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Evaluation of acute toxicity and teratogenic effects of plant growth regulators by *Daphnia magna* embryo assay

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ABSTRACT

This study selected common plant growth regulators (Atonik, Cytokinin, Ethephon, Gibberellic acid and Paclobutrazol) to investigate their biological toxicity to the waters of the important biological indicator *Daphnia magna*. The methods used in this study included traditional neonate acute toxicity test, new *Daphnia* embryo toxicity test, and teratogenic embryo test. The study concluded that the acute toxicity of the five PGRs to *Daphnia* neonate had EC_{50} value range of 1.9–130.5 mg l^{-1}, while acute toxicity of PGRs on *Daphnia* embryo had EC_{50} value range of 0.2–125 mg l^{-1}; the *Daphnia* embryos' LOEC values (0.05–48 mg l^{-1}) for the five PGRs were lower than embryo EC_{50} values. The toxic ratios of 48 h EC_{50} (neonate)/48 h LOEC (embryo) for 5 PGRs were 19–512 times. The study found that teratogenic effects of Paclobutrazol and Cytokinin induced in embryo were higher than those of most other PGRs. Microscopic observation of the teratogenic effects showed that all 5 PGRs induced malformations of the second antenna, rostrum, Malpighian tube, sensory bristles, and tail spine as well as function loss and death.

1. Introduction

Plant growth regulators are environmental hormones that are widely used in agriculture to increase plant growth and reproduction. Plant growth regulators in the water body may endanger aquatic ecosystems; however, related research is extremely limited. The commonly used class of plant growth regulators includes plant growth hormones such as Atonik, Cytokinin, and Gibberellic acid, organic phosphor such as Ethephon, and the Azole class, such as Paclobutrazol. Table 1 shows its classification and uses [1–6]. Many aquatic organisms could be used to detect growth hormones, such as zebra fish embryos, shrimp, rotifers, etc. [7–9]. However, the shortcomings or limitations of the above in research include high cost, large space requirements, long generation time, and difficult manipulation. For example, zebra fish has a long growth cycle and difficult culture conditions and needs a high skills level to obtain embryos.

The Simal-Gándara's research team in Spain, they study new application techniques, for example screened Organochlorine pesticides in stream sediments collected along stream flows close to an industrial area in northwestern Spain, detected by gas chromatography–mass spectrometry [10]. Measure carbofuran sorption kinetics by corn crop soils from Galicia, detected by HPLC [11]. Investigated the behavior of pesticides commonly used on potato in a part of northwestern Spain with a large area devoted to this crop, detected by gas chromatography–mass spectrometry [12]. They investigated the residues of fungicide in vineyard soils and river sediments in Ourense, measured by GC–MSD [13]. Concerns copper levels in the components of soils and river sediments in a river basin partially devoted to vineyards that are regularly treated with copper-bearing grapevine fungicides [14]. They concluded that the effects of processing on the pesticide residues in food is an area where available information should be consolidated and missing information needs to be obtained through further research [10,11,13,15,16]. If these studies could add more than one terminal of the biological tests (toxicity to aquatic organisms such as fish or water fleas) confirmed that the water quality or aquatic safety, who will better to win the trust of local government units.

Daphnia in the aquatic ecology has an important position in the food chain; it consumes algae growth and is the main food for other fish. Daphnids are often used to investigate the toxicity of chemicals in aquatic ecology because of their high sensitivity, short generation time, and ease of manipulation [17,18]. In addition, invertebrates, with special reference to aquatic crustaceans, have been of recent interest as a test model organism because of the need for developing a non-mammalian test system to evaluate toxicity of chemicals, heavy metals, and pesticides, as well as the need for ecotoxicological risk assessment of environmental pollutants to aquatic organism [19–21].

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Table 1
The classification and uses of five plant growth regulators.

PGRs	Chemistry class	Category	Applications
Atonik	Nitrophenolate [1]	Phytohormone	Atonik is the major hormone produced in response to fertilization and plays an important role in fiber production; it can increase fiber length in treated plants [2]
Cytokinin	Purine [1]	Phytohormone	High concentrations of cytokinins can inhibit cell proliferation and induce apoptosis in plants [3]
Ethephon	Tetracyclic diterpenoid	Phytohormone	Gibberellins are widely used to optimize growth in several products, particularly seedless grapes [4]
Gibberellic acid	Organophosphorous compound [1]	Organophosphorous type	Ethylene is a major component of the complex regulatory network that choreographs acclimation responses to submergence [5]
Paclobutrazol	Azole [1]	Azole type	Paclobutrazol is utilized in mango cultivation for growth control and to reduce pruning and tillage [6]

The life-cycle of daphnids contains 4 stages; embryo, neonate, juvenile, and adult stage. The different developmental stages have different sensitivities to environmental pollutants. In addition to the 48 h EC_{50} value of common *Daphnia* neonate, the effects of pollutants on the developmental stages have also been studied, such as life-cycle (21-day tests) effects, the spawning rate of *Daphnia,* and the impact on offspring [22–25,21]. Many studies also showed that embryos had a higher sensitivity to several toxicants than later stages of the life-cycle [26–28]. In addition to *Daphnia* embryo being applied to acute toxicity, it has been applied in a variety of growth hormone induced teratogenic studies in recent years, including insect growth hormone [29–31] and poultry and livestock growth hormone [32–34]. It has also been applied to human growth hormone [35,36]; Mu and LeBlanc [31] reported that vertebrates growth regulator (testosterone) has been shown to cause developmental arrest of embryonic daphnids (*Daphnia magna*); in very low concentrations (8 μM) it can lead to a decrease in the birthrate of female *Daphnia* offspring of 36% with abnormality (severe developmental retardation in shell and antennae) rate of 67%. As mentioned earlier, plant hormones are widely used in agriculture, but its biological effects in water, especially on the more sensitive embryo stage of *Daphnia*, has not been clearly reported to date.

