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Determination of Acute Toxic Effects of Poly (Vinylferrocenium) Supported Palladium Nanoparticle (Pd/PVF$^+$) on *Artemia salina*

Yeşim Dağlıoğlu,[1,*] Mutlu Sönmez Çelebi[2] and Şükrü Önalan[3]
[1]*Biology Department, Faculty of Arts and Sciences, Ordu University, 52200, Ordu, Turkey*
[2]*Chemistry Department, Faculty of Arts and Sciences, Ordu University, 52200, Ordu, Turkey*
[3]*Aquaculture and Diseases of Department, Faculty of Aquaculture, Yüzüncü Yıl Universty, Van, Turkey*

ABSTRACT

Platinum-group elements are released to environment by only anthropogenic activities. It is commonly used in many fields such as technology, medical field and especially catalytic converters. The toxicological effects of Poly(vinylferrocenium) (PVF$^+$), PVF$^+$-supported palladium nanoparticles (Pd/PVF$^+$), and K_2PdCl_4 have been determined on a brine shrimp *Artemia salina* larvae (nauplii). Lethal concentration values of K_2PdCl_4, PVF$^+$ and Pd/PVF$^+$ were 1987.567 mg/L, 242.67 mg/L and 5467.546 mg/L, respectively.We found that toxic effects of K_2PdCl_4, PVF$^+$ and Pd/PVF$^+$ nanoparticles increased with exposure duration. In other words, the toxic effects of these strongly time-dependent toxic effect. Toxicity ranking for 48-h exposure time; PVF$^+$, K_2PdCl_4 and Pd/PVF$^+$, 96-h PVF$^+$, Pd/PVF$^+$ and K_2PdCl_4, respectively. According to ANOVA significant differences were found at the level of $P<0.05$ or $P<0.01$ between mortality rates between time grous and concentrations for these three groups at the end of the exposure duration.

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Authors' Contributions
YD performed the experiments, and statistical analysis, and wrote the article. MSÇ synthesized nanoparticles and helped in experiments. ŞÖ supplied the shrimps and prepared figures for the article.

Key words
***Artemia salina*, palladium nanoparticles, acute toxicity, lethal concentration.**

INTRODUCTION

It is a well known reality that most of the industrial wastes and urban waste water discharges mix with rivers, lakes and coastal waters. The mixing of nano-dimensional industrial products and byproducts to aquatic environments is unavoidable (Daughton, 2004; Donaldson *et al*., 2004). Nanoparticles (NPs) have very different physical and chemical characteristics from the known substances. Since they tend to distribute and precipitate in the aquatic environment depending on their dimension and distribution factors (Limbach *et al*., 2005), mobile systems should be used in investigating their toxic effects in the aquatic phase.

Artemia salina (Crustacea, Anostraca) is involved in significant energy flow in the food chain in ecosystem (Lewan *et al*., 1992; Kanwar, 2007). This shrimp may be used in a laboratory experiment to determine LC_{50} (Meyer *et al*., 1982). The bioassays with *A. salina* have been used for screening of teratological agent (Carballo *et al*., 2002, drugs (Kanwar, 2007) hepatotoxic cyanobacterial strains (Lee *et al*., 1999), food additives (Kerster and Schaeffer, 1983), and plant products (Carballo *et al*., 2002; Silva *et al*., 2007). It has gained popularity as a test organism because of its ease of culture, short generation time, cosmopolitan distribution and the commerical availability of its dormant cysts (Barahona and Sanchez-Fortun, 1999).

Therefore, a testing system that provides a mobile environment for toxicity tests and continuous oxygen support for the testing organism without affecting that organism has been developed. In this study, a comparative evaluation of the toxicity of K_2PdCl_4 from which Pd nanoparticles (NPs) were obtained, PVF$^+$ as the supporter of Pd NPs, and PVF$^+$-supported Pd NPs (Pd/PVF$^+$) has been done on *Artemia salina*.

