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**Original Article**

# In-ovo evaluation of bioremediated malathion

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**Abstract**
Toxic effects of pesticides are diverse. Indiscriminate and extensive use of pesticides has exerted enormous pressure on the environment.Target organisms have developed resistance, whereas many microorganisms have developed detoxification potential too. In this study, three isolates of *Pseudomonas i.e., P. aeruginosa, P. aeruginosa* MY06 and *P. aeruginosa* SWD were employed for degradation of malathion. Media containing different concentrations of malathion, as sole source of carbon were inoculated with the bacteria. The cell free fluids of 192 hours old cultures were then injected into the fertilized eggs of *Gallus domesticus* on third day of incubation and the embryos were recovered on day 7. Non-remediated insecticide preparations induced dose-dependent developmental abnormalities in chick embryos. Whereas cell free culture fluids could produce developmental defects of lesser severity as assessed by morphological and morphometric parameters. Remediated group embryos differed significantly from non-remediated, showing far less drastic effects of the bacterially degraded malathion. The present study indicates bioremediation of malathion with *P. aeruginosa, P. aeruginosa* MY06 and *P. aeruginosa* SWD, reduced its toxicity to a significant extent. Screening of the cell-free culture fluids forembryotoxicity, provides an easily workable *in ovo* toxicity evaluation model.
**Key words:**Biodegradation; detoxification; embryotoxicity; teratogenicity; Malathion; *Pseudomonas*

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## INTRODUCTION

Malathion, an organophosphate pesticide is used in household and agricultural sectors for the control of insects including aphids, thrips, codling moth and mites on vegetables, ornamental flowers and fruit trees (USEPA, 2014). The insecticide interferes with the action of important enzymes, obliterating the insect's nervous system (Costa *et al*. 2008). Blockage of the nervous system pathways causes rapid twitching and paralysis of muscles, which results in death (Flemming*et al.,* 2003). Like other organophosphoruscompounds,malathion is considered relatively safe regarding teratogenicity and embryotoxicity (Nurulain and Shafiullah, 2012) but the non-target damages of malathion, (Walter *et al.,* 1980; Kamrin, 1997; Bofanti*et al*., 2004), and formation of even more toxic malaoxon catalyzed by cytochrome P450 (Buratti*et al.,* 2005), and generation of malathion mono and diacid through carboxylesterase activity (Kutz*et al.,* 1992; USEPA, 2000) are alarming. Impurities in commercial formulations are potent inhibitors of carboxylesterase, allowing a dramatic increase in malaoxon formation (Buratti*et al*., 2005).

Environmental microbiologists routinely isolate pollutant detoxifying microbes from the contaminated environments (Bhadhade*et al.,* 2002; Hashmi*et al*., 2006; Goda*et al*., 2010; Hernandez and Salinas, 2010; Mohamed *et al.,* 2010; Karigar and Rao, 2011; Ibrahim *et al.,* 2014). Many workers have isolated *Pseudomonas sp.* from soil capable of rapid degradation of malathion(Goda*et al*. 2010; Karunyaand Reetha, 2012; Hatit*et al.,* 2013). In a typical procedure the pollutant is made an ingredient of a selective medium. Microbial growth with concomitant degradation of the pollutant, assessed generally by a chemical analytical procedure, is considered to pave a bioremediation process. However, recently it has been known, that some times, the biodegradation products appear more toxic than the intact pollutant. Thus it is very important to verify the toxic effects of microbially degraded products of a pollutant (Aker *et al.,* 2008). The

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present study reports malathion degradation by employing three isolates of *Pseudomonas,* from a contaminated site. Malathion is a proven teratogen (Khera*et al.,* 1978;Solomon and Judith, 1979; Asmatullah and Ijaz, 2004; Cook *et al.,* 2005; Bechan*et al.,* 2013; Prathibha*et al.,* 2014). Assessing embryotoxicity is a very sensitive *in vivo* method for which even smaller doses manifest toxigenic effects on developing fetuses than respective adults. The present research compares embryotoxicity of intact and bacterially degraded malathion. The protocol is a simple and easy to perform but still represents a reliable model to assess the success of a bioremediation process.

