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Acute Toxicity Bioassay Using the Freshwater Luminescent Bacterium *Vibrio-qinghaiensis sp. Nov.—Q67*

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Bioassays are an ideal complement to chemical analyses in evaluating toxicity in the environment. One of the rapid and cost-effective methods for screening purpose is the Microtox® assay system (Bulich, 1979) which is based on the measurement of the reduction in light emission of the luminescent bacteria *Vibrio fischeri* after contact with a toxicant. It has been widely used as a reproducible and sensitive screening method to determine overall toxicity from different types of samples. In China, the most commonly used strain for this kind of bioassay is *Vibrio fischeri T3 sp.* (China-NEPA, 1995). However, the obvious disadvantages of Microtox® assay are that it operates in a relatively narrow pH range (6.5-7.5) (Tarkpea et al., 1986) and because it is a marine species, NaCl has to be supplemented to every test solution to make a final concentration of 2-3% NaCl. Since higher concentrations of NaCl can influence the speciation and subsequent toxicity of certain contaminants in aqueous samples, most notably metals (Hinwood and McCormick, 1987; Vasseur et al., 1986; Ankley et al., 1989), and change the inherent properties of original fresh water samples, there is an apparent need to find freshwater species and develop relevant systems for this type of biological assessment.

A new species of freshwater luminescent bacterium (*Vibrio qinghaiensis sp. Nov.*) was isolated from body surface of *Cymnocypris przewalskii*, an edible fish. This is the major aquaculture product in Qinghai province of China. There is an annual production of about 6000 ton (Zhu et al., 1994; Jin, 1995). This type of freshwater bacterium exhibits a wide pH tolerance and can be luminous in freshwater. Strain *Q67*, among others, was designated as the typical strain of this newly exploited species. In this paper, we propose its use as an assay system and compare the EC_{50}s for selected heavy metals and organic compounds, and toxicity of polluted river water samples, both by assays using *Vibrio qinghaiensis sp. Nov-Q67* (hereafter referred to as *Q67*) and *Vibrio fischeri T3 sp.* (hereafter referred to as *T3*). The latter is the strain recommended for luminescent bioassay by the Chinese Environmental Protection Agency (China-NEPA, 1995). The overall aim is to evaluate the application potential of the newly isolated fresh water luminescent bacterium as a toxicity screening tool.

MATERIALS AND METHODS

Q67 was kindly provided by Professor Wenjie Zhu from East China Normal University. The culture medium consists of 13.6mg KH_2PO_4, 35.8mg $Na_2HPO_4.12H_2O$, 0.25g $MgSO_4.7H_2O$, 0.61g $MgCl_2.6H_2O$, 33.0mg $CaCl_2$, 1.34g $NaHCO_3$, 1.54g NaCl, 5.0g yeast extract, 5.0g tryptone, 3.0g glycerin and 1000ml distilled water. Before each test, the bacterium was inoculated from a stock culture, which is maintained on *Q67* culture medium agar at 4°C, to a fresh agar and cultured at 22±1°C for 24hr. The cells were further grown in liquid culture medium by shaking (120r/min) at 22°C±1°C for 18hr and harvested by centrifuge at 3000

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rpm for 10min. The pellet was resuspended with test medium and centrifuge twice. The bacterial suspension was made by adding test medium until the final relative light unit (RLU) reach 1.2X 10^7. The suspension was kept at 22°C± 1°C for 30 min before the test.

Lyophilized T3 bacterium was obtained commercially from Nanjing Institute of Soil Science, Chinese Academy of Science. The suspension was prepared according to the assay procedure of GB/T 15441-1995 (China-NEPA, 1995). Briefly, 5mg freeze-dried bacteria was revived in 1ml chilled 2.5% NaCl at 4°C for 3min, followed by adding 9ml 3% NaCl. The suspension was incubated at 22°C ± 1°C for 20 min before testing.

Before each test, the RLU of both of *Q67* and *T3* suspension was adjust to 3.5-6.5 million by adding test mediums. The assays of both of the *T3* and *Q67* were carried out by adding 0.1ml bacterial suspension and 0.9ml test medium to a plastic tube (low-background tube, commercially available from Berthod Company). It was thoroughly mixed and the initial relative light unit (RLU) was recorded by a luminometer (Berthod LB9501, Germany). The final RLU was recorded after 20 min incubation at 22°C ± 1 °C. Five concentrations were used for each chemical or water sample. EC_{50}s of chemicals, the concentration leading to a 50% decrease in RLU, were calculated from the plot of log Γ vs. log (concentration), where Γ is defined as the ratio of amount of light loss during incubation time to the amount of light remaining. EC_{50}, corresponds to Γ =1 (Bulich, 1982).

