_c). E_a is the total amount of energy acquired from available total lipid, total protein, glucose and glycogen content. E_c is the activity of electron transport system which can be measured by altering enzyme production in an organism (De Coen & Janssen, 1997).

So far, no reports have been presented about the changes of cellular energy allocation levels of insect pests exposed to TiO_2-NPs. Due to the potential of application of TiO_2-NPs into the formulation of photodegradable pesticides, it was decided to make the ecotoxicological assessment of TiO_2-NPs exposure of an insect pest model species by assessing the sublethal effects of TiO_2-NPs on the energy budget of *Glyphodes pyloalis* Walker.

Materials and methods

Insects

The fifth instar larvae of *G. pyloalis* were collected from infested mulberry orchards (Shine Ichinoise variety) in the vicinity of Rasht, Iran. Mass rearing of insects was carried out in the laboratory on the fresh mulberry leaves (Shine Ichinoise variety), under controlled conditions of 25 ± 1 °C temperature, 70 ± 10% RH, and 16: 8 L: D. Newly-ecdysed fifth instar larvae of *G. pyloalis* were used for bioassay experiments, when at least two generations of *G. pyloalis* were cultured under laboratory conditions.

Synthesis of TiO_2-NPs

TiO_2 nanoparticles were prepared according to the method of Trung *et al*. (2003) by hydrolyzing titanium isopropoxide which was added drop by drop into stock solution (i.e. ethanol and acetic acid in a ratio of 8:3 v/v with glycerol) at 10 °C, followed by rigorous stirring under an argon atmosphere for 3 h. Then, the solutions were heated at 60 °C for 5 h or until the gelling reaction was completed. The dried precipitates were heated at 400 °C for 10 h, at the heating rate of 1 °C/min.

Treatment

According to the results of the bioassay of TiO_2-NPs on the newly ecdysed fifth instar larvae of *G. pyloalis* in the previous study (Memarizadeh *et al*., 2014), treatments were done with concentrations of 290, 380, 475, 565 and 665 mg/L equivalent to LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50} concentrations of TiO_2-NPs, respectively. Mulberry leaf discs (diameter 3.5 cm) were immersed in the dilutions for 45 s. After drying, synchronized fifth instar larvae of *G. pyloalis* were placed on each treated leaf disk. Over three days (i.e. 24 h, 48 h and 72 h) after treatment, representative samples for each concentration were taken randomly from survived larvae. The collected samples were placed in a deep freezer at -20 °C until biochemical assays were performed.

Preparation of samples

To assess the amount of energy reserves (total lipid, glucose and glycogen), each treated larva was homogenized in 50 µl of 2% Na_2SO_4. It was then centrifuged at 8000 × g for 10 min following addition of 1300 µl of chloroform: methanol (1:2, V:V) to the mixture. After centrifugation, total lipid and carbohydrate were assayed from the resultant supernatant and glycogen content from pellet (Yuval *et al*., 1998).

For electron transport system (ETS) assay, each treated larva was homogenized in appropriate buffers which was a solution mixture of 0.1 M Tris-HCl; pH 8.5, 15% poly vinilpyrrolidone, 0.55 mg ml^{-1} $MgSO_4$, 0.2% (v/v) Triton X-100. Following centrifugation at 3000 × g for 10 min, the resulted supernatant was used for assessing the amount of total protein and ETS activity (van Handel & Day, 1988; Yuval *et al*., 1998; Verslycke *et al*., 2004).

Measurement of the energy budget

Cellular energy allocation (CEA) was assayed using the method described by van Handel & Day (1988), and Yuval *et al*. (1998) with minor modifications. By determination of total energy reserves in an insect body as energy available (E_a) and the activity of electron transport system (ETS) as energy consumption (E_c) according to the following formula, CEA was calculated:

E_a = ∑(total lipid, carbohydrate, glycogen and total protein) (joule/insect); E_c = ETS activity (joule/insect); $CEA = E_a/E_c$

Determination of the amount of total lipid

A volume of 125 µl of the resulted supernatant (as described in sample preparation) were transferred to new tube and dried in oven (40 °C for 10 h), samples were then dissolved in 125 µl H_2SO_4 and heated for 10 min at 90 °C. Thereafter, 30 µl of the mixture was reacted with 270 µl vanillin reagent for 30 min and then shacked. The absorbance was measured using a microplate reader (Awareness Technology Inc, Stat Fax 3200) at 545 nm. Vanillin reagent was made by dissolving 600 mg vanillin in 100 ml distilled water and 400 ml 85% H_3PO_4. Standard curve for lipid assay was plotted using cholesterol as the standard (Yuval *et al*., 1998).

