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1 **Application of primary haemocyte culture of *Penaeus monodon* in the assessment of**
2 **cytotoxicity and genotoxicity of heavy metals and pesticides**

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22 **ABSTRACT**

23 Lack of shrimp cell lines has hindered the study of pollutants which adversely
24 affects shrimp health and its export value. In this context a primary haemocyte culture
25 developed from *Penaeus monodon* was employed for assessing the cytotoxicity and
26 genotoxicity of two heavy metal compounds, cadmium chloride and mercuric chloride and
27 two organophosphate insecticides, malathion and monocrotophos. Using MTT assay 12 h
28 IC_{50} values calculated were $31.09 \pm 16.27 \mu\text{M}$ and $5.52 \pm 1.16 \mu\text{M}$ for cadmium chloride
29 and mercuric chloride and $59.94 \pm 52.30 \text{ mg l}^{-1}$ and $186.76 \pm 77.00 \text{ mg l}^{-1}$ for malathion
30 and monocrotophos respectively. Employing Comet assay, DNA damage inflicted by
31 these pollutants on haemocytes were evaluated and the pollutants induced DNA damage
32 in $> 60\%$ of the cells. The study suggested that haemocyte culture could be used as a tool
33 for quantifying cytotoxicity and genotoxicity of aquaculture drugs, management
34 chemicals and pollutants.

35

36 *Keywords:* Aquaculture, *Penaeus monodon*, primary haemocyte culture, heavy metals,
37 organophosphate pesticides, cytotoxicity, genotoxicity, MTT assay, Comet assay

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44 **1. Introduction**

45 Cell lines are widely utilized for assessing the toxicity of major aquatic pollutants
46 such as heavy metals and pesticides. Effluents from industries and runoff from
47 agricultural fields may contain such pollutants, which on reaching aquatic environments
48 lead to impairment of the ecosystem. Needless to say, the bio-accumulation and
49 persistence of these pollutants in the aquatic environment constitute a serious threat to
50 biological life and also to human beings indirectly by virtue of their involvement in the
51 food chain (Binelli and Provini, 2004).

52 Among heavy metals, cadmium and mercury are the most highly toxic elements
53 owing to their persistence in the environment. They are redox inactive which challenge
54 antioxidant defense by binding to thiols in cells such as reduced glutathione (GSH)
55 (Stohs and Bagchi, 1995; Ercal et al., 2001). They cause oxidative and nitrosative stress
56 (Stohs and Bagchi, 1995; Ercal et al., 2001; Pompella et al., 2003) and damage
57 macromolecules in cells. Apoptosis or necrotic cell deaths are the usual consequences
58 (Pulido and Parrish, 2003). Toxicity of pesticides on non - target organisms and
59 ecosystems is of world wide concern (Pimentel et al., 1998). Among pesticides,
60 organophosphate pesticides are stable enough to remain in their toxic form for relatively
61 shorter periods of time (Poovala et al., 1998). They act primarily by inhibiting the
62 enzyme acetylcholine esterase at cholinergic junctions of the nervous system (Gallo and
63 Lawryk, 1991) leading to functional changes at the tissue/organism level (Pope et al.,
64 2005).

65 Heavy metals and pesticides are known genotoxicants, as they have the capacity
66 to interact with and damage the structure of the DNA molecule, often with a concomitant
67 adverse effect on biological integrity (Shugart, 1995; 1998). Many genotoxicants may
68 cause DNA strand breakage by the formation of free radicals or abasic sites which can
69 result in the breakage of phosphodiester linkages within the DNA molecule (Shugart,
70 2000).

71 Attempts to develop cell cultures from marine invertebrates date back about a
72 century (Gomot, 1971; Rannou, 1971). Yet for unidentified reasons all efforts to develop
73 continuous cell lines from these organisms including crustaceans have been ineffective so
74 far (Mothersill and Austin, 2000; Rinkevich, 2005). *Penaeus monodon* is one among the
75 important cultured crustacean in the world. Lack of shrimp cell lines has been hampering
76 progress of research in testing the effects of drugs and management chemicals prior to
77 their administration in aquaculture systems. This has also hindered the study on the
78 effects of pollutants such as heavy metals and pesticides in the aquatic environment,
79 which adversely affects shrimp health and its export value. Even though development of
80 primary cell cultures (Chen et al., 1986; Fan and Wang, 2002; Maeda et al., 2003) and
81 their application in the study of viral pathogens (Wang et al., 2000; Maeda et al., 2004;
82 Jiang et al., 2005) have been reported, their utility in the assessment of cytotoxicity and
83 genotoxicity of heavy metals and pesticides has not been attempted. In the study
84 undertaken here primary haemocyte culture of *P. monodon* was utilized for examining
85 cytotoxicity and genotoxicity of two heavy metal compounds, cadmium chloride ($\text{CdCl}_2 \cdot 2$
86 $\frac{1}{2} \text{H}_2\text{O}$) and mercuric chloride (HgCl_2) and two organophosphate insecticides, malathion
87 ($\text{C}_{10}\text{H}_{19}\text{O}_6\text{PS}_2$) and monocrotophos ($\text{C}_7\text{H}_{14}\text{NO}_5\text{P}$) which reach aquaculture systems

88 through run off. Malathion and monocrotophos are also used as insecticides in paddy
89 fields and aquaculture ponds. Comet assay was employed to evaluate the DNA damage
90 caused by these pollutants. The work was conducted to assess the usefulness of primary
91 haemocyte culture for the study of cytotoxicity and genotoxicity of aquatic pollutants.