MATERIALS AND METHODS

Test chemicals

Poly (vinylferrocenium)-supported pd NPs were prepared according to the procedure described by Çelebi *et al*. (2008). K_2PdCl_4 (≥98.2%) was purchased from Merck.

Preparing test organisms

A. salina cysts (salt lake aqua feed premium artemia cysts) were incubated in artificial seawater, prepared by using artificial ocean salt. The artificial sea water was left to rest for a day in the laboratory and was filtered with a 30-µm milipore cellulosic filter. *Artemia* in

* Corresponding author: yozkan52@gmail.com
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cyst form were hydrated in distilled water at 4°C for 12-h and was washed to separate the sunken cysts from floating cysts. The sunken cysts were washed in deionized waster and collected by a funnel or pipette. 3g of precleaned cysts were incubated in conic plastic bottles containing 1.5 liter seawater at 30±1°C and *artemia* hatched in 24-h. Continuous sunlight was provided with fluorescent lamps. *Artemia* count was done according to the procedure describe by Sorgeloos (1980). For every study to be done, the *Artemia* count was done separately. In short, 100 ml solution containing hatched *Artemia* nauplii were placed in a clean beaker. This solution was constantly mixed to maintain homogeneity, and 1ml of the stock solution was diluted to 100 ml with seawater. This new solution was also continuously mixed; 0.1 ml of this solution was taken and placed on a petri dish for counting. The nauplii count was determined with this volume under a stereo microscope (Leica S8APO). This count was done separately for each elimination, accumulation, and LC_{50} tests.

Preparing the aqueous suspension of Pd/PVF, PVF^+ and K_2PdCl_4

In order to prepare the stock solutions in desired concentrations, the test materials Pd/PVF^+, $PVF^+$and K_2PdCl_4 composites powders were dispersed in deionized water. Then this solution was vortexed for 30 seconds, and ultrasound water bath was used (Bandelin, sonorex) to increase dispersion and to provide maximum distribution of NPs. After these steps, the determined concentrations were obtained by diluting the stock solution.

Experimental setup

NPs have different physical and chemical properties than those of conventional materials. As they are prone to aggregate and sink in the aqueous medium they are kept in motion to study their toxic behavior. For this reason, a new test system that provides motion but does not affect the test subjects needs to be developed. For this purpose, 2 liter conic polyethylene bottles were used in exposure tests. A hole was punctured so that thin plastic tubes could enter. To prevent liquid discharge from the lid, it was sealed with silicone and parafilm. To control the air supply and to ensure easy collection of the *Artemia*, a valve was placed. 100 ml flasks were used for experiments for LC_{50} experiments. In this way, a constant mixing was provided without harming the organisms and constant oxygen was provided for *Artemia.* In order to use the air pump in more than one bottle, a thin tube was put in the motor's exit, the free end of this tube was connected by a T, and a check valve was placed in order to prevent return flow. At the same time, a plastic lid was placed on top of the system to prevent water evaporation. These systems were used in the 24 and 96 h test period. The water wasn't replaced during the experiment and no intervention was done.

LC_{50} value was calculated through the probit analysis.

Determining the acute toxicity of Pd/PVF^+, PVF^+ and K_2PdCl_4

Acute exposure test for *Artemia* nauplii was carried out according to the OECD 202 test guide. The determined concentration of Pd/PVF^+, PVF^+ and K_2PdCl_4 were applied on the *Artemia* culture. The control groups were without the testing compounds. The experiments were carried out in triplicate in conic plastic containers (0.5l). The exposure tests for LC_{50} experiments were carried out in 100 ml volume. For LC_{50}, 50 individuals that were most mobile under the microscope and with the healthiest extremities were chosen. In all concentrations being studied, the dead nauplii were counted under the stereomicroscope at 24, 48, 72 and 96th h. To prevent the NPs sedimentation, aeration throughout the exposure was provided by aeration tube connected to the bottom of the conic bottle. The experiments were carried out in a 16 h light, 8 h dark medium at 24±2°C. The pH of the medium was measured before and after the experiment, and the mean value was calculated as 7.80±2. The *Artemia* were not fed during the exposure tests. After the acute toxicity tests, the changes that took place in the larvae (nauplii) exposed to NPs were recorded with the help of analyses under the phase contrast microscope (Nikon Eclipse 80 i). Every exposed group was compared to the control group and the potential anomalies were recorded.