## MATERIALS AND METHODS

Filter sterilized aqueous concentrations (0.125, 0.25 and 0.5%) of analytical grade malathion (Pestnatal® Sigma-Aldrich-Riedle-de-Haën) were added as sole carbon source to the autoclaved minimal media. The minimal medium contained $K_2HPO_4$:0.1; $MgSO_4$:0.02; $NH_4NO_3$:0.5 and agar; 1.5 $gdl^{-1}$ and 10µl of mineral solution. The mineral solution in turn contained $FeSO_4.7H_2O$:10.0; $CuNO_3$:0.5; Zinc powder: 0.5 and $MnCl_2$;0.5 $gdl^{-1}$. Three strains of *Pseudomonas* designated as *P. aeruginosa, P. aeruginosa* MY06 and *P. aeruginosa*SWD,isolated from insecticide affected soils (Andleeb*et al*. 2013) were cultivated in this selective medium at their predetermined growth optima for 192 hours. Polycultureof three bacterial isolates was also raised similarly.After harvesting mono as well as the polycultures of the *Pseudomonas* sp.were filtered through Millipore filters (0.2µm, Sartorius) to get bacteria free culture fluids (remediated) for their detoxification assessment employing chick embryos.

Fresh fertilized eggs (White leghorn breed) were obtained from Veterinary Research Institute, Lahore. Eggs for each group were selected randomly. They were cleaned with cotton soaked in 70% ethanol. A small window was made in the shell of each egg, except control group (untreated intact). Different concentrations, 0.125, 0.25 and 0.5 µg of malathion in 0.1 ml of autoclaved water (non-remediated), were injected into the yolk sac of the eggs of one group on third day of incubation. Comparable amounts of the filtered fluids remediated by mono as well the poly cultureswere injected similarly into the eggs of the experimental groups. The eggs were then incubated at 37.5±0.5°C. Embryos were recovered on day 7 and were fixed in Bouin's fluid for 48 hours and finally preserved in 80% ethanol for recording morphological and morphometric observations.Morphometric and morphological observations involved recording of crown rump (CR) length and body weight. The gross anatomical observations included the studies of developmental defects of brain, spinal cord, eyes, limbs, heart and beak. The selected embryos were macrophotographed by using camera (Nikon), equipped with telephoto lens.

### *Statistical analysis*

The data were statistically analyzed by making use of Minitab (statistic software) version 16 to find out the means of ten replicates of each parameter of respective groups and effects of different concentrations of non remediatedmalathion. The results were declared highly significant if *P<0.001*, very significant if *P<0.01* and significant if *P<0.05*. Turkey's post hoc tect was applied to compare more than two means for significance at P<0.*05*.

## RESULTS

The control group (un-treated embryos) typically presented stage 31 described by Hamburger and Hamilton (1951), showing well developed body parts of the embryos (Fig.1; Tables I-III). Whereas, the non-remediated group, expressed to 0.125, 0.25 and 0.5 µg/egg of malathionshowed adverse effects on embryonic development (Figs.1 and 2), including a significant ($p > 0.05$) decrease in body weight and CR length as compared to the control group (Tables I-II). The embryos exposed to cell- free culture fluids of malathion inoculated bacteria, showed significant increase ($p>0.05$) in body weight and CR length as compared to those exposed to respective doses of non-remediated malathion (Tables I-II). Some embryos of this group, resembled even with those of control group (Tables I-II). This category of embryos was morphologically well developed too with respect to different parameters (Figs. 1 and 2; Tables I-III). Detailed comparison of morphological characteristics showed severe developmental defects such as microcephaly, micromelia, ectopiacordis, microphthalmia, spina bifida, agnathia and multimelia in non-remediated as compared to controls. However, in few cases of remediated groups, development