92

93 **2. Materials and methods**

94 *2.1. Experimental animals*

95 WSSV and Monodon Baculo virus (MBV) negative *P. monodon* larvae obtained
96 from a local hatchery were stocked and reared in a recirculating shrimp rearing system
97 integrated with nitrifying bioreactors (Kumar et al., 2009) in sea water having a salinity
98 of 15 g l⁻¹. Water quality was maintained by the addition of a probiotic DetrodigestTM
99 (NCAAH, India) to manage detritus and EnterotrophicTM (NCAAH, India) to control
100 *Vibrio*. Water quality parameters measured were pH (7.5 to 8.5), temperature (25 ± 1 °C),
101 NH₄⁺ - N (< 0.1 mg l⁻¹) and alkalinity (60-75 mg l⁻¹). The larvae were fed three times
102 daily with commercially available pelleted feed having 40% protein (Higashimaru, India)
103 made of fish meal, wheat gluten, squid meal, clam meal, wheat flour, yeast, soy lecithin,
104 krill meal, marigold flower petal extract, refined fish oil, cholesterol, minerals and
105 vitamins. They were confirmed WSSV negative by nested PCR (WSSV detection kit,
106 Genei, India) when they grew to 8-12 g after a culture period of three months, and used
107 for all experiments.

108

109 *2.2. Development of primary haemocyte culture*

110 The animals were sacrificed by immersing in crushed ice and disinfected by
111 maintaining in 800 mg l⁻¹ sodium hypochlorite solution prepared in ice cold -sea water
112 (salinity 15 g l⁻¹) for 10 minutes. Subsequently, they were washed 5 times in sterile ice
113 cold sea water, dipped in 70% alcohol and rinsed in ice-cold sea water. Haemolymph was
114 withdrawn aseptically using capillary tubes containing 100 µl anticoagulant (Tris HCl
115 0.01 M (pH 7), Sucrose 0.25 M, Tri Sodium Citrate 0.1 M) from rostral sinus and diluted
116 to obtain 5 x 10⁵ cells ml⁻¹ using modified 2X L-15 (Leibovitz) medium supplemented
117 with 2% glucose, MEM vitamins (1X), tryptose phosphate broth (2.95 g l⁻¹), 20% FBS,
118 N-phenylthiourea (0.2 mM), 0.06 µg ml⁻¹ chloramphenicol, 100 µg ml⁻¹ streptomycin and
119 100 IU ml⁻¹ penicillin, and aliquots of 200 µl were dispensed into 96 well microplates
120 (Greiner Bio-One) and incubated at 25 °C (Jose et al., 2010) for 12 hours. Haemolymph
121 from 5 animals were pooled and diluted to seed a microwell plate.

122

123 2.3. Cytotoxicity of cadmium chloride (CdCl₂·2 ½ H₂O) and mercuric chloride (HgCl₂)

124 Cadmium chloride and mercuric chloride were used as representatives of aquatic
125 pollutants. Stock solutions of cadmium chloride (Qualigens) and mercuric chloride
126 (Merck) were prepared in cell culture medium. A range finding test was carried out
127 before the definitive test and different concentrations of the compounds were added to the
128 wells to get the final concentrations ranging from 0.49 to 500 µM for cadmium chloride
129 and 0.49 to 62.5 for mercuric chloride respectively. Replicates were maintained for each
130 concentration. Cells with the growth medium were kept as control. After 12 hours
131 incubation, wells were observed under phase contrast microscope (Leica, Germany) and

132 MTT assay was performed. Percentage inhibition of cells at each concentration of the
133 compounds was calculated based on the formula,

134 Percentage inhibition of haemocytes = [100- (Average absorbance (MTT assay)
135 of haemocytes at a particular concentration of the compound/Average absorbance of
136 control haemocytes without the compound) x 100].

137

138 *2.4. Cytotoxicity of malathion [S-(1, 2-dicarboethoxyethyl) O, O-dimethyl phosphorodithioate];*
139 *(C₁₀H₁₉O₆PS₂)) and monocrotophos (E)-(dimethyl-1-methyl-3-(methylamino)-3-oxo-1-*
140 *propenyl phosphate); (C₇H₁₄NO₅P))*

141 Commercially available preparations of malathion (50% w/w) and monocrotophos
142 (53% w/w) were used for toxicity studies. Insecticides were dissolved in DMSO and
143 diluted with medium to get stock concentrations of 500 mg l⁻¹ and 1000 mg l⁻¹ of
144 malathion and monocrotophos respectively. Concentrations were fixed based on the
145 range finding test and different concentrations of the compounds were added to the wells
146 to get final concentrations ranging from 0.76 to 185.2 mg l⁻¹ for malathion and 0.98 to
147 1000 mg l⁻¹ for monocrotophos maintaining replicates for each concentration. Cells with
148 the growth medium were kept as control. After 12 hours incubation wells were observed
149 under phase contrast microscope (Leica, Germany) and MTT assay was performed.
150 Percentage inhibition of cells at each concentration of the compounds was calculated.

151

152 *2.5. MTT assay*

153 The assay is a colorimetric method based on the determination of cell viability
154 utilizing the reaction of a tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl
155 tetrazolium bromide, MTT) with the mitochondria of metabolically active cells. The
156 reduction of the tetrazolium salt by nicotinamide adenine dinucleotide dehydrogenase
157 (NADH) and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH)
158 within the cells produces insoluble purple formazan crystals, which are later solubilized
159 yielding a purple-colored solution (Mosmann, 1983).

160 After replacing the medium, 50 μ l of MTT (Sigma) solution (5 mg ml⁻¹ in PBS; 720
161 mOsm) were added to each well and incubated for 5 hours in dark. Blank consisted of
162 medium alone with MTT added. After incubation, the medium was removed and MTT-
163 formazan crystals were dissolved in 200 μ l dimethylsulfoxide. Absorbance was recorded
164 immediately at 570 nm in a micro plate reader (TECAN Infinite Tm, Austria).