RESULTS AND DISCUSSION

Platinum-group elements are released to environment by only anthropogenic activities. It is commonly used in many fields such as technology, medical field and especially catalytic converters. The environment can't sufficiently eliminate these elements and thus they are collected in the environment (Ek *et al*., 2004). The pollution due to platinum-group elements occur in water, mud, soil, roadside dusts and airborne particulate matters. Consequently, a biological collection occurs in the living organisms (Ravindra *et al*., 2004). Platinum-group elements are detected in living and nonliving environments of the cities, and even in the snowy fields of (Barbante *et al*., 2001; Speranza *et al*., 2010).

Pd NPs is administered at very low concentration. One of such studies involved the effect of kiwifruit pollen on *in vitro* pollen performance (Speranza *et al*., 2010).

Table I.- LC/EC values for 96 and 48 hour calculated for nanoparticles Pd/PVF$^+$, K_2PdCl_4 and PVF$^+$.

Points	Pd/PVF$^+$		K_2PdCl_4		PVF$^+$	
	48	96	48	96	48	96
LC/EC 1.00	0.000	0.000	0.000	0.000	0.000	0.000
LC/EC 5.00	0.001	0.000	0.013	0.000	0.000	0.000
LC/EC 10.00	0.034	0.000	0.183	0.000	0.003	0.001
LC/EC 15.00	0.334	0.000	1.081	0.003	0.027	0.002
LC/EC 50.00	5 467.546	0.562	1 987.567	7 849 373.500	242.67	0.150
LC/EC 85.00	89 452 200.000	68 296.547	3 654 593.000	2 034 619.209	2 222 129.25	13.858
LC/EC 90.00	888 231 936.000	1 089 700.750	21 636 450.000	3 432 753.642	19 234 208.00	40.462
LC/EC 95.00	26 646 028 288.000	66 007 232.000	301 668 768.000	6 847 096.107	470 791 552.00	197.932
LC/EC 99.00	1 570 625.290	145 351 426 048.000	42 252 144 640.000	1 058 480.786	189 476 962 304.00	3 887.560

They have compared Pd NPs with $PdCl_2$ in their study and have reported that Pd NPs was very much more toxic. In another study, *in vitro* evaluation of the Pd NPs and its composites designed to evaluate the effects of the automotive pollution by air has been performed in human alveolar carcinoma cell line. The reduction in the epithelial cells and the NPs included in these epithelial cells were detected by TEM. Caspase activation and apoptosis were found in the epithelial cells incubated with high Pd particle whereas those are not observed in the carcinoma cells (Wilkinson *et al*., 2011). In a different study, inhibitory growth effects of the Pd NPs and Pd^{+2} on gram negative *Escherichia coli* and gram positive bacteria *Staphylococcus aureus* cultures have been monitored. It has been also reported as a consequence of this study that antimicrobial activity may demonstrate significant differences depending on dimension, and that Pd NPs shows high antimicrobial activity and that thin-sized differences (<1 nm) may change antimicrobial activity (Adams *et al*., 2013).

In the study of Kanchana *et al*. (2013), dihydrolipoic acid (LA) and photochemical *Solanum trilobatum* (SN) have been used for production of palladium NPs and its nanoformulation was synthesized (SNDP and SNDP-LA NPs). Following, SNDP and SNDP-LA NPs have been evaluated in the therapeutic activities including oxidative stress parameters, analysis of cellular morphology and cell vitality in the human lung cancer cells. A remarkable dose-dependent cytotoxicity due to SNDP-LA NPs have been encountered in the lung cells.