Determination of the amount of total carbohydrate

A volume of 150 µl supernatant (as described in sample preparation), 100 µl distilled water, and 500 µl anthrone reagent were putted in a tube and heated at 90 °C for 10 min. Then absorbance was measured using a microplate reader (Awareness Technology Inc, Stat Fax 3200) at 630 nm. Anthrone reagent was made by dissolving 0.006 g anthrone in 3 ml 85% H_2SO_4. Total carbohydrate in each individual larva was calculated from standard curve using maltose as standard (Yuval *et al*., 1998).

Determination of the amount of total glycogen

The pellets (as described in sample preparation) were washed twice by 400 µl 80% methanol, 250 µl distilled water was then added to each sample and heated at 70 °C for 5 min. The mixture was centrifuged at 8000 × g for 5 min. The anthrone reagent was then added to the supernatant. The samples were heated at

90 °C for 10 min and then cooled down. The absorbance was read at 630 nm. Glycogen was used as the standard (Yuval *et al*., 1998).

Determination of the protein content

Protein concentration was estimated according to the Bradford (1976) method, using bovine serum albumin as the standard by a microplate reader (Awareness Technology Inc, Stat Fax 3200).

Calculation of energy reserves

Different components of energy reserves were transformed to energetic equivalents. Equivalent of these reserves are combustion energy which consisted of 17.5 j mg^{-1} glycogen and carbohydrate, 24 j mg^{-1} protein and 39.5 j mg^{-1} lipid (Gnaiger, 1983).

Determination and calculation of energy consumed

The homogenates of the samples were centrifuged at 4 °C, 3000 × g for 10 min. After centrifugation, 40 µl of extract was added into a 96-well microplate well, together with 120 µl of buffered substrate solution (0.13 M Tris-HCl and 0.3% (w/v) Triton X-100, pH 8.5) containing 1.7 mM NADH (nicotinamide adenine dinucleotide) and 0.23 mM NADPH (nicotinamide adenine dinucleotide phosphate). The reaction was started by addition of 80 µl INT (iodo nitro tetrazolium) (8 mM). Absorbance was measured kinetically using a microplate reader (Awareness Technology Inc, Stat Fax 3200) at 450 nm for 10 min. The amount of formazan formed was calculated based on an extinction coefficient of 15,900 $M^{-1}cm^{-1}$. From a theoretical point of view, formation of 2 µl formazan will use 1 µmol of O_2 and the quantity of consumed oxygen was transformed into energetic equivalents (484 kJ/mol O_2) (Gnaiger, 1983).

Statistical analysis

Three replicates were conducted for all the biochemical assays and data were subjected to analysis of variance (ANOVA). Statistical analyses were performed at $P = 0.05$ by Tukey's test using the SAS software (SAS Institute, 2011).

Results

Results of the present study showed that unlike the control, by increasing the exposure time, a significant reduction was observed in the amount of total lipid in treated insects when all the concentrations of TiO_2-NPs were applied. The lowest amount of total lipid was observed at the LC_{50} concentration of TiO_2-NPs (fig. 1).

There was no significant difference in the amount of carbohydrate content between the exposure times in all concentrations of the TiO_2-NPs and also in the control. But the LC_{30}, LC_{40} and LC_{50} concentrations of TiO_2-NPs caused significant decrease in the total carbohydrate content compared to the control, especially on day 2 and day 3 after treatments (fig. 2). Results also showed that the amount of carbohydrate content in the treated larvae by the LC_{50} concentration was significantly lower than the other treatments (fig. 2).

After the 1st day of the exposure to the LC_{20}, LC_{30} and LC_{40} concentrations, the glycogen content significantly increased and with the LC_{50} concentration, it significantly decreased (fig. 3). On the 2nd day of the treatment, the LC_{10}, LC_{40} and LC_{50} concentrations of TiO_2-NPs decreased the glycogen content. Furthermore, after the 3rd day, the LC_{10}, LC_{20} and LC_{30} concentrations of TiO_2-NPs significantly increased the amount of glycogen. However, the LC_{50} concentration led to reduction of this factor significantly (fig. 3).

The significant reduction in the total protein content compared to the control and over all three days of treatments was observed for LC_{50} concentration of TiO_2-NPs. Results also showed that the total protein content reduced significantly by the LC_{40} concentration after one day. However, the LC_{10} concentration of TiO_2-NPs could significantly increase the total protein content after three days (fig. 4).