165

166 2.6. Comet assay

167 Haemocytes were exposed to cadmium chloride (140 μ M), mercuric chloride (17
168 μ M), malathion (60 mg l⁻¹) and monocrotophos (186 mg l⁻¹) for 4 hours. These
169 concentrations were chosen because more than 80% viability of cells was recorded after 4
170 hour exposure by trypan blue viability assay as dead cells might give rise to false positive
171 results in Comet assay. After the exposure to the compounds, cells were detached using a
172 cell scraper (Greiner Bio-One), pelletised, washed and suspended in PBS. Negative
173 control consisted of cells without any treatment. Added 150 μ l normal agarose solutions
174 (Sigma, USA) (0.75% in PBS) to a frosted slide and covered with a cover slip (22 x
175 50mm) to get a uniform layer, and allowed to solidify. To prepare the second layer, 10 μ l

176 cell suspension was mixed with 70 μ l low melting agarose (Sigma, USA) (0.75% in
177 PBS) and, the suspension was layered over the one prepared initially after removing the
178 cover slip. The preparation was kept undisturbed after replacing the cover slip until
179 agarose got solidified. A third layer of low melting agarose of the above strength was
180 applied over the second layer after removing the cover slip and replaced the same
181 subsequently. The slides were treated with pre chilled lysis solution (2.5 M NaCl, 100
182 mM EDTA, 10 mM Tris, 1.5 M NaOH, pH 10 with 1% Triton X 100 and 10% DMSO)
183 for 1 hour at 4 °C after removing the cover slips. On completion of the process, the slides
184 were incubated in electrophoresis buffer (300 mM NaOH and 1mM EDTA, pH>13) for
185 20 min at 4 °C for unwinding of DNA, and were electrophoresed for 40 min at 25 V and
186 300 mA in the same buffer. Subsequently, the slides were neutralized in 0.4 M Tris (pH
187 7.5), stained with ethidium bromide (2 μ g ml⁻¹) and observed under fluorescent
188 microscope (Olympus, Germany) at 400X magnification, and the cells were
189 photographed. For each sample, 25 cells from each slide were analysed for DNA damage
190 using Comet ScoreTM (TriTek Corp., Summerduck, USA), of the Comet scoring software.
191 Experiments were repeated four times. Percentage of nuclei with tails, tail length,
192 percentage DNA in the tail and tail moment (arbitrary units) were used to estimate DNA
193 damage. Tail moment is the tail length multiplied by fraction of DNA in the tail.

194

195 2.7. Statistical analysis

196 The results in the figures are average values of 4 replicates \pm standard deviation.

197 The results of the cytotoxicity assay were analyzed by probit analysis using the SPSS

198 software (SPSS Inc., USA). The effects of treatments were statistically analyzed by
199 analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

200

201 3. Results

202

203 3.1. Cytotoxicity of cadmium chloride and mercuric chloride

204 Control haemocytes appeared spherical or elliptical initially, and within a few
205 hours of culturing two distinct morphological types such as round to elliptical and small
206 and large spindle shaped cells, with granules, could be seen in the modified 2X L-15
207 medium (Fig. 1). At higher concentrations of cadmium chloride ($>125 \mu\text{M}$) and mercuric
208 chloride ($>15.6 \mu\text{M}$) round cells were few and spindle shaped cells were totally absent.
209 More round cells and few spindle shaped cells were present at lower concentrations
210 ($<15.6 \mu\text{M}$ for cadmium chloride and $<1.95 \mu\text{M}$ for mercuric chloride) (Fig 1). At still
211 lower concentrations number of spindle cells was higher. Toxicity of cadmium chloride
212 and mercuric chloride in primary haemocyte culture in terms of percentage inhibition of
213 cells determined through MTT assay is presented in Figs 2 and 3. IC_{50} values were 31.09
214 $\pm 16.27 \mu\text{M}$ and $5.52 \pm 1.16 \mu\text{M}$ ($7.10 \pm 3.72 \text{ mg l}^{-1}$ and $1.50 \pm 0.31 \text{ mg l}^{-1}$) for cadmium
215 chloride and mercuric chloride respectively .

Fig 1

216

217 3.2. Cytotoxicity of malathion and monocrotophos

218 At higher concentrations of malathion and monocrotophos ($>61.7 \text{ mg l}^{-1}$ and >166
219 mg l^{-1} respectively) only round cells were present and the number of spindle shaped cells

Fig 2,
3, 4,

220 increased as the concentrations decreased (Fig 1). Toxicity of malathion and
221 monocrotophos in primary haemocyte culture in terms of percentate inhibition of cells
222 determined hrough MTT assay is presented in Figs 4 and 5. IC₅₀ value of malathion was
223 found to be $59.94 \pm 52.30 \text{ mg l}^{-1}$ and of that of monocrotophos $186.76 \pm 77.00 \text{ mg l}^{-1}$.
224 Malathion was found to be non toxic at $\leq 6.86 \text{ mg l}^{-1}$ and monocrotophos at $\leq 15.6 \text{ mg l}^{-1}$.

225 3.3. Genotoxicity of cadmium chloride, mercuric chloride, malathion and monocrotophos

226 All pollutants tested induced DNA strand breakage and thereby Comets in the
227 haemocytes (Fig 6). In the negative control most of the nuclei were circular without any
228 visible tail while short tails were observed in $11.46 \pm 7.89\%$ of cells (Table 1).
229 Percentage of cells with tails were significantly higher ($P < 0.05$) than the control for
230 cadmium chloride, mercuric chloride, malathion and monocrotophos ($> 60\%$). Tail length
231 of the Comets in mercuric chloride treatment was the lowest (23.09 ± 6.83 pixels) while
232 for cadmium chloride, malathion and monocrotophos length of the tail ranged from 28-33
233 pixels. Percentage tail DNA for all the pollutants were significantly higher ($P < 0.05$) but
234 no significant differences ($P > 0.05$) were obtained between that of cadmium chloride and
235 mercuric chloride, and malathion and monocrotophos. Tail moment was found to be
236 lesser for mercuric chloride (11.82 ± 5.50) ($P < 0.05$) when compared to the other three
237 and the values were highest for cadmium chloride (17.17 ± 6.22). In the control tail
238 moment was significantly lower (0.04 ± 02) ($P < 0.05$).