To optimize the assay conditions, initial cell density (with RLU from 1.7 to 13.4 million) of *Q67* suspensions were exposed to $CuSO_4$solution. Light intensity was recorded after 5, 20, 35 and 55 min and the conditional EC_{50}s were obtained.

To verify the influence of major cations and anions in natural fresh waters on bioluminescence, the corresponding chloride and sodium salts of the major cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, SO_4^{2-}, HCO_3^-, NO_3^-) were added to the test medium and stimulation and inhibition on bioluminescence were recorded. The test medium was prepared based on the average concentration of major cations and anions in natural freshwater (Stumm and Morgan, 1995a). The composition of the test medium is 42.0 mg/l of $NaHCO_3$, 4.2 mg/l of KCl, 11.1 mg/l of $CaCl_2$, and 28.6 mg/l of $MgSO_4 \cdot 7H_2O$. The ionic strength of test medium was adjusted to 0.05 mol/l $NaNO_3$, according to the studies on the influence of ionic strength on bioluminescence (I = 0, 0.025, 0.05, 0.1 mol/l, respectively).

Stock solutions of heavy metals were made up using chloride salts in established test medium. Chlorophenols and pesticides were dissolved in DMSO and diluted in two fold increments with test medium before an assay. The organic solvents in all tests were kept below 1%(v/v).

River water samples were collected from the Le An River which is severely polluted by the largest opencast copper mine in China (Wang, et al., 1994). The samples were kept in sealed glass jars after sampling and were assayed for acute toxicity immediately after transport to laboratory. Water samples were filtered through a 0.45 μm Millipore filter before testing.

The assay was conducted in triplicates on *Q67* and *T3* following the method mentioned above. The toxicity of water samples was expressed as percentages of inhibition (%H) and acute toxic units which is the reciprocal of the original water concentration in the test medium (in percentage) that causes 50% inhibition in bioluminescence.

RESULTS AND DISCUSSION

Fig.1 shows the influence of cell density and incubation time on EC_{50}s of $CuSO_4$to *Q67*. The EC_{50} varies with cell density and it was relatively stable when in the range of 3.5-6.5 million

RLU, which was chosen as an optimal suspension density. The cell density of the test organism and the testing volume are the two most important parameters affecting the bioassay results of microorganisms and algae. This results from the fact that exposure per capita decreases with increase of total number of organism and decrease of test volume (Stratton and Giles, 1990). It can be observed that EC_{50}, decreased slowly after 20 min at high cell density, therefore, an incubation time of 20 min was adapted as the optimal for the *Q67* bioassay, as shown in Fig. 1.

The influence of pH on the bioluminescence of the bacterium *Q67* was verified at pHs from 4.0 to 10.0. These were compared with that at pH 7.0 (as 100%). The loss of RLU from *Q67* is less than 10% from pH 5.0 to pH 9.0. This result demonstrated that the optimal bioluminescence of *Q67* could be obtained at pH 5.0-9.0 (Tab.1) which is within the range of natural waters (Chen et. al., 1987). In comparison with the conventionally used *V. fischeri* bioassay of which optimal pH is 7.3-7.5 (Svenson and Zhang, 1995), *Q67* exhibited a much wider pH tolerance. Therefore, it is not necessary to adjust the pH before the *Q67* assay for most of the samples (pH 5.0-9.0) and toxicity variation due to the change of pH could be avoided.

The influence of major cations and anions in natural waters showed significant influence on bioluminescence only when at higher concentration, except for calcium. Calcium enhanced the bioluminescence at concentrations higher than 20 mg/l. There should be no significant influence of other cations or anions expected when *Q67* bioassay is applied to water samples with concentration variation within the concentration ranges observed in natural waters (Tab.1). *Q67* is therefore suitable for bioassays using samples from most of the fresh water bodies. The influence of calcium on bioluminescence needs to be considered when this test is applied to waters of varying of calcium concentrations.

In the test medium without ion strength adjustment, RLU decreased rapidly with incubation time (Fig. 2). This resulted from lower osmotic strength of the test medium. In the solutions of which the ionic strength values are higher than 0.05mol/l $NaNO_3$, RLU decreased much more slowly, especially after 30 min of incubation. Since the bioluminescence is stable enough in medium of 0.05 mol/l $NaNO_3$, this is recommended as the standardized ion strength for *Q67* bioassay. The test should be performed 30 mins after the bacterial suspension was prepared.