The main purpose of this study was to investigate the effects of plant growth regulators on *Daphnia* neonate and the embryo stage. Five kinds of plant growth regulators (Atonik, Cytokinin, Ethephon, Gibberellic acid and Paclobutrazol) were selected. The aims of this study were to (a) investigate the biological acute toxicity of PGRs on neonate and embryo of *D. magna*, (b) evaluate PGRs induced teratogenic effect on *D. magna*, and (c) microscopic observation of PGRs' effects on organ development and teratogenic response of *D. magna* embryo.

2. Materials and methods

2.1. Chemicals

Five different kinds of plant growth regulators (PGRs) were prepared, namely Atonik (CAS No. 67233-85-6), purity > 97.0%, purchased from Meryer Chemical Co. (Shanghai, China); Cytokinin (CAS No. 525-79-1), purity 99%, purchased from Changzhou Share Chemical Co. (Jiangsu, China); Ethephon (CAS No. 16672-87-0), purity 98%, purchased from Meryer Chemical Co. (Shanghai, China); Gibberellic acid (CAS No. 77-06-5), purity 90%, purchased from Meryer Chemical Co. (Shanghai, China); Paclobutrazol (CAS No. 76738-62-0), purity > 97%, purchased from QingDao TengLong WeiBo Technology Co. (QingDao, China). The chemical properties of each PGR are listed in Table 2. The molecular structures of the 5 PGRs are shown in Fig. 1. Simulated high hardness medium [37] was used to prepare the series concentrations of dilution water for the acute toxicity test on neonates and embryos.

2.2. Neonate acute toxicity tests

Daphnia magna (*D. magna*) have been maintained parthenogenetically in Chung Shan Medical University, Taiwan since 2001. They have been kept in 10-l tanks on a windowsill of the laboratory at approximately 20 °C. The tanks generated enough green alga (*Chlorella vulgaris*) to sustain a colony of several hundred for at least 6 months. The tanks were topped up alternatively with dechlorinated and conditioned tap water to replenish water lost by evaporation and then aerated with filtered air.

In order to evaluate the neonate acute toxicity of each of the five PGRs, randomly selected neonates from laboratory cultures (<24 h old, ≧third-brood) were used. At each concentration of the five PGRs, four replicates with five neonates per replicate were employed according to US EPA procedure [38]. The immobilization and mortality for the individuals in each container were assessed under a low-magnification microscope (10–63 times magnification, Nikon SMZ800, Japan). Simulated high hardness medium [37] was used to prepare the series concentrations of dilution water for the acute toxicity test on neonates. Tests were performed in 50 ml of medium in 100 ml glass beakers. The beakers with the

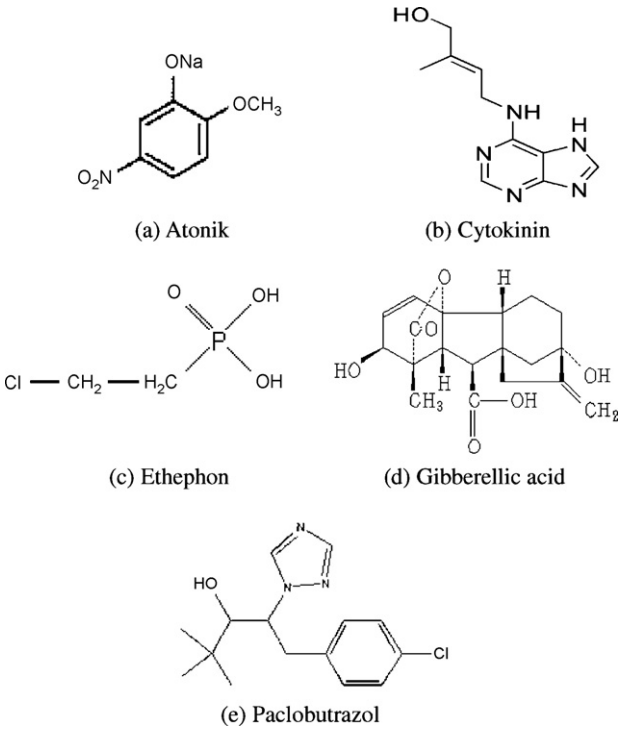


Fig. 1. Molecular structure of five plant growth regulators studied.

Table 2
Plant growth regulators' chemical properties.