Fig
6

Table
1

239

240

4. Discussion

241 Deleterious effects of environmental pollutants may result from direct toxic action on the
242 tissues or from subtle alterations in the homeostatic mechanisms such as the immune
243 system (Auffret and Oubella, 1997). The immune defense of shrimp is comprised of
244 cellular and humoral - mediated mechanisms, in which haemocytes play a key role and
245 this makes haemocyte culture an ideal system for evaluating the effects of environmental
246 pollutants. Primary cell cultures of penaeid shrimp have been developed from different
247 tissues such as lymphoid (Chen and Wang, 1999; Lang et al., 2002), heart (Tong and
248 Miao, 1996; Chen and Wang, 1999), ovary (Shimizu et al., 2001; Maeda et al., 2003) and
249 embryo (Toullec et al., 1996; Fan and Wang, 2002) while haemocyte culture was
250 attempted only by a few researchers in *P. vannamei*, *P. aztecus* (Ellender et al., 1992), *P.*
251 *japonicus* (Itami et al., 1999) and *P. chinensis* (Jiang et al., 2005). In the primary
252 haemocyte culture developed, round to elliptical cells could be regarded as hyaline cells
253 and small and large spindle shaped cells with granules could be regarded as granular or
254 semi-granular cells based on the morphology and presence of granules. The metabolic
255 activity of the culture was almost the same during the second, third and fourth days and
256 the activity started declining only subsequently. The culture remained viable for upto 8
257 days. Even though dividing cells were not observed, DNA synthesis was detected in $22 \pm$
258 7% of cells by means of 5-Bromo-2'-deoxyuridine (BrdU) assay at 24 hours (Jose et al.,
259 2010). Studies on the application of haemocyte culture or any other primary cultures of
260 shrimp in cytotoxicity and especially the genotoxicity of drugs, management chemicals
261 and pollutants in aquaculture systems have been rarely cited in literature. The *P. monodon*
262 haemocyte culture has been applied for assessing cytotoxicity of N- methyl-1-
263 hydroxyphenazine, a proposed aquaculture drug (Preetha et al., 2009) and benzalkonium

264 chloride, an aquaculture management chemical (Jose et al., 2010) employing MTT assay.
265 Sung et al. (2003) examined the effects and toxicity of phthalate esters on the isolated
266 haemocytes of *Macrobrachium rosenbergii* utilizing haemocytic adhesion, pseudopodia
267 formation, superoxide anion production and phenoloxidase activity.

268 Toxicity of cadmium chloride and/or mercuric chloride has been evaluated in vivo on
269 different aquatic organisms such as fishes (Pereira et al., 2009), sea urchin (Filosto et al.,
270 2008), shrimp (Lee et al., 2000; Espericueta et al., 2001) and crab (Botton, 2000). In our
271 study toxicity was tested in vitro on primary haemocyte culture of *P. monodon* and 12
272 hour IC₅₀ values for cadmium chloride and mercuric chloride were $31.09 \pm 16.27 \mu\text{M}$ and
273 $5.52 \pm 1.16 \mu\text{M}$, respectively. Mercuric chloride was found to be more toxic than
274 cadmium chloride and concentration dependent increase was observed in the toxicity of
275 both heavy metals in haemocytes. Espericueta et al. (2001) reported the LC₅₀ values as
276 2.49 and 1.23 mg l⁻¹ for cadmium and mercury, respectively in post larvae of
277 *Litopenaeus vannamei* after 96 hour exposure while in *Limulus polyphemus* embryos IC₅₀
278 was reported to be >1000 mg l⁻¹ and 12.80 mg l⁻¹ for Cd and Hg after 24 hour exposure
279 (Botton, 2000). In the present study, MTT assay which measured the metabolic activity
280 as well as viability of cells was utilized to estimate the cytotoxicity rather than trypan
281 blue assay which measured only the viability. IC₅₀ value of malathion was found to be
282 $59.94 \pm 52.30 \text{ mg l}^{-1}$ and that of monocrotophos was $186.76 \pm 77.00 \text{ mg l}^{-1}$. Cytotoxicity
283 increased with concentration for both the pesticides, and malathion was more toxic when
284 compared to monocrotophos. Similarly, concentration dependant increase in the toxicity
285 of malathion was detected in grass carp cell line (Chen et al., 2006) and human liver
286 carcinoma cell line (Moore et al., 2010). IC₅₀ of malathion ranged from $37.94 \pm 1.93 \text{ mg}$

287 l^{-1} for 12 h to $3.04 \pm 0.27 \text{ mg } l^{-1}$ for 72 hours in grass carp cells (Chen et al., 2006). In the
288 nauplii (48 h old) of *Artemia salina* 24 h LC_{50} of monocrotophos was estimated to be
289 $262.68 \pm 17.30 \text{ mg } l^{-1}$ (Rao et al., 2007). These values are comparable to those in the
290 present study. Semi-granular or granular cells were more sensitive to heavy metals and
291 pesticides than the hyaline cells and the number of these cells increased as the pollutant
292 concentration decreased. According to Holmblad and Soderhall (1999) semi-granular
293 cells appear to be the most sensitive and react first during an immune response, by
294 degranulating. This degranulation stimulates the granular cells also to degranulate as
295 well. The advantage of using haemocyte culture over explant cultures for cytotoxicity
296 studies is the easiness in quantification of cells to be seeded to maintain uniform cell
297 number in micro plates, a prerequisite for quantal assays, and the rapidity in obtaining
298 monolayer of cells.

299 Different methods have been developed for detecting DNA strand damage such as
300 micronucleus and sister-chromatid exchange assays which are based on the enumeration
301 of downstream aberrations after DNA damage (Lee and Steinert, 2003). A more recent
302 method is the Comet assay which detects the DNA strand breaks and alkali labile sites by
303 measuring the migration of DNA from immobilized nuclear DNA (Singh et al., 1988). A
304 large number of studies report that Comet assay is more sensitive when compared to
305 sister chromatid exchanges or micronucleus test (Mitchelmore and Chipman, 1998; Lee
306 and Steinert, 2003; Frenzilli et al., 2009). The main advantages of the Comet assay
307 include the collection of data at the level of the individual cell, the need for a small
308 number of cells per sample and the sensitivity for detecting DNA damage (Lee and
309 Steinert, 2003; Jha, 2004). Tail length is the basic parameter used for quantifying DNA

310 damage in the Comet assay. However, the tail length levels off after migrating to a
311 certain distance. After the maximum length is attained, the Comet tail will not be
312 extending in length, however grow in intensity as compared to that with the head portion
313 (Bowden et al., 2003). Therefore, in addition to tail length, percentage tail DNA and tail
314 moment were also calculated using Comet scoring software.