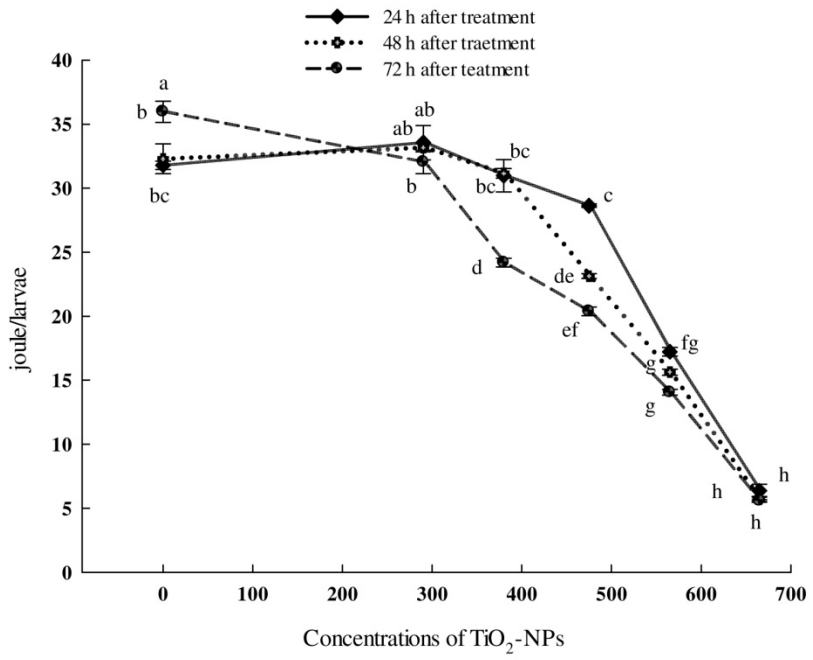

Fig. 1. Average energy equivalent (joule/larvae) of lipids in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of TiO_2-NPs. Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

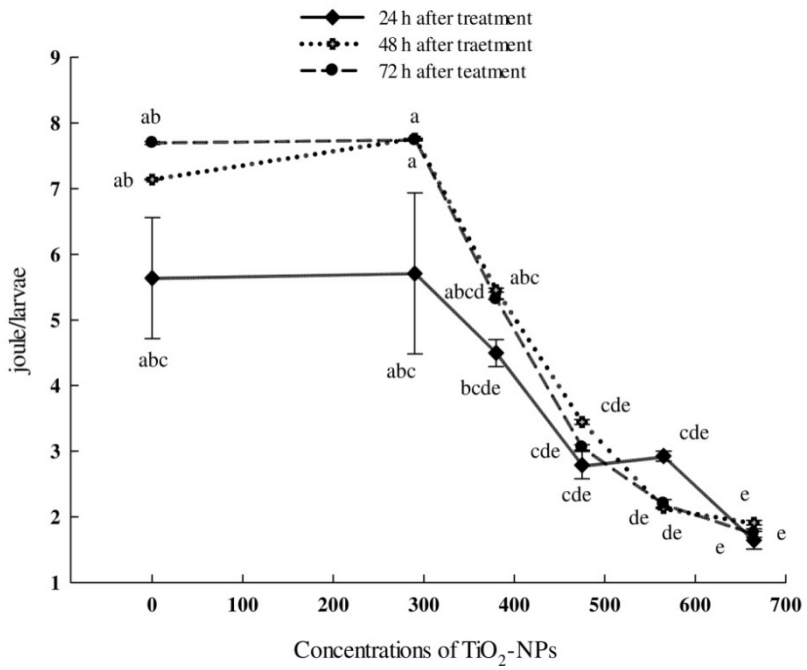

Fig. 2. Average energy equivalent (joule/larvae) of carbohydrate in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of TiO_2-NPs. Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

