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Application of *Vibrio qinghaiensis* sp. Q67 for Ecotoxic Assessment of Environmental Waters – A Mini Review

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ABSTRACT

Ecotoxicity assessment using luminescent bacteria has been wildly used because it is rapid, sensitive and cost effective for screening water and wastewater quality. This mini-review focused on the application of *Vibrio qinghaiensis* sp. Q67 (abbreviated as "Q67"), a natural freshwater luminescent bacteria strain discovered in China. The characteristics of the bioassay using Q67 were firstly reviewed with comparison to that using the *Vibrio fischeri*, a widely used marine luminescent bacteria strain. In addition to the principal of bioassay using luminescent bacteria, attention was paid to the applications of Q67 to the toxicity assessment of organic and inorganic substances, and practical water samples. With its advantage for direct evaluation of freshwater samples without salt addition, Q67 toxicity test can be a good alternative of *Vibrio fischeri* for toxicological study of environmental waters.

Keywords: *Vibrio qinghaiensis* sp. Q67; *vibrio fischeri*; bioassay; environmental water

1. INTRODUCTION

Aquatic environmental assessment is usually based on the analyses of a wide spectrum of environmental pollutants. With the development of analytical technologies, more and more pollutants from organic and inorganic sources can be quantitatively detected from various environmental waters even in trace concentrations. This has made it possible to carry out a thorough screening of environmental pollutants and a comprehensive evaluation of the water quality from the viewpoint of environmental protection. Such kind of water quality screening and assessment are fully dependent on national environmental standards. For example, in the United States, the National Recommended Water Quality Criteria (USEPA, 2013) specifies 58 water quality items for the protection of aquatic life, 121 items for the protection of human health, and 27 items for the control of organoleptic effects (e.g., taste and odor) in surface water, while in China, the Environmental Quality Standards for Surface Water, GB 3838-2002 (MEP & GAQSIQ, 2002) specifies 24 items for all surface waters and 85 additional items for source water of potable supply. Other standards or regulations such as those for domestic/industrial effluent discharge can also provide legal basis for the assessment according to the study objectives. By comparing the monitoring data with the applicable standard and/or guideline values, estimation can be obtained on the conformity of the water for certain water use and/or the major water quality problems related to individual pollutants.

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However, in many cases the objective of water quality assessment may not be to screen fully the water quality parameters but to conduct a general evaluation of the harmful or toxic effects from known and/or unknown pollutants in the water. For such purposes, bioassays are found to be very useful as supplementing methods for water quality assessment and providing information about the comprehensive toxic effects of various pollutants on aquatic ecosystems.

Of the methods for ecotoxicity assessment, bioassays using luminescent bacteria are widely applied in water toxicity tests for its advantages of good sensibility, reproducibility, flexibility and low cost (Parvez et al., 2006; Mendonça et al., 2009). The luminescent bacteria assays often show good correlations to toxicity bioassays using other flora and fauna such as algae, crustacean and fish (Girotti et al., 2008). The most commonly used luminescent bacterium is *Vibrio fischeri* as has been standardized by International Standard Organization for determination of inhibitory effect of water samples on the light emission (ISO 11348-2008). Because *Vibrio fischeri* is a marine strain, it needs a salty environment for conducting the ecotoxicity test (Jones et al., 2011; An et al., 2012). Therefore, when *Vibrio fischeri* is used for freshwater samples, NaCl addition is required to adjust the salt concentration of the testing samples to 2-3%. One concern on the application of *Vibrio fischeri* in such a way is that the addition of high concentration salt may change the inherent property of the testing sample such as an increase in the insolubility of organic substances and/or a decrease in the bioavailability of metal ions (Farré and Barceló, 2003). As an alternative luminescent bacterium, *Vibrio qinghaiensis* sp. Q67 (abbreviated as "Q67") has been isolated from the body surface of *Cymnocypris przewalskii*, one of the edible fish in Qinghai Province, China (Zhu et al., 1994). It is verified that Q67 has similar property to other luminescent bacteria in light emission and inhibition, and can be used for the bioassay under a freshwater environment which much benefits the toxicity tests in most cases (Ma et al., 1998). Nowadays, both bioassays using *Photobacterium phosphoreum* T_3 spp. (marine strain) and Q67 are recommended as applicable methods for ecotoxicity tests in China (Wei, 2002).