315 Comet assay has become one of the most popular tools for detecting DNA strand
316 breaks in aquatic animals (Frenzilli et al., 2009) and has been employed to assess the
317 DNA damage in aquatic vertebrates such as *Cyprinus carpio* (Arabi et al., 2004),
318 *Carassius auratus* (Masuda et al., 2004), *Danio rerio* (Kosmehl et al., 2008) and *Liza*
319 *aurata* (Pereira et al., 2009), and invertebrates such as *Limnoperna fortunei* (Villela et al.,
320 2007), *Dreissena polymorpha* (Juhel et al., 2007), *Polycelis feline* (Horvat et al., 2005),
321 *Palaemonetes pugio* (Lee et al., 2000; Lee et al., 2008), *Mytilus edulis* (Rank and Jensen,
322 2003; Cheung et al., 2006) and *Crassostrea virginica* (Bissett et al., 2009). For in vivo
323 studies in aquatic invertebrates such as oysters, mussels, clams and shrimp, cells from
324 haemolymph, embryos, gills and digestive glands were used for Comet assay (Lee and
325 Steinert, 2003). In vitro studies of genotoxicants were reported in haemocytes, gill cells
326 and digestive gland cells from various mollusks namely *Mytilus edulis*, *Nassarius tegula*
327 and *Musculista senhousia* (Sastre et al., 1997; Mitchelmore et al., 1998; Wilson et al.,
328 1998). Studies of genotoxicants in shrimp cell cultures were not reported while Lee et al.
329 (2000) studied the effects of genotoxicants such as chromium (III) chloride, sodium
330 chromate, mercuric chloride, and 2-methyl-1,2-naphthoquinone (MNQ) on grass shrimp,
331 *Palaemonetes pugio*, embryos employing Comet assay. DNA damage in a variety of
332 aquatic animals has been associated with reduced growth, abnormal development and

333 reduced survival of embryos, larvae and adults (Steinert, 1999; Lee et al., 2000). In grass
334 shrimp embryos Comet assay was used to provide a sensitive measure of DNA strand
335 breakage exposed to genotoxicants, highway run off sediments, sediments with coal ash
336 and phototoxicants (Lee et al., 2000; 2008; Lee and Kim, 2002; Kim and Lee, 2004).

337 In the concentrations tested cadmium chloride and mercuric chloride induced
338 Comets in 72% and 61% of the cells respectively after an exposure over a period of 4
339 hours. Genotoxicity of cadmium was reported in rat hemispheres and cerebellum
340 (Klimova and Misurova, 2002) in rat Leydig cells (Yang et al., 2003), in *Palaemonetes*
341 *pugio* embryos (Hook and Lee, 2004), in *Mytilus edulis* (Pruski and Dixon, 2002;
342 Emmanouil et al., 2007) and in *Litopenaeus vannamei* (Chang et al., 2009). The
343 mechanism of metal induced carcinogenesis is still unknown, but one possible pathway
344 may involve the interaction of metals with DNA, either directly or indirectly (Hartmann
345 and Speit, 1994). Cadmium induces an oxidative DNA damage leading to DNA strand
346 breaks, DNA-protein cross-linking and inhibitions of DNA repair (Dally and Hartwig,
347 1997; Valverde et al., 2001). Genotoxicity of mercury was reported in *Cyprinus carpio*
348 gill cells (Arabi et al., 2004), blood cells of *Liza aurata* (Pereira et al., 2009), human
349 salivary gland tissue cells and lymphocytes (Schmid et al., 2007) and *Palaemonetes*
350 *pugio* embryo (Lee et al., 2000). HgCl_2 have a deleterious effect on the membrane
351 integrity and glutathione (GSH) content of gill cell suspensions of carp, *Cyprinus carpio*
352 L. and mercury caused DNA strand breaks at 3000 μM (Arabi et al., 2004). HgCl_2 has
353 been reported to cause DNA damage in *P. pugio* embryos at concentrations as low as
354 0.003 μM after a 12 hour exposure period (Lee et al., 2000). DNA damage was reported
355 by Pereira et al. (2009) in golden grey mullet, *Liza aurata* due to environmental exposure

356 of mercury. Clastogenic effects mostly associated with the spindle mechanism
357 disturbance and the generation of reactive oxygen species, accompanied by glutathione
358 depletion contribute to the genotoxicity of mercury (de Flora et al., 1994). The present
359 study demonstrates the potential of cadmium chloride and mercuric chloride to induce
360 DNA damage in primary haemocyte culture.

361 Giri et al. (2002) evaluated the genotoxicity of malathion using chromosome
362 aberration, sister chromatid exchange and sperm abnormality assays in mice.
363 Genotoxicity was detected in human liver carcinoma cells at 24 mM exposure of
364 malathion. In *Channa punctatus*, sublethal concentrations of malathion (Kumar et al.,
365 2010) and monocrotophos (Ali and Kumar, 2008) induced dose dependant DNA damage
366 in gill, kidney and lymphocytes were reported during a study period of 1-29 days and 1-
367 21 days respectively. Jamil et al. (2005) reported genotoxic effect of monocrotophos in
368 human lymphocytes in vitro employing Comet assay. A concentration dependant increase
369 in the DNA damage was detected in the erythrocytes of *Tilapia mossambica* (Banu et al.,
370 2001) and the gill cells of the bivalve, *Meretrix ovum* (Revankar and Shyama, 2009) after
371 the exposure to monocrotophos. Both malathion and monocrotophos are
372 organophosphorous compounds capable of phosphorylation and alkylation. Although
373 phosphorylation is responsible for the inhibition of acetylcholineesterase and produces
374 corresponding neurotoxicity in target and non-target species, other biological activities
375 such as mutation appear to be due to the alkylation properties (Eto and Ohkawa, 1970).
376 The heterocyclic bases of the nucleic acids may serve as nucleophilic agents for such a
377 reaction, and alkylation may take place on nitrogen atoms possessing high electron
378 density. Alkylation is more likely with the methyl ester group than ethyl and higher alkyl