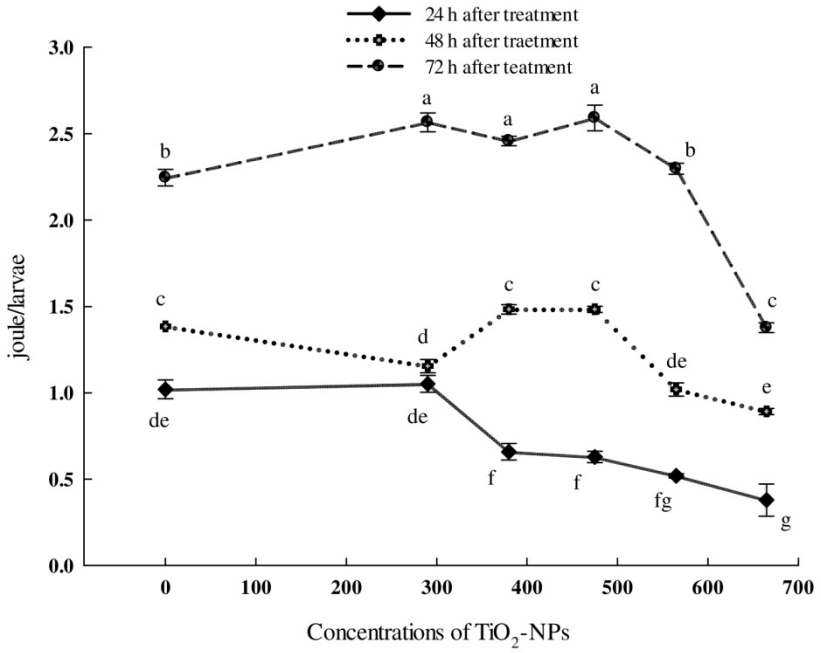

Fig. 3. Average energy equivalent (joule/larvae) of Glycogen in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of TiO_2-NPs. Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

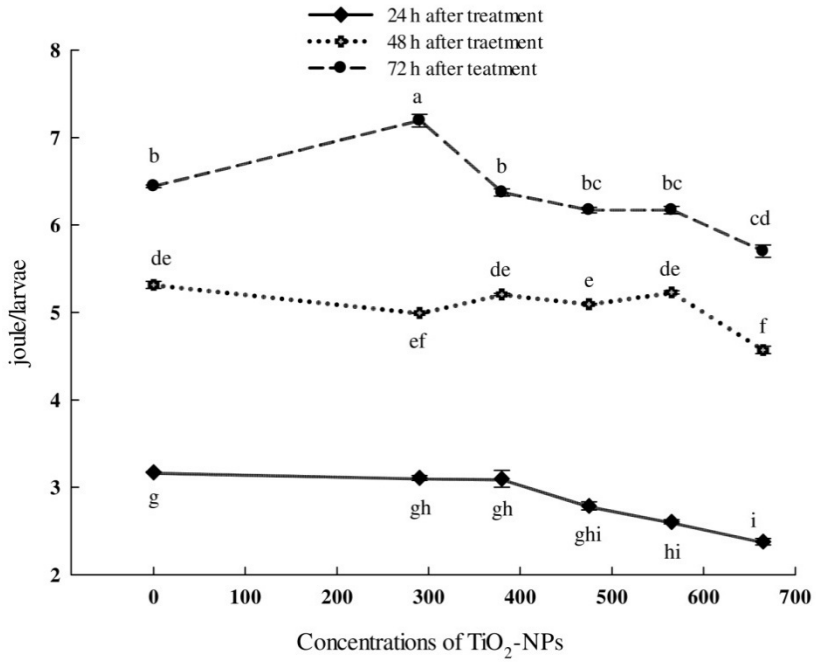

Fig. 4. Average energy equivalent (joule/larvae) of protein in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of TiO_2-NPs. Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

Reserve energy was calculated by summation of total lipids, protein, carbohydrate and glycogen contents (fig. 4). In the case of untreated larvae (control) the E_a significantly increased, from 1st day to 3rd day of the fifth instar larvae. However, E_a significantly increased only in treated larvae by LC_{10} and LC_{20} concentrations and just after 2nd day of the treatment. Results also showed that higher concentrations of TiO_2-NPs affected the energy reserves and the E_a roughly decreased in a dose-response related manner and over all time points (fig. 5).

The energy consumption during the exposure times was calculated based on the mitochondrial ETS activity and the results are presented in the fig. 6. Results showed that the E_c was increased applying the LC_{20}, LC_{30} and LC_{40} concentrations over all three times of exposure; however the LC_{50} concentration significantly decreased the E_c on 1st and 2nd after treatment. Results also showed that the E_c was not affected by the LC_{10} concentration of TiO_2-NPs, over all three points of the exposure time (fig. 6).

As shown in fig. 7, in contrast to the E_c, CEA was significantly decreased as the concentration increased and at all time points. Hence, LC_{10} concentration showed no significant effect on CEA, but maximum effects were obtained for LC_{40} and LC_{50} concentrations (fig. 7).

In general, analysis of variance (split-plot in the time) showed that the amount of lipid, glycogen, protein, Ec, Ea and CEA in treated larvae significantly affected by: (1) the TiO_2-NPs concentrations, (2) time of exposure to TiO_2-NPs and (3) interplay effect of concentration and exposure time. This means that the effect of TiO_2-NPs concentrations on the amount of lipid, glycogen, protein, Ec, Ea and CEA is correlated to the length of exposure time. However, analysis of variance of the amount of carbohydrate in treated larvae showed that this factor only was affected by the TiO_2-NPs concentrations.