PGRs	Chemical name	Molecular formula	M.W. ($g\,mol^{-1}$)	CAS No.	Purity	Water solubility	Purchase from
Atonik	5-nitroguaiacol sodium salt	$C_7H_6NNaO_4$	191.12	67233-85-6	>97.0%	Soluble	Meryer Chemical Co. (Shanghai, China)
Cytokinin	9H-purin-6-amine, N-(2-furanylmethyl)-	$C_{10}H_9N_5O$	215.21	525-79-1	99%	Soluble	Changzhou Share Chemical Co. (Jiangsu, China)
Ethephon	2-chloroehtyl phosphonic acid	$C_2H_6ClO_3P$	144.49	16672-87-0	98%	131 $mg\,l^{-1}$ (20 °C)	Meryer Chemical Co. (Shanghai, China)
Gibberellic acid	2,4a,7-Trihydroxy-1-methyl-8-methylenegibb-3-ene-1,10-dicarboxylic acid 1, 4a-lactone	$C_{19}H_{22}O_6$	346.3	77-06-5	90%	5 $g\,l^{-1}$	Meryer Chemical Co. (Shanghai, China)
Paclobutrazol	(+/-)-R*,R*-beta-((4-Chlorophenyl) methyl)-alpha-(1,1-dimethylethyl)-1H-1,2,4-triazol-1-ethanol	$C_{15}H_{20}ClN_3O$	293.79	76738-62-0	>97%	350 $mg\,l^{-1}$	QingDao TengLong WeiBo Technology Co. (QingDao, China)

neonates were placed in a constant growth chamber (temperature 20 ± 2 °C, under a 16/8-h light/dark cycle). Immobility was used as the endpoint for determining acute toxicity; daphnids showing no movement within 15 s after gentle stirring were defined to be immobile. The EC_{50} values were calculated by probit analysis [39] based on nominal concentrations.

2.3. Embryo acute toxicity tests

For these tests, the series of concentrations used was designed on the basis of the EC_{50} values obtained from the neonate acute toxicity test. The endpoint was defined on the basis of the ratio of live neonates hatched to the original number of exposed *D. magna* embryos.

After the dispensed eggs settled to the bottom of the well, the medium in each well was removed carefully and replaced with 300 μl of fresh simulated high-hardness medium containing chemicals at varying concentrations. Plates were kept at 20 ± 2 °C under a 16/8-h light/dark cycle and covered to minimize evaporation of the culture medium. The eggs were observed daily to check developmental differentiation and the incidence of premature death was recorded. In addition, hatched *D. magna* were inspected for gross morphological abnormalities under a low-magnification microscope (10–63 times magnification, Nikon SMZ800, Japan). Experiments were repeated 4 times. The 48 h EC_{50} values and 95% confidence limits were calculated by probit analysis [39] on the basis of nominal concentrations.

2.4. Embryo developmental teratogenic assay

The design of the series concentrations was based on embryo 48 h-lowest observed effect concentrations (LOEC) of those five plant growth regulators for embryo developmental teratogenic assay, which, in turn, were based on the above-mentioned data of the embryo acute toxicity test. In addition, hatched *D. magna* were inspected for gross morphological abnormalities under a low-magnification microscope (10–63 times magnification, Nikon SMZ800, Japan). The observation time was extended to 72 h because the PGRs would have affected embryo growth. In addition, gross morphological abnormalities (endpoints) including the formation of carapace, first and second antenna, ocellus, compound eye, rostrum, digestive caecum, Malpighian tube, brood chamber, sensory bristles, pigmentation in the body, and tail spine were studied. Meanwhile, using EDI (embryo development inhibition rate) [27] the development of embryos was observed at different times (24, 48, and 72 h) of teratogenic rate [40,41].

3. Results and discussion

3.1. D. magna neonate acute toxicity of plant growth regulators doses

In this study, the acute toxicity of PGRs, as defined by the 24 and 48 h EC_{50} value, was determined using neonatal daphnids, while the control groups, showing no mortality, were cultured in simulated high hardness medium (Table 3). The 24 h exposure indicated that Cytokinin is the most toxic to neonatal daphnids (24 h EC_{50} = 2.3 $mg\,l^{-1}$), followed by Gibberellic acid, Paclobutrazol, Atonik, and Ethephon as the least toxic (149.7 $mg\,l^{-1}$). Prolonged exposure time to 48 h showed a decline in the EC_{50} values of neonatal daphnids for the five PGRs, meaning that PGRs' toxicity increased with time. The 48 h EC_{50} values of neonatal daphnids for the five PGRs were Cytokinin (1.9), Gibberellic acid (22.5), Paclobutrazol (25.6), Atonik (36.2), and Ethephon (130.5 $mg\,l^{-1}$). Similarly, 48 h exposure revealed that Cytokinin is the most toxic to neonatal daphnids, while Ethephon is the least toxic. Of the five PGRs, the toxicity ratios of 24 h EC_{50}/48 h EC_{50} were in the range 1.1–1.5, which indicates that PGRs' acute toxicity to neonatal daphnids were mainly in the first 0–24 h, and continued to rise during 24–48 h.