Shoaib *et al*. (2012) reported LC_{50} of methyl parathion 0.00011 ppm against mysids and 0.11 ppm againt *Artemia*. Similarly the LC_{50} of fenvalerate was found to be 0.0004 ppm for mysids and 0.18 ppm for *Artemia*.

Acute toxicity/LC_{50}

LC_{50} at 48 h and 96 h for the same concentrations of Pd/PVF$^+$ NPs, K_2PdCl_4 and PVF$^+$ were comparatively evaluated. 5467.546 mg/L and 0.562 mg/L; 1987.567 and 7849373.500 and 242.67 mg/L and 0.150 mg/L, respectively. These values demonstrate that toxic effect depends on exposure duration (Table I).

In the comparison of acute toxic effects conducted on the same concentrations of Pd/PVF$^+$ NPs, PVF$^+$ and K_2PdCl_4 powders at 48th and 96th h; the results revealed that most toxic agent at 48th h was PVF$^+$. LC_{50} calculations showed that PVF$^+$ NPs was 22-fold and 8-fold more toxic than Pd/PVF$^+$ particle and K_2PdCl_4, respectively while K_2PdCl_4 was 3-fold more toxic than Pd/PVF$^+$ NPs. For the exposure at 96-h, PVF$^+$ NPs (0.150 mg/L) was the most toxic and Pd/PVF$^+$ particle was coming after while K_2PdCl_4 was found the least toxic. LC_{50} values for 96th h demonstrated very interesting results. PVF$^+$ powder was 3-fold more toxic than

Pd/PVF$^+$ NPs while toxic effect of K_2PdCl_4 decreased significantly by the prolonged exposure duration (789373.500 mg/L). This value is incomparably higher than the others. We have found when we interpreted these results that to toxic effect of K_2PdCl_4 decreased by time or further it lost toxic effect in contrast to toxic effects of NPs Pd/PVF$^+$ and PVF$^+$ powder. Similarly, toxic effect of Pd/PVF$^+$ NPs increased the most by increasing exposure time.

Analysis of exposure durations and concentration groups

According to ANOVA results between mortality rates due to different concentrations of Pd/PVF$^+$ at the end of the exposure duration, significant differences at the level of P<0.05 were found between concentration intervals 0*-0.01 mg/L and 0*-0.1 mg/L whereas another significant difference at the level of P<0.01 was found between concentrations of 0* and 1 mg/L. Between mortality rates due to different exposure duration of Pd/PVF$^+$; significant difference at the level of P<0.05 were found between exposure durations of 96th h and 72th h and whereas significant differences at the level of P<0.01 was found between concentrations exposure duration intervals of 96th-24th h and 96th and 48th h (Table III) . Exposure duration and concentration of Pd/PVF$^+$ NPs for *Artemia salina* the mortality rates increased with each increased concentration (0.01; 0.1 and 1 mg/L) for different time periods. However, this increase was not directly proportional and has proceeded slowly in the first hours. Mortality rates at the end of exposure of Pd/PVF$^+$ for 96 h were 18%, 20% and 27%, respectively (Figs. 1, 2).

In Table III, according to ANOVA results between mortality rates due to concentration groups of K_2PdCl_4 at the end of the exposure duration, significant differences at the level of P<0.01 were found between concentration intervals 0*mg/L – 0.1 mg/L and 0*-1 mg/L whereas significant differences at the level of P<0.05 was found between concentrations of 0.01mg/L-0*mg/L and 0.01mg/L and 1mg/L. Between mortality rates due to different exposure duration of K_2PdCl_4; significant differences at the level of P<0.05 were found between exposure durations of 24th-72th h and 48th and 96th h whereas a significant difference at the level of P<0.01 was found between concentrations exposure duration interval of 24th-96th h. Mortality rate of *Artemia salin*a depending on exposure duration and concentration increased by time, however that increase was directly proportional for 0.1 mg/L concentration whereas no mortality was reported for 0.01 mg/L and then mortality rate increasingly continued until 72 h and decreased after that hour. For 1 mg/L concentration; mortality rate increased for especially between 48 h and 72-h (Figs.1,2).