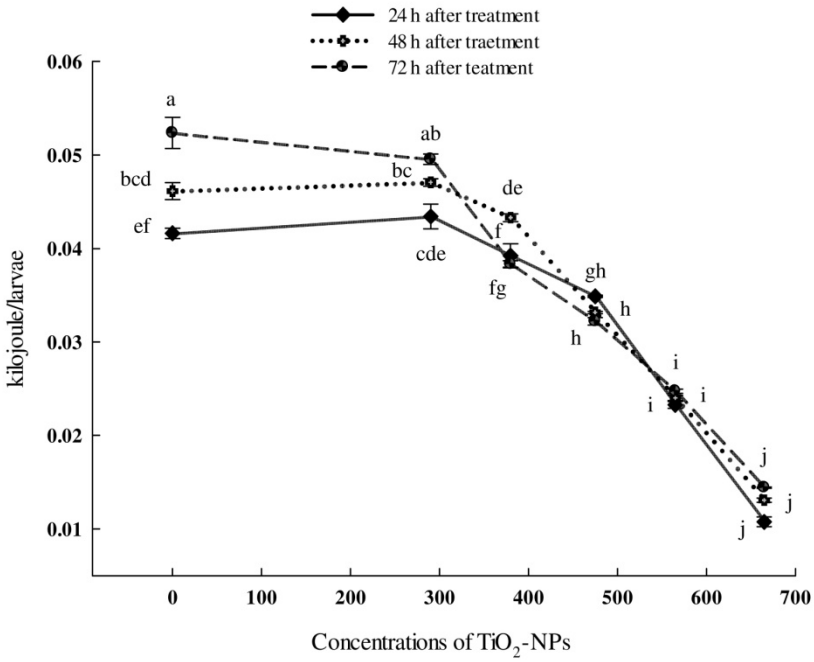

Fig. 5. Average energy equivalent (Kilojoule/larvae) of energy reserves (E_a) in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of TiO_2-NPs. Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

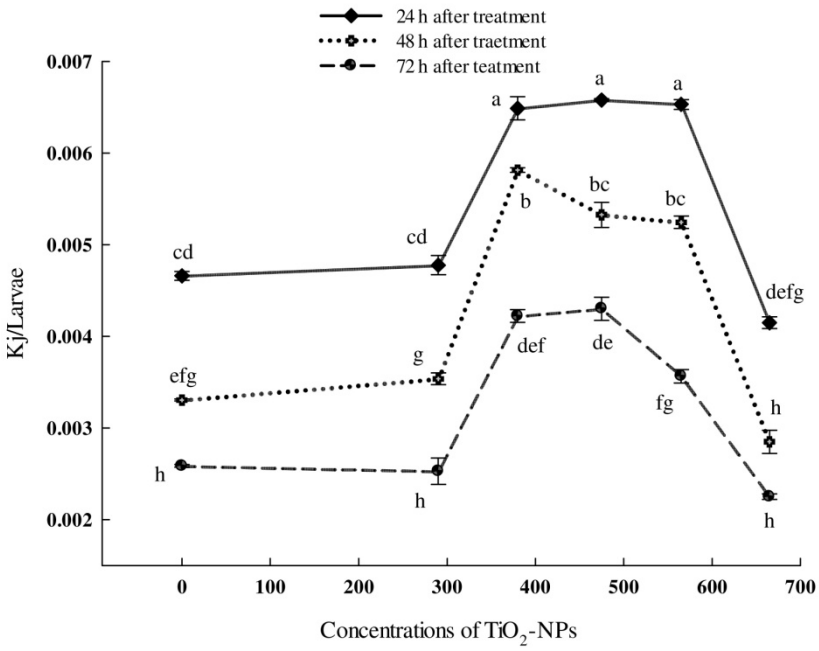

Fig. 6. Average energy equivalent (Kilojoule/larvae) of energy consumed (E_c) in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of TiO_2-NPs. Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