The acute toxicity of six heavy metals was assayed using *Q67* and *T3* and the results are shown in Tab.2. Strain *Q67* is more sensitive to Hg, Cu and Zn, less sensitive to Cd and Ni, while strain *T3* is very sensitive to Hg and less sensitive to Cu and Zn. The same sequence for the toxicity of the Hg, Cu, Zn and Cd on these two strains was observed, that is Hg > Cu > Zn > Cd. Obviously strain *Q67* is more sensitive than strain *T3* for the tested heavy metals except for Hg. The difference in sensitivity of the two species may be attributed to the different susceptibility of organisms and, in part, to the complex formation of metals with chloride. For example, $HgC1_4^{2-}$ is the dominant species in inorganic sea water while $Hg(OH)_2$ is the dominant species in inorganic fresh water (Stumm and Morgan,1995b). The latter is a lipophilic complex whose toxicity may not follow the free-ion activity model (Campbell, 1995).

The toxicity of six commonly used pesticides in China, including both germicides and insecticides, was evaluated with *Q67* bioassay and the results were shown in Tab.3. As expected, *Q67* and *T3* are more sensitive to germicides than to insecticides, because they are closely related to the target organisms of germicides. The toxicity sequences of the tested germicides assayed both by *Q67* and *T3* are the same, that is fosethy-Al > quintozene > triadimefon. The toxicity sequence is different for the tested insecticides and *T3* was less susceptible than *Q67*.

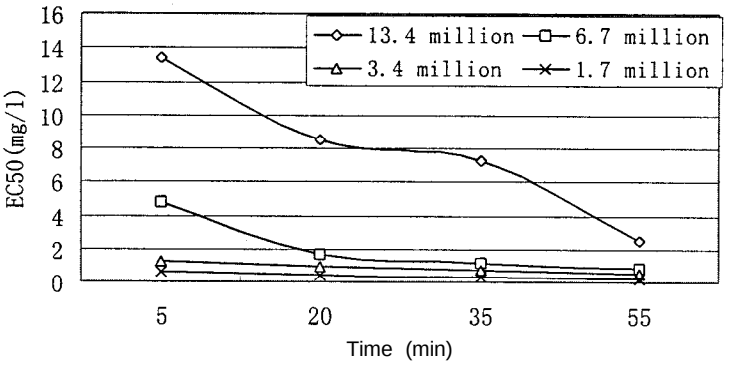

Figure 1. Influence of cell density and incubation time on $CuSO_4$ EC_{50} values of *Q67*

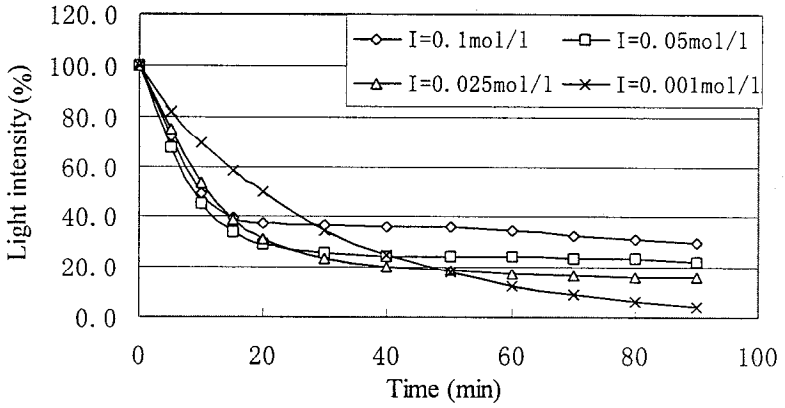

Figure 2. Light intensity change of *Q67* in solutions of different ionic strength

Table 1. pH and concentration range of major cations and anions in natural fresh waters and no observed effect concentration (NOEC, mg/l) for *Q67* bioassay

	Concentration range in natural fresh waters [a]	NOEC for *Q67* [b]
K^+	0.5-10	100
Na^+	0.7-25	100
Ca^{2+}	2-120	20
Mg^{2+}	0.4-6	100
HCO_3^-	6-19	100
Cl^-	1-35	100
SO_4^{2-}	0.2-40	1000
NO_3^-	0.002-1.8	100
pH	6.5-8.5	5.0-9.0

[a] Data source: Stumm and Morgan, 1995

[b] NOEC is defined as maximum concentration of cations or anions added to the test medium that produce an inhibition or stimulation of bioluminescence less than 10% of the control.

The toxicity of five chlorophenols was assayed and the results are shown in Tab. 4. The results were compared with those obtained from bioassay using *NRRL-B-1117*, a strain of

Vibrio fischeri (Kaiser and Devillers, 1994). The same toxicity sequence with increased chlorine substitution could be noted for both strains, but *Q67* is more sensitive to those chemicals. It is also showed that increasing lipophilicity corresponds to an increase in toxicity. In fact, the EC_{50}s of chlorophenols could be predicted by their lipophilicity. For *Q67*, the relationship between $lgEC_{50}$s and lgP is $lgEC_{50}$(mol/l)=-1.18 lgP-1.24 (R^2=0.981), but for NRRL-B-1117, it is $lgEC_{50}$(mol/l)=-0.977 lgP-1.41 (R^2=0.956).