D. magna, a small freshwater crustacean, is a keystone species in ecological food webs and a model species for toxicant exposure [42]. Most of the tests on zooplankton are performed with *Daphnia* (both *D. magna* and *D. pulex*). Tests on both species were included as input data for zooplankton acute toxicity where the endpoint was 48 h EC_{50} on immobilization [43]. Naturally occurring cytokinins are N_6-substituted adenine derivatives that generally contain an isoprenoid derivative side chain. These hormones influence numerous aspects of plant development and physiology [44]. Cytokinins have a delaying effect on leaf senescence and their levels have been observed to decline in senescing leaf tissues [45]. Cytokinin ribosides can induce apoptosis upon their phosphorylation in tobacco BY-2 cells [46]. Studies on the effects of cytokinin for daphnids and insects are limited. It is possible that cytokinin could inhibit *Daphnia* neonate growth or cause molted sheaths in the *Daphnia*, and it has been reported that high concentrations of cytokinins could inhibit cell proliferation and induce apoptosis in plants [45,46,3,47].

Paclobutrazol (PBZ) is a triazole compound [48], which is a soil acting growth retardant effective on a wide range of grass species and dicotyledonous weeds. Persistence of paclobutrazol residues in soil may result in contamination of nearby water bodies, which in turn may also be a hazard to human and animal health [49]. Using indicator plant bioassay, soil residues of paclobutrazol have been shown to be present 20 months after its application in sweet

Table 3
Toxicity values of plant growth regulators on *D. magna* neonates after 24 h and 48 h exposure ($n = 20$).

PGRs (mg l^{-1})	24 h EC_{50}	48 h EC50	24 h EC_{50}/48 h EC50
Control	–	–	–
Atonik	50.1 ± 3.5	36.2 ± 2.5	1.4
Cytokinin	2.3 ± 0.1	1.9 ± 0.3	1.2
Ethephon	149.7 ± 7.1	130.5 ± 3.2	1.1
Gibberellic acid	32.1 ± 3.3	22.5 ± 2.0	1.4
Paclobutrazol	38.3 ± 2.7	25.6 ± 1.7	1.5

Table 4
Toxicity values of plant growth regulators on *D. magna* embryos after 48 h exposure ($n = 20$).

PGRs (mg l^{-1})	Neonate 48 h EC_{50}	Embryo 48 h EC50	Embryo 48 h LOEC[a]	Neonate 48 h EC_{50}/ Embryo 48 h EC_{50}	Neonate 48 h EC_{50}/ Embryo 48 h LOEC
Control	–	–	–	–	–
Atonik	36.2 ± 2.5	26.1 ± 1.8	7.6 ± 0.5	1.4	4.8
Cytokinin	1.9 ± 0.3	0.64 ± 0.1	0.1 ± 0.0	3.0	19
Ethephon	130.5 ± 3.2	125 ± 6.3	48 ± 2.3	1.1	2.7
Gibberellic acid	22.5 ± 2.0	19.4 ± 1.1	8.5 ± 0.5	1.2	2.6
Paclobutrazol	25.6 ± 1.7	0.24 ± 0.0	0.05 ± 0.0	106	512

[a] Embryo 48 h-lowest observed effect concentration.

Table 5
Comparison of LOEC[a] abnormality rate of *D. magna* embryos caused by each plant growth regulator after 24 h, 48 h, and 72 h exposure ($n = 20$).

D. magna organs	Atonik				Cytokinin				Ethephon				Gibberellic acid				Paclobutrazol			
	24[b]	48	72	48/72	24	48	72	48/72	24	48	72	48/72	24	48	72	48/72	24	48	72	48/72
Second antennae	0	25	40	63	0	30	50	60	0	25	25	100	0	20	40	50	0	20	60	33
Rostrum	0	20	25	80	0	10	20	50	0	0	0	–	0	10	10	100	0	25	30	83
Malpighian tube	0	30	80	38	0	30	80	38	0	20	50	40	0	30	60	50	0	30	80	38
Sensory bristles	0	20	30	67	0	20	45	44	0	10	15	67	0	25	40	63	0	20	50	40
Tail spine	0	20	60	33	0	30	70	43	0	10	25	40	0	30	60	50	0	50	80	63
EDI rate	0	35	DG	ND	0	40	DG	ND	0	15	DG	ND	0	20	DG	ND	0	40	DG	ND

EDI: embryo development inhibition, DG: deformed growth, ND: no detection.
[a] Lowest observed effect concentration values of PGRs; atonik (7.6 mg l^{-1}), cytokinin (0.1 mg l^{-1}), ethephon (48 mg l^{-1}), gibberellic acid (8.5 mg l^{-1}), paclobutrazol (0.05 mg l^{-1}).
[b] Time exposed; 24 h, 48 h and 72 h.