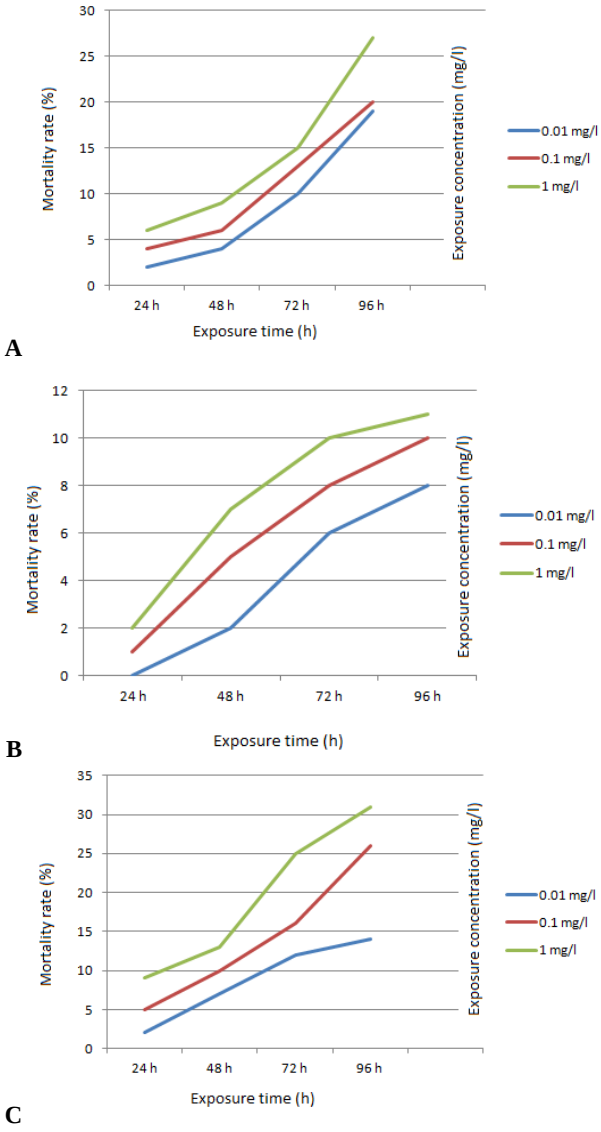

Fig. 1. Effect of different concentrations of Pd/PVF$^+$ administration on mortality rate of *Artemia* nauplii exposed to different concentration of Pd/PVF$^+$ of for 96 h..

In Table III, according to ANOVA results between mortality rates due to concentrations of PVF$^+$ at the end of the exposure duration, significant differences at the level of P<0.05 were found between concentration intervals 0.01-1 mg/L and 0.01-0* mg/L whereas another significant difference at the level of P<0.01 was found between concentrations of 0* mg/L and 1 mg/L. Between mortality rates due to different exposure duration of PVF$^+$; significant differences at the level of P<0.05 were found between exposure durations of 24-72 h, 24-96 h