Table 2. EC_{50}s of six heavy metals by *Q67* and *T3* bioassays

Compound[a]	EC_{50}(mg/l)	
	T3	*Q67*
Hg	0.05±0.0025	0.26±0.02
Cu	4.37±0.29	0.65±0.04
Zn	6.08±0.52	0.81±0.05
Cd	22.40±0.54	5.71±0.39
Ni	171.00±15.40	6.91±0.48
Mn	139.00±2.22	29.87±2.39

[a] Metal chlorides were used

Table 3. EC_{50}s of six pesticides by *Q67* and *T3* bioassays

	Trade Name	Chemical Name	EC_{50}(mg/l)	
			T3	*Q67*
germicide	fosethyl-Al	Aluminum tris (o-ethyl phosphonate)	4.98±0.29	8.59±0.34
	quintozene	pentachloronitrobenzene	12.40±0.37	11.04±0.55
	triadimefon	1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-butanone	55.80±3.90	121.80±2.44
insecticide	CCU[a]	1-(2-chlorobenzoyl)-3-(4-chlorophenyl) urea	572.70±22.91	170.10±15.31
	aldicarb	2-methyl-2-(methythio) propionaldehydeomethylcarbamoyloxime	530.70±42.46	649.20±51.94
	DMA[a]	N-(2,4-dimethylphenyl)-N' -methylformamidine	266.90±8.01	37.64±2.63

[a] Commercially available only in China

Both *Q67* and *T3* assays were applied to assess the toxicity of river water samples from the Le An River, which is severely polluted by multi-metals discharges from mines along the river. The river receives metal pollutants mainly from A04 and A07 and finally flows into Poyang Lake (A14). The results are shown in Tab.5. The *Q67* bioassay indicates the toxicity originated from two main pollution sources along the river, as expected as they were seriously polluted by heavy metals (Lin and Li, 1994). It was obvious that toxicity at A07 was much stronger than that at A04 and other locations. Bioassay of *T3* indicated only toxicity discharge from A07 and showed stimulation for sample of A04 for unknown reason. Toxicity of river waters decreased downstream as indicated by both bioassays.

This investigation indicated that the bioassay results obtained using strain *Q67* are comparable with those obtained by strain *T3*. Performance of the bioassays with this new freshwater species (*Q67*) to various chemicals and water samples reveals that it is as effective and reliable as the conventional *V. Fischeri* bioassay. In certain circumstances, bioassay with *Q67* may be preferable as it gave more reasonable explanation to the results obtained.

This study provided evidence that this species of freshwater luminescent bacterium is potentially a very useful species as a screening level test. This study suggested that the choice of test medium could profoundly affect the bioavailability of the pollutant and the resulting toxicity. Therefore, the *Q67* assay is preferred for freshwater bioassay because the composition of the test medium is most closely related to the nature of fresh water. It is apparent that wide pH tolerance is another advantage of *Q67* assay, because the toxicity change due to pH adjustment may be avoided.

It must be emphasized that toxicity is species specific and chemical specific. Variations in the sensitivity of organisms to toxicants can be quite large. No single species can be proven unquestionably superior to all others. The small battery of bioassays presently available is too restricted to fully cover the potential toxic impact of complex wastes on all types of aquatic biota. Using several species simultaneously in multispecies bioassays, the likelihood of making an erroneous conclusion may be reduced.

Table 4. EC_{50}s of five chlorophenols by *Q67* and Vibrio fischeri *NRRL-B-11177* bioassays

Chemical	Log P[a]	EC_{50}(mg/L)	
		Q67	*NRRL-B-1117*[b]
3-CP (mg/L)	2.48	9.43±0.28	14.1±0.71
2,3-DCP (mg/L)	3.09	2.09±0.19	4.92±0.34
2,5-DCP (mg/L)	3.24	1.87±0.07	9.38±0.19
2,4,5-TCP (mg/L)	4.10	0.11±0.007	1.27±0.08
2,3,4,5-TCP (mg/L)	4.71	0.06±0.0048	0.18±0.01

[a] Octanol-Water Partition coefficients from Xie et al. (1984)
[b] Data was cited from Kaiser and Devillers (1994)

Table 5. Toxic evaluation of water samples from Le An River by *Q67* and *T3* bioassays

Sample site	*Q67*		*T3*	
	%H[a]	TU[b]	%H[a]	TU[b]
A01	6.39		8.75	
A04	30.32		-16.79	
A05	12.67		-1.12	
A07	83.56	4	56.50	1.2
A08	29.32		5.69	
A13	25.80		8.92	
A14	29.40		1.75	

[a] Percentages of inhibition in bioluminescence for original water samples
[b] Acute toxicity units defines as the reciprocal of original water concentration in test medium (in percentage) that causes 50% inhibition in bioluminescence

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