of micrognathia, displaced forelimb and twisted hind limbs were encountered when treated with *P.aeruginosa* (Fig. 1).Whilst no apparently detectable anomaly when treated with*P.aeruginosa*MYO6 (Fig, 2), butmultimelia and twinning of head forembryos treated when 0.25µg malathion remediated with *P.aeruginosa*SWD strain were observed (Figs.3 and 4). The poly-culturing of bacteria resulted into reduced embryotoxicity, with lowest dose of malathion (0.125 µg ) twinning of head, spina bifida and hydrocephaly were observed while amelia and ectopiacordisresulted for the higher doses of the treated malathion(Fig. 5, Table I-III).

**Table I: Effects of different concentrations of intact (A) and bio-remediated (B) malathion on body weight (mg) of 7 days old chick embryos. The fluids were injected into the eggs on day 3rd of incubation.**

| Concentration (%) | | *P. aeruginosa* | *P. aeruginosa* MY06 | *P. aeruginosa SWD* | Poly-culture |
|---|---|---|---|---|---|
| **Control** | | 702.80±68.05$^{a}$ | 702.80±68.05$^{a}$ | 702.80±68.05$^{a}$ | 702.80±68.05$^{a}$ |
| 0.125 | A | 345.45±86.06$^{b}$ | 345.45±86.06$^{b}$ | 345.45±86.06$^{b}$ | 345.45±86.06$^{b}$ |
| | B | 480.80±72.27$^{b}$ | 601.00±148.08$^{a}$ | 631.00±53.00$^{a}$ | 502.00±108.88$^{c}$ |
| 0.25 | A | 343.00±79.75$^{b}$ | 343.00±79.75$^{b}$ | 343.00±79.75$^{b}$ | 343.00±79.75$^{b}$ |
| | B | 530.30±90.62$^{b}$ | 595.00±187.94$^{a}$ | 624.60±91.29$^{a}$ | 759.00±121.36$^{a}$ |
| 0.5 | A | 379.90±47.83$^{b}$ | 379.90±47.83$^{b}$ | 379.90±47.83$^{b}$ | 379.90±47.83$^{b}$ |
| | B | 596.60±94.12$^{c}$ | 706.00±164.69$^{a}$ | 478.00±97.75$^{b}$ | 762.00±149.38$^{a}$ |

A: Fluid from medium without inoculation of bacteria (non-remediated group); B: Fluid after 192 hrs of the bacterial growth (remediated group); Means ± S.E.M. of 10 replicates values within the same column with same alphabets did no differ significantly (*P>0.05*). Here $^{***}$and $^{**}$ represent significance at *P<0.001* and *P< 0.01*, respectively.

**Table II: Effects of different concentrations of intact (A) and bio-remediated (B) malathion on CR length (mm) of 7 days old chick embryos. The fluids were injected into the eggs on 3rd day of incubation.**