379 ester groups, and phosphate esters such as monocrotophos are more reactive than the
380 phosphorothionate esters such as malathion. As organophosphorous insecticides are
381 chemical alkylating agents (Wild, 1975), alkylation of DNA bases either directly or
382 indirectly via protein alkylation is probably involved in the DNA damage (Mohan, 1973).
383 According to Wild (1975) the phosphorous moiety in the organophosphates appears to be
384 a good substrate for nucleophilic attack and it causes phosphorylation of DNA, which is
385 an instance of DNA damage. Malathion used in the present study is a commercial grade
386 product and may contain impurities formed during manufacturing and storage. Among
387 the impurities are malaoxon formed from malathion by oxidation and isomalathion
388 formed by isomerization (Berkman et al., 1993). Malaoxon and isomalathion have been
389 reported to be mutagenic (Flessel et al., 1993; Pluth et al., 1996, Blasiak et al., 1999).
390 These two compounds also could contribute to the genotoxicity of malathion. In the
391 present study malathion and monocrotophos, which are the two commonly used
392 insecticides in India, have proved to be genotoxic to *P. monodon* primary haemocyte
393 culture.

394 In conclusion, the study has demonstrated the use of primary shrimp haemocyte
395 culture for testing the cytotoxicity of aquatic pollutants such as heavy metals and
396 pesticides employing MTT assay. The primary cell culture system could also be
397 employed to assess the DNA damage induced by genotoxic pollutants in the aquatic
398 environment. The present study confirms the genotoxic potential of two heavy metal
399 compounds, cadmium chloride and mercuric chloride and two organophosphate
400 insecticides, malathion and monocrotophos, and this forms the first report of the
401 demonstration of genotoxicity of pollutants using shrimp cell culture as a model system.

402

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407

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ACCEPTED MANUSCRIPT

Research Highlights

- Primary haemocyte culture was developed from *Penaeus monodon*.
- Cytotoxicity and genotoxicity determination of aquatic pollutants was conducted.
- IC₅₀ values were calculated using MTT assay.
- All heavy metals and pesticides tested induced DNA strand breakage in haemocytes.

Table 1

DNA damage induced by heavy metals and pesticides in the primary haemocyte culture of *P. monodon*. Mean values and standard deviation obtained from average of 25 cells per experiment (Total 4 experiments for each compound).

Parameters	Control	Cadmium chloride (140 μM)	Mercuric chloride (17 μM)	Malathion (60 mg l^{-1})	Monocrotophos (186 mg l^{-1})
% cells with tail	11.46 \pm 7.89	72.20 \pm 7.46	60.83 \pm 17.72	67.75 \pm 13.55	61.55 \pm 12.98
Tail length (pixels)	1.3 \pm 0.50	33.47 \pm 9.24	23.09 \pm 6.83	29.13 \pm 7.75	28.17 \pm 7.87
% DNA in tail	2.04 \pm 1.25	44.21 \pm 4.76	44.72 \pm 7.25	40.81 \pm 9.10	38.85 \pm 13.68
Tail moment	0.04 \pm 0.03	17.17 \pm 6.23	11.82 \pm 5.50	14.36 \pm 5.56	13.86 \pm 5.74

Fig.1. Primary haemocytes after 12 hour exposure to A) Cadmium chloride (15.63 μM), B) Mercuric chloride (1.95 μM), C) Malathion (61.7 mg l^{-1}), D) Monocrotophos (100 mg l^{-1}), and E) Control.