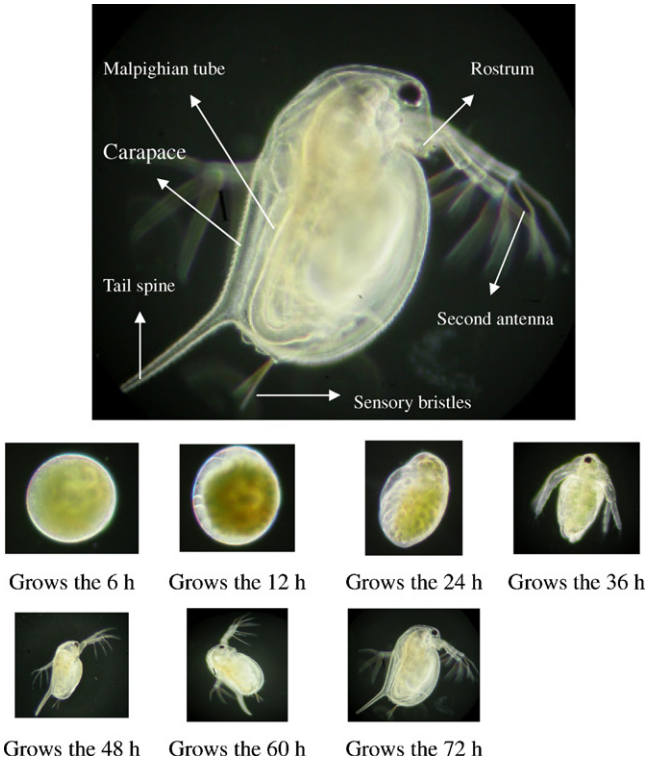
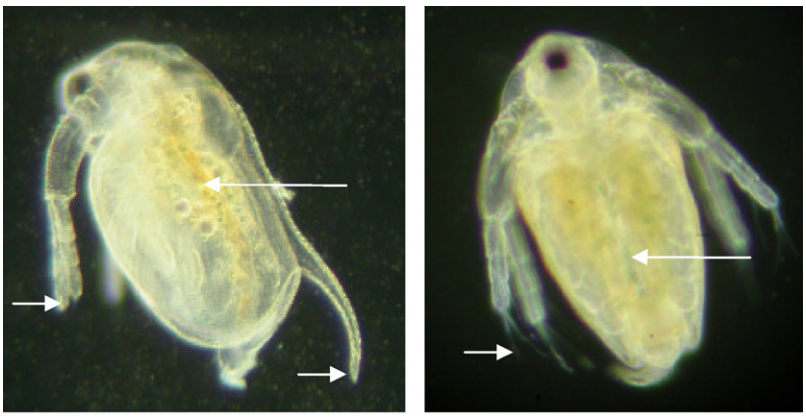


Fig. 2. Stages of *D. magna* early development *in vitro* (—: scale bar; 100 μm).

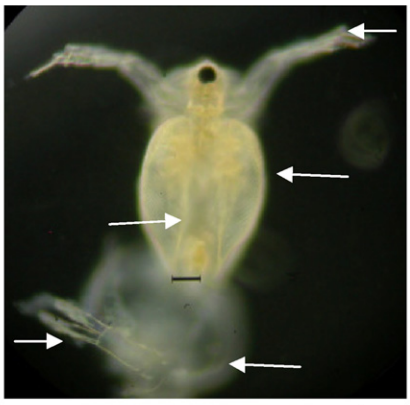
cherry orchards [50] and for almost 3 years after its application as trunk drench in apricot trees [51]. Thus, in areas where paclobutrazol is applied regularly, there may be a risk of environmental contamination due to its residues persisting in soil for a very long time. Silva et al. [6] reported that the problem with paclobutrazol utilization would be in its application for long periods, as it could accumulate in soil and harbor microflora due to its low degradation. This implies that paclobutrazol accumulates in *Daphnia* and is not easily removed through metabolism and decomposition; therefore, *Daphnia* neonate is poisoned. Previous studies reported that the half life ($T_{1/2}$) of [^{14}C]-paclobutrazol in aqueous suspension at 25 °C was 4.5 years [52], and soil metabolism studies indicated that N6-Benzyladenine has a half-life of 7–9 weeks [53]. Because cytokinin and paclobutrazol result in high acute toxicity in *Daphnia* and have a long half-life in water, future research should focus on the roles of cytokinin and paclobutrazol in other aquatic biological hazards.

3.2. *D. magna* embryo acute toxicity of plant growth regulators doses

The embryo acute toxicity of PGRs, as defined by the 48-h EC_{50} value, was determined using *D. magna* embryos, while the control groups, showing no mortality or growth retardation, were cultured with simulated high hardness medium (Table 4). The 24 h exposure indicated no *Daphnia* embryo mortality because it is not easy to determine since only the body surface and compound eye have been formed. According to the EC_{50} values of *D. magna* embryo, prolonged exposure time of up to 48 h revealed that, for the five PGRs, Paclobutrazol is the most toxic to embryos (0.24 mg l^{-1}),

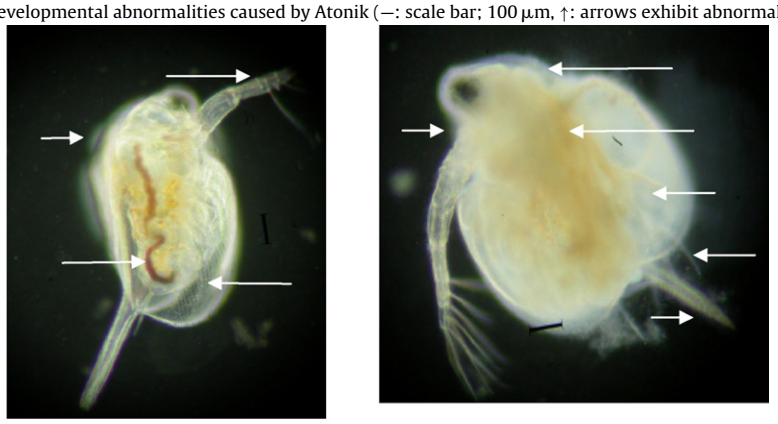


(a) Exposed the 48 h (19 mg l^{-1}). (b) Exposed the 72 h (15 mg l^{-1}).