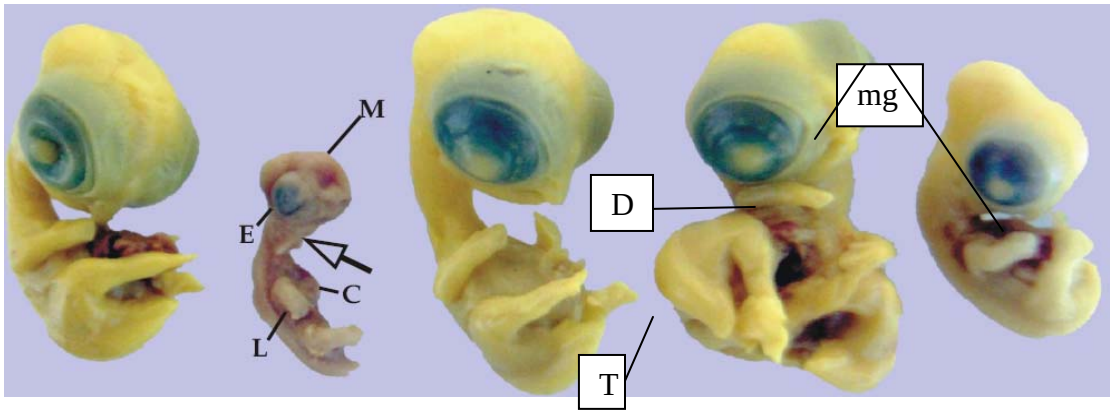
| Concentration (%) | | *P. aeruginosa* | *P. aeruginosa* MY06 | *P. aeruginosa*SWD | Poly-culture |
|---|---|---|---|---|---|
| **Control** | | 18.7±1.48$^{a}$ | 18.7±1.48$^{a}$ | 18.7±1.48$^{a}$ | 18.7±1.48$^{a}$ |
| 0.125 | A | 14.00±1.62$^{b}$ | 14.00±1.62$^{b}$ | 14.00±1.62$^{b}$ | 14.00±1.62$^{b}$ |
| | B | 15.50±1.36$^{c}$ | 17.70±1.90$^{a}$ | 18.10±1.64$^{a}$ | 15.88±1.52$^{a}$ |
| 0.25 | A | 14.00±0.89$^{b}$ | 14.00±0.89$^{b}$ | 14.00±0.89$^{b}$ | 14.00±0.89$^{b}$ |
| | B | 15.44±0.83$^{c}$ | 18.70±3.10$^{a}$ | 17.00±2.28$^{a}$ | 17.60±1.62$^{a}$ |
| 0.5 | A | 15.00±0.79$^{c}$ | 15.00±0.79$^{b}$ | 15.00±0.79$^{b}$ | 15.00±0.79$^{b}$ |
| | B | 16.88±1.44$^{c}$ | 17.60±2.28$^{a}$ | 15.88±1.60$^{b}$ | 18.80±3.34$^{a}$ |

A: Fluid from medium without inoculation of bacteria (non-remediated); B: Fluid after 192 hrs of the bacterial growth(remediated group); Means ± S.E.M. of 10 replicates values within the same column with same alphabets did no differ significantly (*P>0.05*). Here $^{***}$and $^{**}$ represent significance at *P<0.001* and *P< 0.01*, respectively.

**Table III: Embryotoxicity (%) induced by different concentrations of intact (A) and bio-remediated (B) malathion in 7 days old chick embryos. The fluids were injected into the eggs on 3rd day of incubation.**

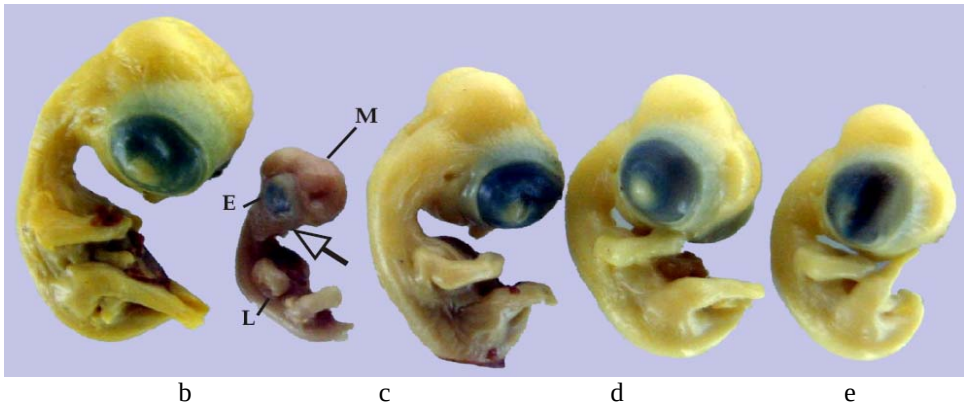
| Concentration (%) | | *P. aeruginosa* | *P. aeruginosa* MY06 | *P. aeruginosa*SWD | Poly-culture |
|---|---|---|---|---|---|
| **Control** | | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.125 | A | 50.00 | 50.00 | 50.00 | 50.00 |
| | B | 40.00 | 9.00 | 10.00 | 24.00 |
| 0.25 | A | 60.00 | 60.00 | 60.00 | 40.00 |
| | B | 30.00 | 20.00 | 50.00 | 15.0 |
| 0.5 | A | 70.00 | 60.00 | 60.00 | 70.00 |
| | B | 40.00 | 16.66 | 14.28 | 20.00 |