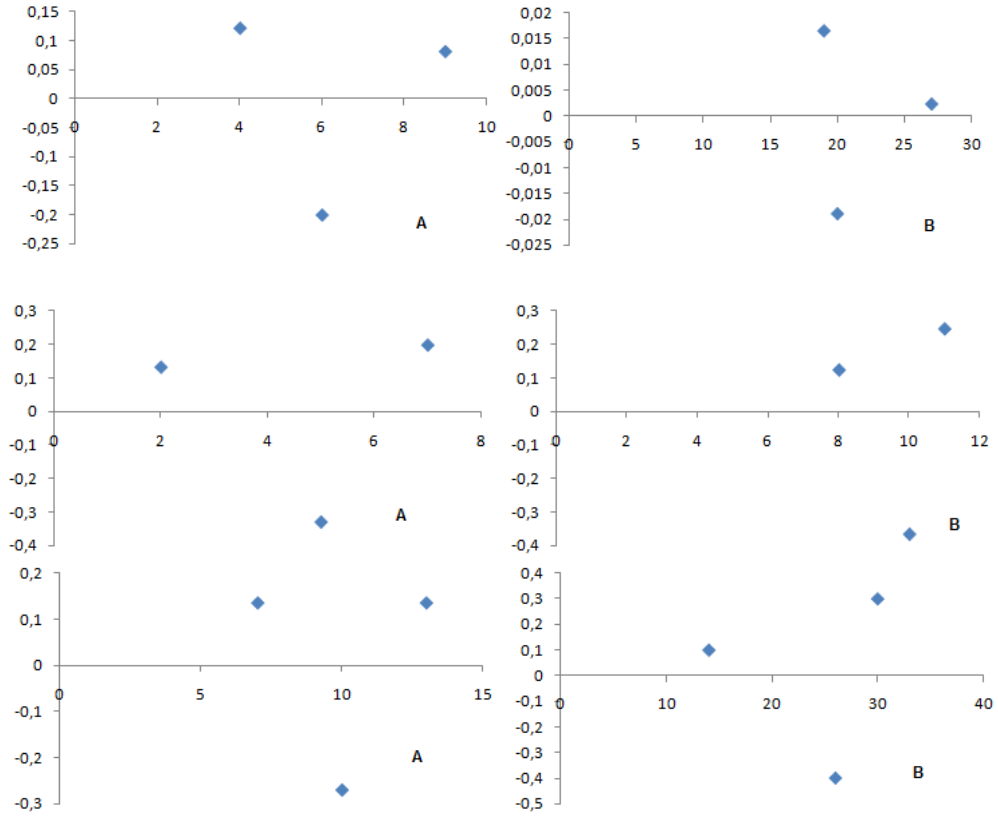

Fig. 2. Regression distributions for Pd/PVF+ nanoparticles (top), K_2PdCl_4 nanoparticles (middle), and PVF+ (bottom) for 48-h (A) and for 96-h (B).

Table II.- The results of Tukey's test with respect to differences between mortality rates based on varying concentration and exposure durations to Pd/PVF+, K_2PdCl_4 and PVF+.

Time (h)	N	Subset for alpha = 0.05			Con. (mg/L)	N	Subset for alpha = 0.05		
		1	2	3			1	2	3
Pd/PVF+									
24	12	0.0567[a]			*0	12	0.0000[a]		
48	12	0.0967[a]			0.01	12		0.1750[b]	
72	12	0.1850[a]			0.1	12		0.2133[b]	
96-h	12		0.3283[b]		1	12		0.2783[b]	
Sig.		0.074	10.000		Sig.		10.000	0.206	
K_2PdCl_4									
24	12	0.0167[a]			0*	12	0.0000[a]		
48	12	0.0683[ab]	0.0683[ab]		0.01	12		0.1733[b]	
72	12		0.1150[bc]	0.1150[bc]	0.1	12		0.2817[cb]	0.2817[cb]
96	12			0.1433[c]	1	12			0.3933[c]
Sig.		0.229	0.312	0.715	Sig.		10.000		0.188
PVF+									
24	12	0.0783[a]			0*	12	0.0000[a]		
48	12	0.1500[ab]	0.1500[ab]		0.01	12		0.1733[b]	
72	12		0.2633[bc]	0.2633[bc]	0.1	12		0.2817[cb]	0.2817[cb]
96	12			0.3567[c]	1	12			0.3933[c]
Sig.		0.728	0.366	0.535	Sig.		1.000		0.188

0* mg/L, control group; con., concentration.