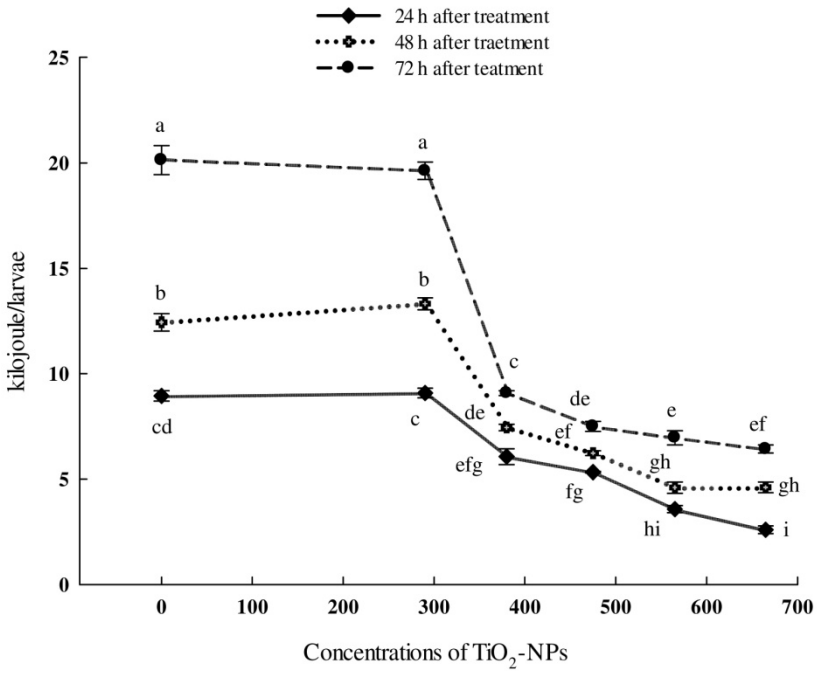

Fig. 7. Average energy equivalent (Kilojoule/larvae) of cellular energy allocation (CEA) in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of TiO_2-NPs. Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

Discussion

Metabolic balance of the treated organism can be assessed via the CEA test (De Coen *et al*., 1995). The electron transport activity in the homogenates is equivalent to the energy consumption (E_c); while reserve energy (E_a) for metabolism is calculated by summation of total lipids, protein, carbohydrate, and glycogen contents and energy available for growth is shown through the difference between E_a and E_c (De Coen *et al*., 1995; De Coen & Janssen, 2003; Rueda-Jassoa *et al.*, 2004).

Results of the present study showed the reduction of total lipid in the exposure to TiO_2-NPs in a dose-response related manner. This reduction is due to the fact that lipids are used as the first energy source when exposed to the environmental contaminations (De Coen & Janssen, 1997; Amorim *et al*., 2012). Additionally, this decrease in the amount of lipid can also be a result of oxidative damage on the cell membranes in addition to increased levels of lipid peroxidation (Novais *et al*., 2013).

Determination of the amount of total carbohydrate showed that LC_{30} and LC_{40} concentrations of TiO_2-NPs led to significant decrease in the total carbohydrate contents compared to the control on 2nd and 3rd day after treatments (fig. 2). Results also showed that LC_{50} concentration had the highest effects on the amount of carbohydrate content over all three time points. Depending on the physiological conditions, lipid and carbohydrate could be stored in the insect's body or could be used as an energy source (Miranda *et al*., 2003). Therefore, it is likely that reduction in carbohydrate content indicates a cost for detoxification of TiO_2-NPs.

Lower concentrations of TiO_2-NPs (i.e. LC_{10}, LC_{20} and LC_{30} concentrations) led to increase, and higher concentrations (i.e. LC_{40} and LC_{50} concentrations) led to decrease in the glycogen content. The reduction of glycogen levels under high concentration of TiO_2-NPs can refer to the costs for physiological regulation.

Proteins play very important role in the synthesis of detoxifying enzymes to detoxify the toxicants when entering into the insect's body (Jain *et al*., 2011). Thus, reduction of amount of total protein can be due to inhibition of some detoxification enzymes (i.e. general esterases and glutathione S-transferases) over treatments by TiO_2-NPs (Memarizadeh *et al.*, 2014).

The reduction of energy reserves could be due to both decreased food consumption and/or increased metabolic activity (De Coen & Janssen, 2003; Novais *et al*., 2013). Results of Li *et al*. (2011) showed that TiO_2- and Al_2O_3-NPs could disrupt the energy of assimilation and consumption of *Ceriodaphnia dubia* (Branchiopoda) dramatically. Furthermore, they reported that the energy consumption was increased also with the increase in the concentration of nanomaterials. Therefore, the study of TiO_2 and Al_2O_3 on the *C. dubia* is in accordance with our results and clearly demonstrates the importance of energy disruption in determination of the toxicity of nanomaterials. Present results also showed a reduction in the available energy reserves over three days of exposure and an increased ETS activity. This increased metabolic activity can be a cost to deal with TiO_2-NPs detoxification and also may be the main reason for the decrease in energy reserves.

The CEA was significantly decreased as the concentration increased at all time points. Although, this factor was not affected by LC_{10} concentration of TiO_2-NPs; however, CEA interestingly decreased by exposure to LC_{40} and LC_{50} concentrations. Lipid and carbohydrate contents were mainly responsible for the decrease in the CEA when exposured to different concentrations of TiO_2-NPs, along with the increased ETS activity (Miranda *et al*., 2003). This decrease in the net energy budget indicated that energy was spent to overcome the toxicity of TiO_2-NPs, especially for the LC_{40} and LC_{50} concentrations and thus there will be less energy available for other physiological functions (Novais *et al*., 2013).