A: Fluid from medium without inoculation of bacteria (non-remediated group)
B: Fluid after 192 hrs of the bacterial growth (remediated group)




a b c d e

**Figure 1A Composit photograph of 7 days chick embryos administered with cell-free fluids of culture of*P. aeruginosa*a)control(non-treated); b)experimental control(intact malathion) and c-e) experimental treated with (0.125, 0.25 and 0.5% of malathion, respectively) ]Note: Adversely affected chick embryos, showing microcephaly (M) agnathia (arrow), microophthalmia (E), micromelia (L), ectopiacordis (C), agnathia (arrow), micrognathia(mg), displaced forelimb (D), and twisted hind limb(T).**




a b c d e

**Figure 2A Composit photograph of 7 days chick embryos administered with cell-free fluids of culture of*P. aeruginosa* MYO6: a) control (non treated); b) experimental control (intact malathion) and c-e) experimental treated (0.125, 0.25 and 0.5% of malathion ,respectively).Note: Adversely affected chick embryos, showing microcephaly (M) agnathia (arrow), microophthalmia (E) and micromelia (L)**

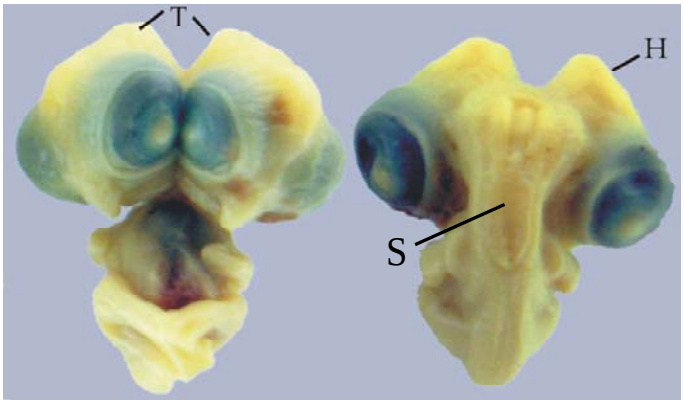
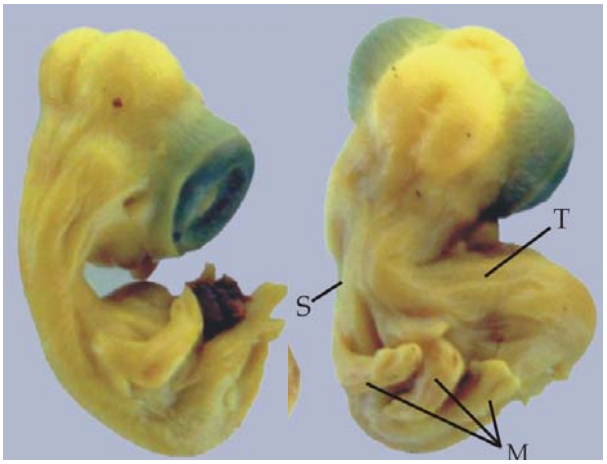
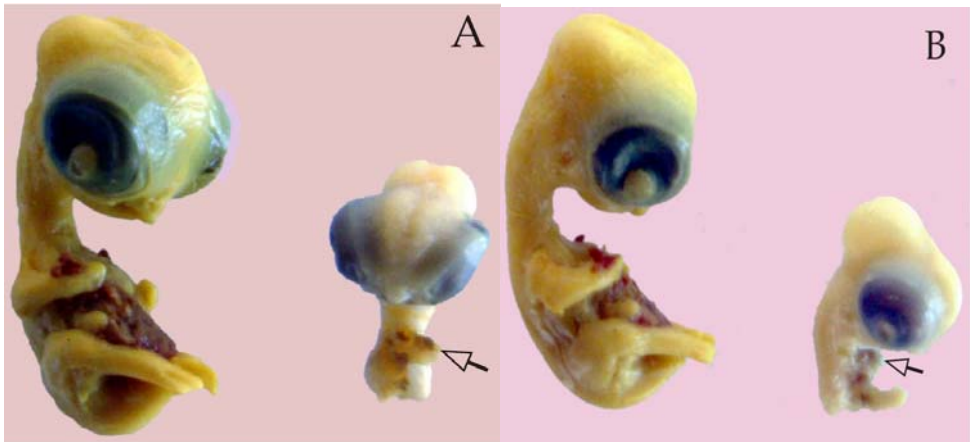