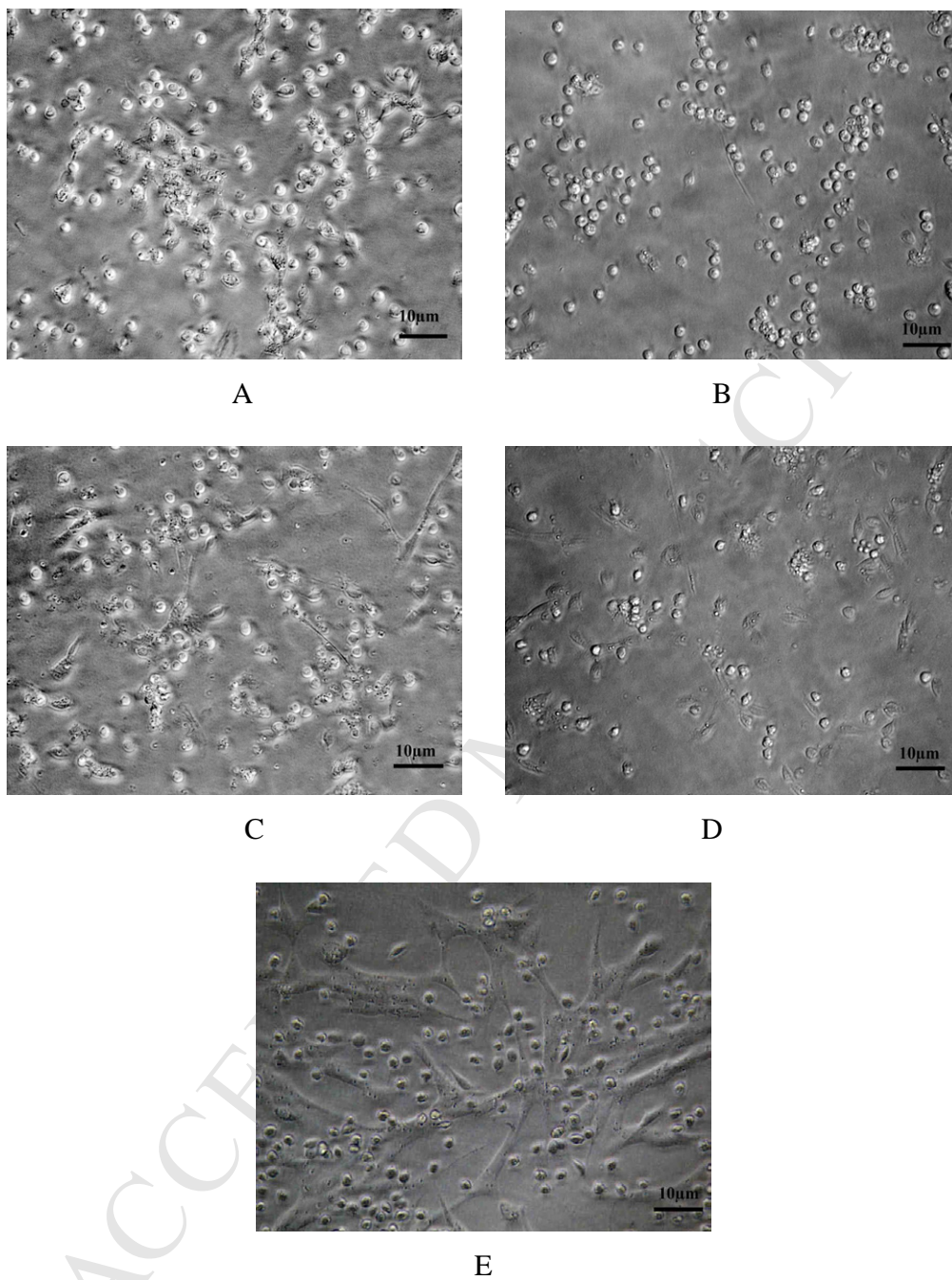
Fig.2. Toxicity of cadmium chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

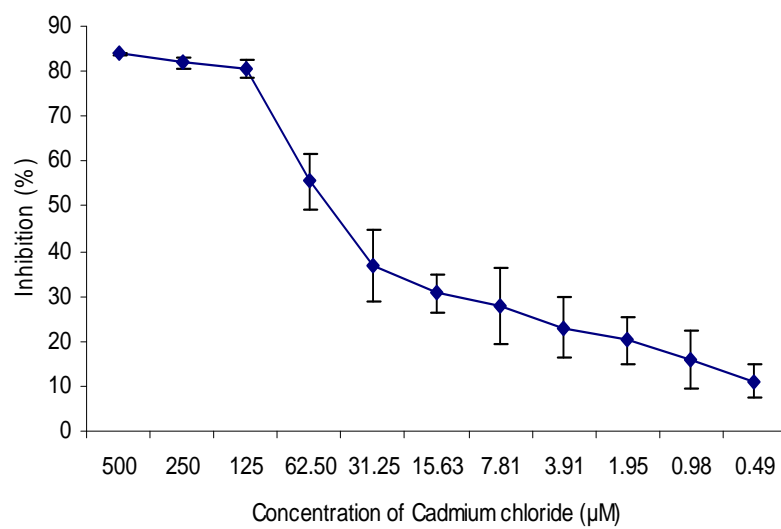
Fig.3. Toxicity of mercuric chloride in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

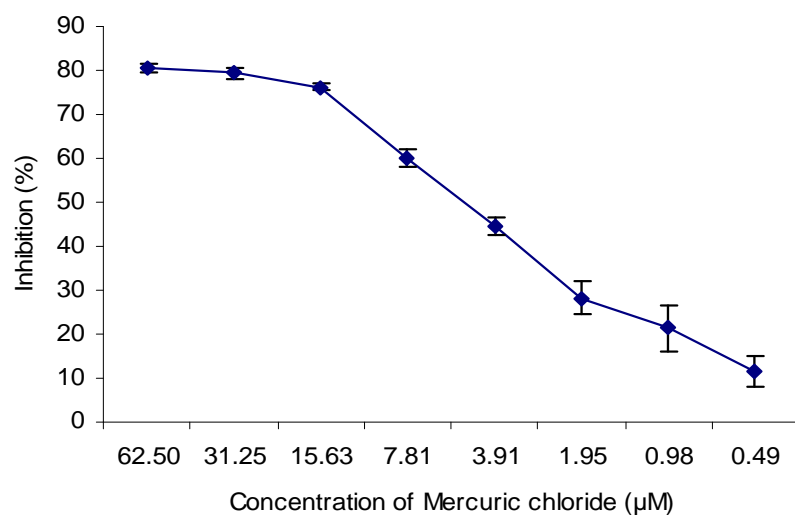
Fig.4. Toxicity of malathion in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

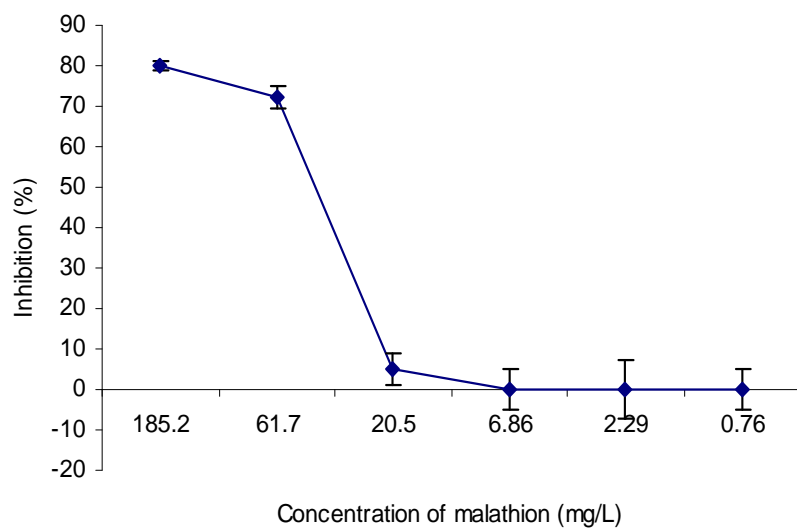
Fig.5. Toxicity of monocrotophos in primary haemocyte culture in terms of percentage inhibition of cells determined through MTT assay (n=4).