The energy consumption (E_c) and the energy reserves available for metabolism (E_a) can be used as an indicator of the metabolic conditions (Rueda-Jassoa *et al*., 2004). Results showed that energy metabolism of *G. pyloalis* was affected by TiO_2-NPs, especially by LC_{40} and LC_{50} concentrations, and thus energy reserves were reduced and mitochondrial electron transport system activity changed due to increased cellular respiration. Reduced energy budget resulted in the extra energy requirements for detoxification (Amorim *et al*., 2012). Therefore, the increased ETS activity and expenditure of energy suggest the additional energy requirement to handle TiO_2-NPs toxicity. Consequently, it will be possible to use the CEA as an appropriate early biomarker for the impacts of TiO_2-NPs and it will be more informative in predicting physiological impacts and future toxicity of them.

Acknowledgment

Authors would like to thank Iran National Science Foundation for their financial support of this project.

References

Amorim, M. J. B., Gomes, S. I. L., Soares, A. M. V. M. & Scott-Fordsmand, J. J. (2012) Energy basal levels and allocation among lipids, proteins, and carbohydrates in *Enchytraeus albidus*: changes related to exposure to Cu salt and Cu nanoparticles. *Water, Air, and Soil Pollution* 223, 477-482.

Bagheri, F., Talebi, Kh. & Hosseininaveh, V. (2010) Cellular energy allocation of pistachio green stink bug, *Brachynema germari* Kol. (Hemiptera.: Pentatomidae) in relation to juvenoid pyriproxyfen. *African Journal of Biotechnology* 9(35), 5746-5753.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Buzea, C., Blandino, I. I. P. & Robbie, K. (2007) Nanomaterials and nanoparticles: sources and toxicity. *Biointerphases* 2(4), 117-172.

Callow, P. & Sibly, R. M. (1990) A physiological basis of population processes: ecotoxicological implications. *Functional Ecology* 4, 283-288.

Clemente, Z., Castro, V. L., Jonsson, C. M. & Fraceto, L. F. (2012) Ecotoxicology of nano-TiO_2 – an evaluation of its toxicity to organisms of aquatic ecosystems. *International Journal of Environmental Research* 6(1), 33-50.

De Coen, W. & Janssen, C. R. (1997) The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. *Journal of Aquatic Ecosystem Stress and Recovery* 6, 43-55.

De Coen, W. & Janssen, C. R. (2003) The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental Toxicology and Chemistry* 22, 1632-1641.

De Coen, W., Janssens, C. R. & Persoone, G. (1995) Biochemical assessment of cellular energy allocation in *Daphnia magna* exposed to toxic stress as an alternative to the conventional ‘‘Scope for Growth’’ methodology. *Proceedings International Symposium on Biological Markers of Pollution. Association Nationale de Protection des Plantes, Chinon, France.*

Federici, G., Shaw, B. J. & Handy, R. D. (2007) Toxicity of titanium dioxide nanoparticles to rainbow trout (*Onchorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. *Environmental Toxicology* 84(4), 415-430.

Ferin, J., Oberdorster, G. & Penney, D. P. (1992) Pulmonary retention of ultrafine and fine particles in rats. *American Journal of Respiratory Cell and Molecular Biology* 6, 535-542.

Gnaiger, E. (1983) Calculation of energetic and biochemical equivalents of respiratory oxygen consumption. pp. 337-345 in Gnaiger, E. & Forstner, H. (Eds) *Polarographic oxygen sensors*: *aquatic and physiological applications*. 370 pp. Springer Verlag, Berlin.

IARC (2010) *IARC* (*International Agency for Research on Cancer*) *monographs on the evaluation of carcinogenic risks to humans*: *carbon black, titanium dioxide, and talc*. Vol. 93, 460 pp. World Health Organization, Lyon, France.

Ireland, M. P. & Richards, K. S. (1977) The occurrence and localisation of heavy metals and glycogen in the earthworms *Lumbricus rubellus* and *Dendrobaena rubida* from a heavy metal site. *Histochemistry* 51, 153-166.

Jain, R., Ahi, J., Rai, S. & Jain, N. (2011) N-nitroso-N-methylurea induced changes in protein level of ovary and fatbody of the insect *Periplaneta americana. Asian Journal of Experimental Biological Sciences* 2(3), 533-536.