Since the ecotoxicity test using Q67 is based on the inhibitory effect of the testing water sample on the light emission, principally any substance which can inhibit the luminescence may be evaluated as to be "toxic" to the Q67 bacterium. On the other hand, the inhibition of the water sample on the light emission may also be affected more or less by its chemical composition. Therefore, it is still unclear that to which extent the toxic effect from individual toxic substances can be truly evaluated and how reliable the Q67 test will be. In order to answer such questions, this article provided a mini-review of the literatures on the application of Q67 in recent years in the ecotoxicity tests for water samples with known organic substances, inorganic substances, as well as practical water and wastewater including the authors' own experiences. The review was focused on the literatures on the use of natural Q67 strains but excluded those on the application of genetically modified strains and biosensors.

2. PRINCIPLE OF BIOASSAY USING LUMINESCENT BACTERIA

The bioassay using a luminescent bacterium principally relies on the bioluminescent enzyme system which consists of a NAD(P)H:FMN oxidoreductase and a luciferase. The reduced flavin mononucleotide ($FMNH_2$) plays an important role in the bioluminescence reaction. Upon reaction with the reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) in the pres-

ence of NAD(P)H:FMN oxidoreductase enzyme, FMN is reduced to $FMNH_2$ following a reaction shown in Eq. (1).

$$NAD(P)H + FMN + H^+ \xrightarrow{\text{NAD(P)H:FMN oxidoreductase}} NAD(P)^+ + FMNH_2 \quad (1)$$

Reduce $FMNH_2$ gets oxidized into FMN and H_2O upon reaction with molecular oxygen In the presence of long chain aldehyde and luciferase enzyme, $FMNH_2$ is then oxidized into FMN (Inouye, 1994) following a reaction shown in Eq. (2) which is accompanied by the emision of blue-green light of 420 nm in wavelength.

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} FMN + RCOOH + H_2O + h\nu \quad (2)$$

Any factors that may affect the abovementioned bacterial metabolism will disturb the luminescent light emission. By measuring the light intensity of the testing sample and comparing with that of the blank control, the inhibition (%) can be calculated for characterizing the toxic effect of the pollutants in the sample. In most cases a series testing samples with varied concentrations should be prepared for the luminescent bacteria tests, and the effective concentration (EC_{50}) which is the concentration corresponding to the inhibition value of 50% can be obtained from the relationship between concentration and inhibition. By definition, the higher the EC_{50} value, the lower the ecotoxic effect. For the convenience of comparison following the common sense, the toxicity units (TU) or toxicity impact index (TII_{50}) can be introduced for quantifying the toxicity following Eqs. (3) and (4) (Farré and Barceló, 2003; Araújo et al., 2005).

$$TU = (EC_{50})^{-1} \times 100 \quad (3)$$

$$TII_{50} = (EC_{50})^{-1} \times 100 \quad (4)$$

Although TU and TII_{50} are calculated in the same way, terminologically they are different parameters. TU is defined as the toxicity units related to the amount of a known substance which may not be comparable with the toxicity of other substances, whereas TII_{50} is defined as the toxicity impact index related to the amount of a mixture of unknown composition which is expressed in percentage and allows comparison of the toxicity between different waters (Farré et al., 2001).

Concerning the toxicity assays, the luminescent bacteria can be used both for short- and long-term tests. The short-term tests, usually 5-30 min and thus called the acute toxicity tests, are based on the change of light intensity due to a disturbance on the photosensitization activity by toxic substances, while the long-term tests, usually 12-24 h and called the chronic toxicity test, can be used to examine the changes in viability or growth rate of the bacteria.

As the toxicity analysis is based on the measurement of luminescent light intensity, any substance that can increase or adsorb the light may interfere with the analysis and lead to erroneous results. For example, the color in a water sample may increase the light intensity at the wavelength of the luminescent light emitted by the bacteria while suspended particles may disperse or adsorb the luminescent light. All these may much disturb the determination of the true inhibitory effect of the toxic substances in the water sample. Therefore, sample pretreatment and light intensity calibration are often required in conducting the toxicity tests. For the removal of turbid substances, centrifugation or filtration is a common pretreatment stage before the luminescent bacteria test (Hernando et al., 2006). In particular, some inorganic matter containing in a water sample, such as nutrient salts, may promote the metabolic activity of the luminescent bacteria, and stimulate light emission (Rosal et al., 2010). In order to eliminate the stimulating effect of inorganic salts, many pretreatment methods have been utilized. When organic substances are the targets of

ecotoxicity test, liquid-liquid extraction (Pérez et al., 2009; Cao et al., 2009), resin adsorption (Liška, 2000; Reginatto et al., 2009) and solid-phase extraction (Pessala et al., 2004; Smital et al., 2011; Ma et al., 2011) are the common pretreatment methods for effective extraction of organic substances and elimination of all inorganic interferences. If heavy metals become the target substances, passive sampling can effectively concentrate the ionic metals by utilizing the diffusive gradient in thin-films and semi-permeable membrane devices (Roig et al., 2011).