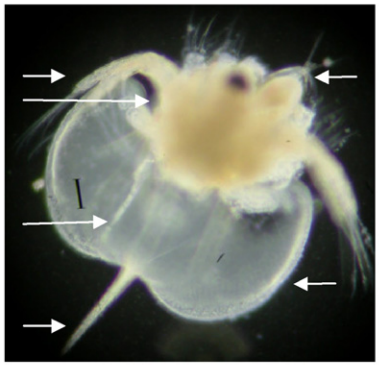


(c) Exposed the 72 h (19 mg l^{-1}).

Fig. 3. Developmental abnormalities caused by Atonik (—: scale bar; 100 μm, ↑: arrows exhibit abnormalities).



(a) Exposed the 48 h (1.5 mg l^{-1}). (b) Exposed the 72 h (0.6 mg l^{-1}).



(c) Exposed the 72 h (1.5 mg l^{-1}).

Fig. 4. Developmental abnormalities caused by Cytokinin (—: scale bar; 100 μm, ↑: arrows exhibit abnormalities).

followed by Cytokinin (0.64), Gibberellic acid (19.4), Atonik (26.1), and Ethephon (125).

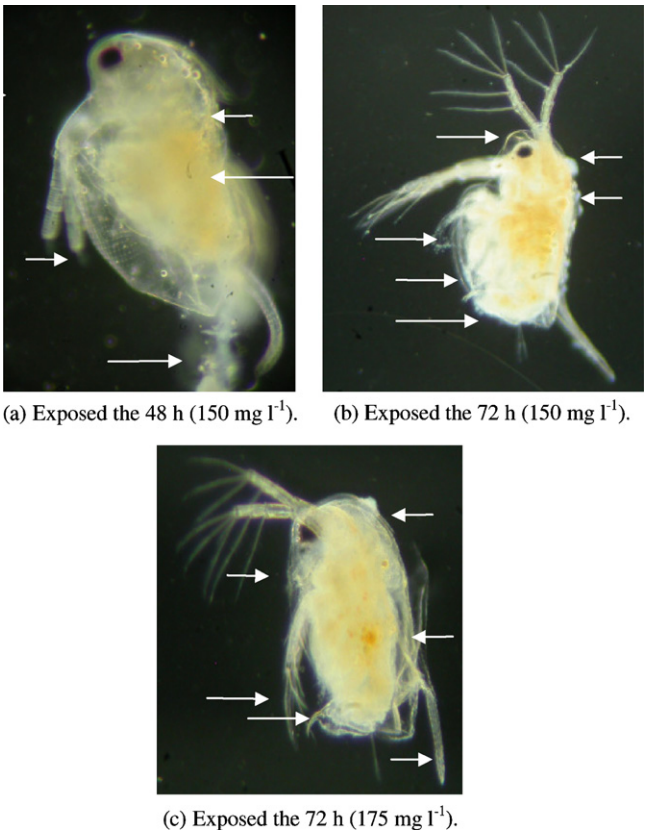
Table 4 also shows 48-h LOEC values of PGRs on *Daphnia* embryo; the 48-h LOEC values are significantly lower than the 48 h EC_{50}'s. This study revealed that Paclobutrazol is the most toxic to *Daphnia* embryo (48 h LOEC = 0.05 mg l^{-1}), followed by Cytokinin (0.1 mg l^{-1}), and the least toxic is Ethephon (48 mg l^{-1}). For the five PGRs, the toxic ratios of neonate 48 h EC_{50}/embryo 48 h EC_{50} revealed that Cytokinin and Paclobutrazol were more toxic for embryos than for neonates (3.0 and 106 times, respectively), while Atonik, Ethephon, and Gibberellic acid showed toxic ratios in the range 1.1–1.4. Further comparison of the toxic ratios of neonate 48 h EC_{50}/embryo 48 h LOEC for the five PGRs similarly revealed that Cytokinin and Paclobutrazol were more toxic for embryos than for neonates (19 and 512 times), while Atonik, Ethephon, and Gibberellic acid showed toxic ratios in the range 2.6–4.8. From these results can be seen that the neonate stage of *Daphnia* has more toxicity resistance than the embryo stage, which means embryos were markedly more sensitive to some PGRs than neonates. The toxic ratios of Cytokinin (19) and Paclobutrazol (512) were higher than those of the other three, which were under 10.