**Figure 3 Photographs of abnormal chick embryo administered with 0.125 µg/egg of cell free culture fluid of the poly-culture. Note: Twinning of head (T), spina bifida (S), and hydrocephaly (H)**




a b

**Figure 4 Photographs of, a) standard control and, b) abnormal chick embryo administered with 0.5 µg /egg of cell-free culture fluid of*P.aeruginosa* SWD. Note: Twinning of spinal cord (S), Twisting of spinal cord (T), Polymelia (M), and Twisted hind limbs(W).**




**a b c d**

**Figure 5:Photographs of chick embryos. (A) treated with 0.25 µg of malathion/egg experimental control (a) and with cell free fluid of the poly-culture (b)]; (B) treated with 0.5 µg/egg of experimental control (c) and with cell free fluid from the poly-culture (d),Note: ectopiacordis (arrow) and amelia (a).**

## DISCUSSION

Besides its advantages, malathion has been found teratogenic when bioassayed against chick embryos.Its different concentrations causemicromelia, overall growth retardation, sparse plumage and beak defects (Greenberg and LaHam 1969; Wyttenbach and Thompson, 1985; Lenselink 1992; Pourmirza, 2000). In the present study, significant increase in morphological parameters of body weight, CR length and marked decrease in embryotoxicity in remediated group indicates the cogent potential of detoxification of *Pseudomonas* as compared to non-remediated group.

Several studies have proven the efficiency of microorganisms to remediate the pollutants successfully (El Deeb*et al.,* 2000; Bhadade*et al.,* 2002), powered by various enzymes like oxido-reductases and hydrolases (Karigar and Rao, 2011). Among them, the genus *Pseudomonas* has a history of showing strong potential to degrade a variety of toxins (Guerin and Boyd, 1995; Prijambada*et al.,* 1995; Duetz*et al.,* 1996; Foster and Bia, 2004; Mclaughlin*et al.,* 2006; Goda*et al.,* 2010; Ajao*et al.,* 2011; Singh *et al.,* 2013; Patel *et al*.,2014). All isolates of *Pseudomonas*, employed during the course of present study, degraded different concentrations of malathionfollowing their cultivations upto 192 hrs.