Kahru, A., Dubourguier, H. C., Blinova, I., Ivask, A. & Kasemets, K. (2008) Biotests and biosensors for ecotoxicology of metal oxide nanoparticles: a minireview. *Sensors* 8(8), 153-517.

Li, M., Czymmekb, K. J. & Huanga, C. P. (2011) Responses of *Ceriodaphnia dubia* to TiO_2 and Al_2O_3 nanoparticles: a dynamic nano-toxicity assessment of energy budget distribution. *Journal of Hazardous Materials* 187, 502-508.

Linhua, H., Zhenyu, W. & Baoshan, X. (2009) Effect of sub-acute exposure to TiO_2 nanoparticles on oxidative stress and histopathological changes in juvenile carp (*Cyprinus carpio*). *Journal of Environmental Sciences* 21, 1459-1466.

Lovern, S. B. & Klaper, R. (2006) *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C_{60}) nanoparticles. *Environmental Toxicology and Chemistry* 25(4), 1132-1137.

Memarizadeh, N., Ghadamyari, M., Adeli, M. & Talebi, K. (2014) Biochemical biomarkers of *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) in exposure to TiO_2 nanoparticles. *Invertebrate Survival Journal* 11, 47-53.

Miranda, J. E., Bortoli, S. A., Takahashi, R. & Silva, A. F. (2003) Nutritional indexes of silkworm (*Bombyx mori* L.) treated with juvenile hormone analogues. *Revista do Centro de Ciencias Rurais* 8, 32-38.

Moore, M. N. (2006) Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environment International* 32(8), 967-976.

Novais, S. C., Amadeu, M. V. M. S., De Coen, W. & Amorim, M. J. B. (2013) Exposure of *Enchytraeus albidus* to Cd and Zn – changes in cellular energy allocation (CEA) and linkage to transcriptional, enzymatic and reproductive effects. *Chemosphere* 90, 1305-1309.

Rueda-Jassoa, R., Conceic, L. E. C., Diasc, J., De Coen, W., Gomesc, E., Reese, J. F., Soaresb, F., Dinisb, M. T. & Sorgeloos, P. (2004) Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. *Aquaculture* 231, 417-433.

SAS Institute (2001) *SAS user's guide*: *statistics, version 9*. SAS Institute, Cary, NC.

Smolders, R., Bervoets, L., De Coen, W., & Blust, R. (2004) Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. *Environmental Pollution* 129, 99-112.

Trung, T., Cho, W. J. & Ha, C. S. (2003) Preparation of TiO_2 nanoparticles in glycerol-containing solutions. *Materials Letters* 57, 2746-2750.

van Handel, E. & Day, J. F. (1988) Assay of lipids, glycogen and sugars in individual mosquitoes: correlations with wing length in field collected *Aedes vexans*. *Journal of the American Mosquito Control Association* 4, 549-550.

Verslycke, T., Roast, S. D., Widdows, J., Jones, M. B. & Janssen, C. R. (2004) Cellular energy allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison. *Journal of Experimental Marine Biology and Ecology* 360, 1-16.

Wang, H. H., Wick, R. L. & Xing, B. S. (2009) Toxicity of nanoparticulate and bulk ZnO, Al_2O_3 and TiO_2 to the nematode *Caenorhabditis elegans*. *Environmental Pollution* 157, 1171-1177.

Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J., Li, Y., Jiao, F., Zhao, Y. & Chai, Z. (2007) Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicology Letters* 168, 176-185.

Warheit, D. B., Hoke, R. A., Finlay, C., Donner, E. M., Reed, K. L., Sayes, C. M. & Finlay, C. (2007) Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. *Toxicology Letters* 171, 99-110.

WHO (1969) Toxicological evaluation of some food colours, emulsifiers, stabilizers, anti-caking agents and certain other substances. *Food and Agriculture Organization* (*FAO*) *Nutrition Meetings Report Series*, No. 46A

Widdows, J. & Donkin, P. (1992) Mussels and environmental contaminants: bioaccumulation and physiological aspects. pp. 383-424 in Gosling, E. M. (Ed.) *The mussel Mytilus*: *ecology, physiology, genetics and culture*. 590 pp. Elsevier, Amsterdam.

Yuval, B., Kaspi, R., Shloush, S. & Warburg, M. S. (1998) Nutritional reserves regulate male participation in Mediterranean fruit fly leks. *Ecological Entomology* 23, 211-215.

Zhu, X. S., Zhu, L., Duan, Z. H., Qi, R. Q., Li, Y. & Lang, Y. P. (2008) Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health, Part A, Toxic/Hazardous Substances and Environmental Engineering* 43, 278-84.

Received: 19 December 2013
Accepted: 9 February 2014