As mentioned earlier, the life-cycle of daphnids contains 4 stages; embryo, neonate, juvenile, and adult stage; exposure of any stage to PGR pollutants is possible at any time. In the neonate acute toxicity test, immobility or mortality were expressed as study endpoints, but in the embryo acute toxicity test, inhibited embryo development, organ deformities, or mortality were expressed as study endpoints because PGRs inhibit continued growth of embryos and neonates. Until now, only a few investigations have been undertaken to determine the effects of environmental pollutants on the developmental stages of daphnids, whose embryos are commonly used in ecotoxicological research. In one study, exposure of Hg at 10 μg l^{-1} for 24–72 h induced abnormal development in the cephalic and body regions and undeveloped formation of second antennae of *D. carinata* [28]. The agricultural fungicide fenarimol (10 μM) can cause *D. magna* embryo developmental arrest and incomplete development of antennae and shell spines [54]. In another study, 4-nonylphenol (800 μg l^{-1}) caused *D. magna* embryo developmental arrest and deformities of shell spines and antennae [55]. Baird et al. [27] showed that embryos had a higher sensitivity to several toxicants than later stages of the life-cycle. Abe et al. [26] also supported the same point that embryo assay was more sensitive to aniline derivatives than acute juveniles immobilization assay. Reports further noted that ratios of 48 h EC_{50} (juvenile)/72 h EC_{50} (embryo) for eight anilines were greater than 5.0. EC_{50}s for embryos and juveniles were poorly correlated. This indicated that the sensitivities of the two life stages to the effects of anilines were different [26]. In this study, *Daphnia* embryos are more sensitive to Paclobutrazol and Cytokinin than neonates. Hence, these results suggest that future assessment of the effect of these two PGRs on aquatic organisms should receive special attention.

3.3. *D. magna* embryo developmental affects of plant growth regulators doses

In order to evaluate the lowest observed effect concentration of PGRs on *D. magna* embryos, gross morphological abnormalities (e.g., carapace, second antennae, rostrum, Malpighian tube, sensory bristles, and tail spine) were expressed as study endpoints. For PGRs' effects resulting in *Daphnia* embryo growth retardation, observation time was extended to 72 h.

First, the control group is discussed. Fig. 2 shows the growth of the control group of *D. magna* embryos in different stages. Fig. 2a illustrates the growth at 6 h when embryonic differentiation has not occurred and the embryo is still round. Fig. 2b presents the growth at 12 h when cells in embryos start to differentiate. Fig. 2c shows the growth at 24 h when the body surface and compound eye have formed gradually. Fig. 2d illustrates the growth at 36 h when the ocellus and compound eye are already separated, the second antenna has appeared, and the tail spine has formed without breaking away from the tail-end. Fig. 2e shows the growth at 48 h when the Malpighian tube has matured with straight tail spine. Fig. 2f presents the growth at 60 h when the first and second antennas have developed completely. Fig. 2g shows the growth at 72 h when embryos have developed into mature zooids.

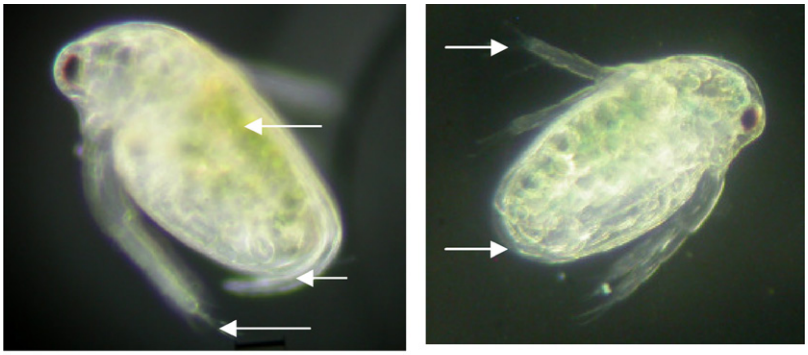

(a) Exposed the 48 h (150 mg l^{-1}). (b) Exposed the 72 h (150 mg l^{-1}).

(c) Exposed the 72 h (175 mg l^{-1}).

Fig. 5. Developmental abnormalities caused by Ethephon (—: scale bar; 100 μm, ↑: arrows exhibit abnormalities).

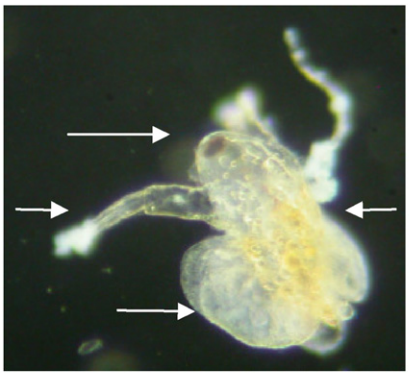
The summary of *D. magna* embryos exposed to PGRs at 24, 48, and 72 h, causing embryo developmental arrest or teratogenesis, are presented in Table 5. The 24 h exposure indicated no abnormalities in embryonic development because it is not easy to determine since only the body surface and compound eye have been formed. The 48-h EDI rate of PGRs on embryo developmental progression was 15–40%. At 48 h the rate of PGRs on the teratogenic embryo endpoint was 0–50%. At 72 h, Atonik, Cytokinin Gibberellic acid, and Paclobutrazol had high teratogenic rates (60–80%) on the Malpighian tube and tail spine of neonatal daphnids, while that of Ethephon was 25% on the tail spine, which were higher than those on other organs (second antennae, rostrum, and sensory bristles). These results clearly demonstrate that PGRs induced growth retardation and had teratogenic effects on *D. magna* embryos. The ratios of 48 h/72 h indicate that rates of Ethephon and Gibberellic acid on the second antennae and rostrum, respectively, were 100%. In addition, rates of Atonik and Paclobutrazol on the rostrum were 80–83% (Table 5). In this study, the second antennae and rostrum may be interpreted as early stage developmental organs.