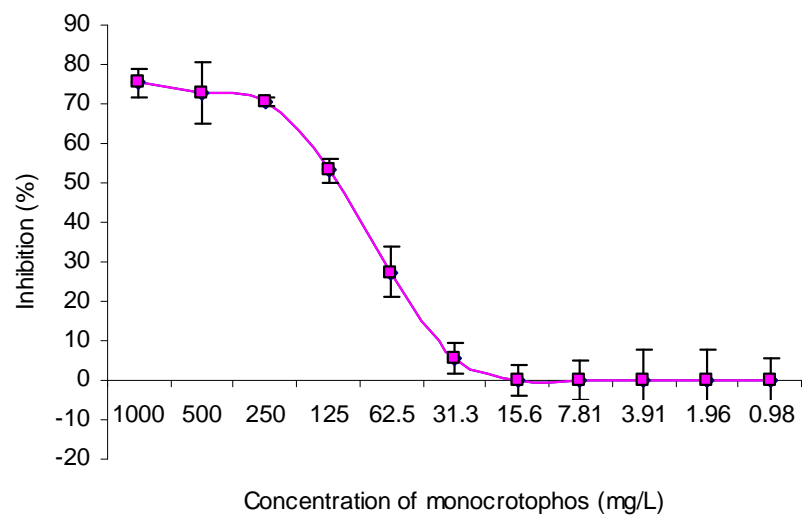
Fig.6. Comets formed by haemocytes after exposure to A) Cadmium chloride, B) Mercuric chloride, C) Malathion, D) Monocrotophos, and E) Control.

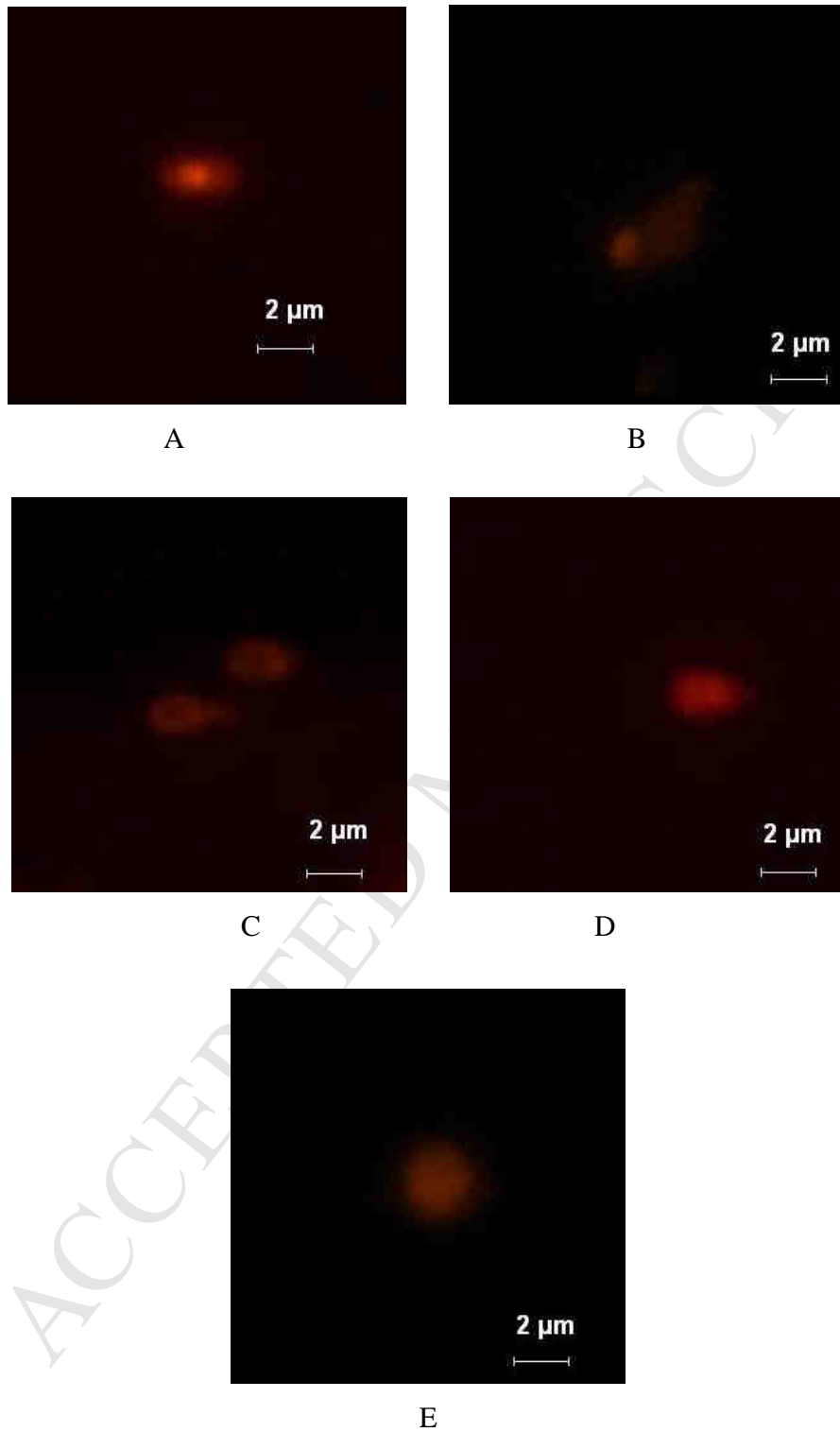
**Fig.1.**

**Fig.2.**

**Fig.3.**

**Fig.4.**

**Fig.5**

**Fig.6.**