3. CHARACTERISTIC OF Q67 FOR TOXICITY ASSAYS

3.1 Toxicity assays for known organic substances

In recent years, the Q67 test of organic compounds mainly focused on herbicides, insecticides, ionic liquids (ILs) and phenolic compounds. The toxicity of single substances and mixtures were studied using the short- and long-term inhibition assay on Q67 for predicting the mixture toxicities by interaction models.

Water pollution by herbicides and insecticides has constituted a serious environmental problem due to potential toxicity and bioaccumulation. The interaction mechanisms of their combinations were studied using Q67 with microplate format. By Q67 tests, the overall toxicity of the multiple component mixtures of ten compounds, including three herbicides and seven insecticides, were found to be in very good agreement with those predicted by the concentration addition (CA) model (Zhou et al., 2010) as below:

$$\sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = 1 \qquad (5)$$

where, n is the number of components in the mixture, EC_{xi} is the concentration of the i-th component that provokes x% effect when applied individually and c_i is the concentration of the i-th component in the mixture.

In order to investigate whether the CA model could predict the combined toxicity of herbicides and insecticides, five herbicides and one organophosphorus (OP) insecticide were selected as the test components, and the results indicated that the combined toxicity was predictable by the CA model (Liu et al., 2009). The overall toxicity of the multiple component mixtures of six OP insecticides could also be predicted by the CA model (Zhang et al., 2008). For some organic mixtures, an independent action (IA) model was also applicable especially at the low- concentration range (Zhang et al., 2008). The most widely used mathematical equation for IA can be expressed as (Zhou et al., 2010):

$$E(c_{mix}) = 1 - \prod_{i}^{n} [(1 - E(c_i)] \qquad (6)$$

where, $E(c_{mix})$ is the total effect of the mixuture and $E(c_i)$ is the effect of the i-th component. In contrast to CA, the concept of IA is based on an assumption of a dissimilar mechanism of action for all mixture components. Although the mechanisms that explain these interactions are yet experimentally verified, the CA and IA models can provide useful tools for the prediction of toxicity of the pesticides mixtures based on the toxic effect of individual component determined by Q67 test.

By short- and long-term Q67 toxicity analyses for six triazine herbicides, it was found that comparing with the short-term toxicity test, the long-term toxicity test, though time consuming, could provide additional information on the toxicity of toxicants with different modes of action, and from the concentration-time-effect surface dynamic analysis of the toxicity could be conducted for investigating the toxicity development over the incubation time (Zhu et al., 2009).

Although ionic liquid (IL) is a class of salts in the liquid state at room temperature, it typ-

ically consists of a bulky organic cation in combination with various anions and the structure of organic-base has a great effect on its physicochemical properties. Therefore, the studies on the toxicity of ILs can also be put into the category of organics. As green solvents, ILs are widely applied in various fields for their special physicochemical properties. In some studies, ILs were found to be toxic to algae, daphnia magna, earthworm and fish (Pretti et al., 2009; Luo et al., 2010). In Q67 microplate toxicity analysis, most of the IL mixtures displayed the classical addition while some IL mixture exhibited antagonism or synergism (Zhang et al., 2011). In a study on the toxicity of eight ILs, four consisting of 1-ethyl-3-methylimidazolium ([emim]) and the others of 1-butyl-3-methylimidazolium ([bmim]), using Q67, the toxicities of [emim]-based ILs were found to be lower than those of [bmim]-based ILs, and the mixture of [emim]-based ILs exhibited synergism while [bmim]-based ILs resulted in antagonism (Zhang et al., 2012a). The joint toxicity between IL and pesticide was also analyzed using Q67. The results indicated that all the binary mixtures between IL and pesticide exhibited a similar toxicity action rule, i.e., displayed a synergistic interaction in a high concentration region, an additive action in a medium concentration region, and an antagonistic interaction in a low concentration region (Zhang et al., 2009). The hormetic effect of ILs on Q67 was found to depend not only on the range and spacing of exposure concentration but also on their structure components (Wang et al., 2011). By a long-term toxicity tests of four 1-alkyl-3-methylimidazolium bromides ([amim]Br), it was also indicated that [amim]Br with shorter side chains such as 1-ethyl and –butyl could cause obvious hormetic time-dependent effect because they significantly induced FMN, NADH, superoxide dismutase and catalase (Zhang et al., 2012b).