Microscopy showed that exposure of *D. magna* embryos to different doses of PGRs solution at different times led to the



(a) Exposed the 48 h (12 mg l^{-1}).

(b) Exposed the 48 h (15 mg l^{-1}).

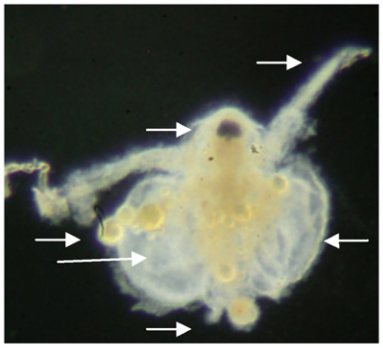


(c) Exposed the 72 h (15 mg l^{-1}).

Fig. 6. Developmental abnormalities caused by Gibberellic acid (↑: arrows exhibit abnormalities).

(a) Exposed the 48 h (0.1 mg l^{-1}).

(b) Exposed the 48 h (0.2 mg l^{-1}).



(c) Exposed the 48 h (0.8 mg l^{-1}).

Fig. 7. Developmental abnormalities caused by Paclobutrazol (↑: arrows exhibit abnormalities).

phenomenon of variations in neonatal organs, including: (i) underdeveloped second antennae (Figs. 3–7), (ii) breakage or damage of Malpighian tube (Figs. 4–7), (iii) curved and unextened tail spine (Figs. 3, 5, 6, 7), (iv) incomplete shedding of body shell (Figs. 3, 5, 7), and (v) abnormal development in the cephalic and body regions (Figs. 4, 5, 7). In this study, the effects on growth mostly occurred in the late stage (stage 4, 5 and 6) of organogenesis. It should be noted that permeability of compounds into embryos at early stages of development might be low because the embryo is covered with a thin transparent egg shell until stage 4. This phenomenon was also observed by Ohta et al. [56] who used *D. magna* embryos treated with teratogenic compound ethylenethiourea. Some studies have shown that embryonic developmental abnormalities mostly concern the compound eyes, carapace, and second antennae of *D. magna* [17,56,57].

In this study teratogenic effects were most significant at 48–72 h; they were not significant before 48 h. For example, Malpighian tube and tail spine deformities caused by PGRs were significant at 72 h. Second antennae deformity caused by Ethephon and rostrum deformity caused by Gibberellic acid were significant at 48 h. In addition, PGRs also showed teratogenic phenomena in other *Daphnia* embryo endpoints. The present study clearly demonstrated that PGRs may cause both acute toxicity and teratogenic effects in neonate and embryo of *D. magna*. In this study, *D. magna* embryo teratogenic effects were used to detect the toxicity of PGRs, which provide indicators of sensitivity and teratogenic. The parthenogenetic development stages of *Daphnia* embryo have the potential to be used as an important research tool for the evaluation of the effects of related environmental hormones on aquatic ecosystems. In addition, studies on the effects of PGRs on other aquatic organisms are limited to date. It is recommended to further investigate the biological effects of PGRs on other water bodies.

4. Conclusions

Results from the present study demonstrate that acute toxicity of PGRs on *D. magna* embryo assay system is more sensitive than generally used 24-h old neonate bioassay. The toxic ratios of neonate 48 h EC_{50}/embryo 48 h LOEC revealed that PGRs are more toxic on embryos than on neonates (2.6–512 times). The 48 h LOEC values of PGRs on *Daphnia* embryos indicated that Paclobutrazol and Cytokinin are most teratogenic (0.05 and 0.1 mg l^{-1}, respectively) followed by Atonik, Gibberellic acid, and Ethephon with threshold teratogenesis concentrations of 7.6, 8.5, and 48 mg l^{-1}, respectively. The LOEC values of PGRs on *Daphnia* embryo endpoints (carapace, second antennae, rostrum, Malpighian tube, sensory bristles, and tail spine) indicated that PGRs have teratogenic effect on *Daphnia* embryos, with the exception that Ethephon had no teratogenic effect on the rostrum. In this study, PGRs' teratogenic effect increased with increasing exposure time (0–72 h). Ethephon caused second antennae deformity and Gibberellic acid caused rostrum deformity that could be observed significantly at 48 h exposure. PGRs caused significant Malpighian tube and tail spine deformities (these showed high teratogenic rate of 60–80%) at 72 h exposure as opposed to its teratogenic lowest, namely the rostrum (teratogenic rate of 0–30%). These suggest that the development of the rostrum of *Daphnia* embryos is affected the most by acute toxicity, and its teratogenic effect is the least. The present study clearly demonstrated that PGRs may cause acute toxicity and teratogenic effect in both neonate and embryo of *D. magna*. In addition, studies on the effects of PGRs on other aquatic organisms are limited to date. It is recommended that the biological effects on other water bodies be further investigated.

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