Table III.- The Difference between the Pd/PVF$^+$, K_2PdCl_4, K_2PdCl_4 concentration and time groups according to ANOVA multiple comparison test.

(I) Con. mg/L	(J) Con. mg/L	Mean difference (I-J)	Standartd error	Sig.	95% Confidence interval	
					Lower bound	Upper bound
Pd/PVF$^+$						
0*	0.01	-0.17500*	0.05184	0.008	-0.3134	-0.0366
	0.1	-0.21333*	0.05184	0.001	-0.3518	-0.0749
	1	-0.27833**	0.05184	0.000	-0.4168	-0.1399
(I) Time (h)	(J) Time (h)					
96	24	0.27167**	0.05141	0.000	0.1344	0.4089
	48	0.23167**	0.05141	0.000	0.0944	0.3689
	72	0.14333*	0.05141	0.038	0.0061	0.2806
K_2PdCl_4						
0.01	1	-0.06667*	0.02419	0.041	-0.1313	-0.0021
	0*	0.08000*	0.02419	0.010	0.0154	0.1446
0*	0.01	-0.08000*	0.02419	0.010	-0.1446	-0.0154
	0.1	-0.11667**	0.02419	0.000	-0.1813	-0.0521
	1	-0.14667**	0.02419	0.000	-0.2113	-0.0821
(I) Time (h)	(J) Time (h)					
24	72	-0.09833*	0.02670	0.003	-0.1696	-0.0270
	96	-0.12667**	0.02670	0.000	-0.1980	-0.0554
48	96	-0.07500*	0.02670	0.036	-0.1463	-0.0037
PVF$^+$						
1	0.01	0.22000*	0.05466	0.001	0.0740	0.3660
	0*	0.39333**	0.05466	0.000	0.2474	0.5393
0*	0.01	-0.17333*	0.05466	0.014	-0.3193	-0.0274
	0.1	-0.28167**	0.05466	0.000	-0.4276	-0.1357
(I) Time (h)	(J) Time (h)					
24	72	-0.18500*	0.06897	0.049	-0.3691	-0.0009
	96	-0.27833*	0.06897	0.001	-0.4625	-0.0942
48	96	-0.20667*	0.06897	0.022	-0.3908	-0.0225

0* mg/L, control group; con., concentration; sig., significance.

and 48-th and 96 h groups. When we interpreted mortality rates of PVF$^+$ on *Artemia salina* depending on time and duration, it can be stated that mortality rate of 0.01 mg/L continuously increased until 72th h and then continuously decreased after 72th h. In contrast to that, mortality rate continuously increased after 72th h. Mortality rate for 1 mg/L increased in especially exposure time intervals between 48th h and 96th h (Figs. 1, 2).

When we have comparatively evaluated the differences between mortality rates at the end of exposure durations depending on exposure duration (time interval/h) and concentration groups for Pd/PVF$^+$ NPs, PVF$^+$ and K_2PdCl_4 powders conducted on the same concentrations on *Artemia salina*, most significant differences were encountered at 96th h for these three substances during the distinct time interval groups. Between the concentration groups; 1 mg/L demonstrated highest difference for each of three substances (Table II).

The variation between mortality rates for Pd/PVF$^+$, PVF$^+$ and K_2PdCl_4 essentially originated from the control group (0*mg/L). For Pd/PVF$^+$ NPs, there was a difference between control group and other concentration groups whereas differences were observed between control group and other groups and concentration groups of 0.01 mg/L and 1 mg/L for K_2PdCl_4 and PVF$^+$.

As a conclusion, acute toxic effect of Pd/PVF$^+$ NPs, PVF$^+$ and K_2PdCl_4 powders occur at the end of 96th exposure duration and no significant difference was found between the concentration groups.

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