Genotoxicity as well as dose and age dependent mortality of chick embryo by malathionhas also been observed by Jira*et al*. (2012).On the other hand in a similar study, increased trend towards body weight and developmental anomalies were observed in non remediated conditions for other insecticides like dimethoate, S-metolachlor and benfluralin(Keseru*et al.,* 2004). Malformations such as those of skeletal structures have been observed for diverse pesticides such aschlorpyrifos, cypermethrin,spinosad and bendiocarbtreated chick embryos (Petrovová*et al.,* 2010; Ugginie*t al.,* 2012).Carbosulfan caused musculoskeletal deformities in skin, limbs,head, neck, skull, lower body and overall reduction in ossification of skeleton in developing chick(Mathur*et al.,* 2013). In the present study, somemalformations found in the embryos of remediated group might be due to the byproducts of malathion such as butendioic acid, malaoxon, phoratoxonsulfone, and ethyl methyl methylphosphonate, produced as a result of bioremediation (Andleeb and Qazi, 2014).Some of the degradation products are even more toxic than malathion(Giri*et al.,* 2002). Organophosphate pesticides are degraded by a number of bacteria such as *Pseudomonas* sp. through hydrolysis and/or microbial cleavage utilizing phosphatase, oxidoreductases, phosphatases, esterase, hydrolase, and oxygenase into a variety of metabolites like malaoxon, diethylphosphorothioate with subsequent conversion into salt of succinic acid (Rathore and Nollet, 2012; Abo-Amer,2007;Turnbull, 2013; Andleeb and Qazi,2014). In another study, malathion monocarboxylic acid (MMA), malathiondicarboxylic acid(MDA) and various phosphothionatesyielded by *Pseudomonas* and other bacterial species have also been documented (Thabit and Naggar, 2013).

As far as remediated group is concerned lesser levels of embryotoxicity induced by monoculture of *Pseudomonas aeruginosa*MYO6, might be due to non production of malaoxon by the isolate, which has been reported even more toxic than the parent compound (Burattie*t al.,* 2005). Malformations developed in the embryos treated with the cell free culture fluids remediated by monoculture of *Pseudomonas aeruginosa* SWD, cannot be blamed ethyl methyl methylphosphonateandbutanedioic acid; commonly known as succinate for interfering the normal development, as these metabolites represent intermediates of TCA cycle (Song and Lee, 2006).However, toxicity of ethyl methyl methylphodphonate could be expected like with those of its similar compounds;dimethylmethylphosphonate (DMMP) and diethyl ethylphosphonate (DEEP) known to cause kidney tumors in male rats (Blumbache*t al.,* 2000).

Embryotoxicity caused by *Pseudomonas aeruginosa* in remediated groupmight be due to degradation associated formation of all four kinds of metabolites including phoratoxonsulfone, as mentioned earlier, in such a combination which affected the normal development adversely.Phoratoxonsulfone is also produced as metabolite of phorate, another pesticide (Bowman andCasida, 1958) and has been reported as causative agent of decreased body weight, tremors, excessive salivation, decreased motor activity, hunched posture, impaired righting reflex and laboured breathing (Lochry, 1990b) but with no birth defects in rat

(USEPA 1985; Wayne, 1992).Results of embryotoxicityof poly-cultured treated malathion might be due to non production of succinate. However, lower toxicity among embryos treated with cell free culture fluids of the mixed bacterial cultures, as compared to that caused by monocultural fluids of *Pseudomonas aeruginosa* could be explained for possibility of efficient biodegradation of the insecticide into relatively safe concentrations of metabolites. Similar trends had also been observed for bioremediation of malathion by mixed bacterial culture of three strains of *Bacillus* (Singh *et al.,* 2013), *Pseudomonas aeruginosa*and *Bacillus subtilis (*Ajao *et al.,*2011*)* and for other pesticides (Roberts *et al.,* 1993; Sutherland *et al*., 2000; Kumar and Philip, 2006). Thus, in designing the bioremediation process for environmental cleaning, the levels of pollutant degradation assessed by chemical analyses should not be taken as sole attribute, but the processed effluents must be evaluated *in vivo* for estimating their potential hazards. Although, many detoxification testing models are available, viz., *in vitro* and *in vivo*, it is generally accepted that tissue culture model is not only expensive but its results also deviate from *in vivo* trials. The present *in ovo* procedure is relatively low cost, easy to handle and provides a reliable toxicity assessing model. The aforementioned discussion regarding the diversified detoxification potential of *Pseudomonas* genus is suggestive of consideration of these isolates for degradation of more pollutants.

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