In a study on seven phenolic compounds in aqueous solutions using Q67 bacteria, it was illuminated that the mixture toxicity of phenolic compounds well followed both the CA and IA models at any concentration ratios and the concentration levels under consideration, indicating that either similar action mechanism or dissimilar action mechanism would be valid for multi-component mixtures (Huang et al., 2011).

Regarding four organic compounds frequently encountered in domestic and/or industrial wastewater, namely ciprofloxacin, acetaminophene, linear alkybenzene sulphonate (LAS) and phenol, the authors compared their toxic effects on Q67 in terms of EC_{50} on the basis of total organic carbon (TOC) concentration as 57.97, 627.66, 10.62 and 169.95 mg/L, respectively (Ma and Wang, 2013). This indicated that LAS, as a typical surfactant commonly used everywhere and usually existing in rather high concentration in wastewater, was much toxic than the pharmaceutical products (ciprofloxacin and acetaminophene) and phenol.

Table 1 Toxicity assays of known organic substances using Q67 bacteria

Organic substances	References
Herbicides	Zhang et al. (2009); Liu et al. (2009); Zhu et al. (2009); Zhou et al. (2010)
Insecticides	Zhang et al. (2008); Liu et al. (2009; Zhou et al. (2010)
ILs	Zhang et al. (2009); Wang et al. (2011); Zhang et al. (2011); Zhang et al. (2012a); Zhang et al. (2012b)
Phenolic compounds	Liao et al. (2010); Huang et al. (2011)
Herbicides and pesticides combinations	Liu et al. (2009); Zhou et al. (2010)
Insecticides and ILs combinations	Zhang et al. (2009)

Table 1 summarizes the major studies conducted in recent years for toxicity assays of known organic substances using Q67 bacteria.

3.2 Toxicity assays for inorganic substances

For inorganic substances that are suspicious of being ecologically toxic, most of the research works have focused on heavy metals, including Q67 toxicity studies on individual heavy metals, and their additive, synergistic or antagonistic relations.

Table 2 summarizes the reported toxicity values in term of EC_{50} for individual heavy metals commonly encountered in water environment based on existing Q67 toxicity studies. There are apparently large differences between the EC_{50} values of the same heavy metal reported by different researchers, possibly due to the different chemical compositions of the testing samples prepared, especially the coexisting anions in the solution. However, if the ranges of the EC_{50} values for each of the heavy metals listed in the table are compared, Hg seems to be most toxic on Q67, and then Cu and Cd. A reliable detailed comparison of the toxicities of different heavy metals will much depend on a precise control of chemical composition of the testing sample, as well as the condition for the Q67 bioassay.

In study the combined toxicity of on Q67, the synergistic effects were found among four heavy metals, i.e., Cu, Zn, Cd and Hg, for most of the paired mixtures, except for the Zn-Cd mixture which acted antagonistically and the Cu-Zn mixture which acted additively (Liu et al., 1997). The additive effect of the binary mixture of Cu-Zn was also identified in another study, while the binary mixtures of Cu-Hg, Cu-Cd, Cu-Ni were found to show antagonistic actions (Gao et al., 2003). As the number of constituents in the mixtures increased to three or more, the combined effects tended to be consistent with an additive mode (Liu et al., 1997). However, antagonistic effects were reported for the equitoxic mixture of nine heavy metals (Deng et al., 2007). Similar to organic substances, both the CA and IA models have been found to be useful for predicting the combined effects of various heavy metals in many of the studies cited above. Anyway, according to the models applied the interpretations on the interactions among heavy metals in combined mixtures would differ from each other.

Table 2 EC_{50} values of heavy metals from published literatures

Compound	EC_{50} (mg/L)
Cu	0.211 (Liu et al., 1997), 0.212 (Gao et al., 2003), 9.392 (Deng et al., 2007), 2.74 (Ma and Wang, 2013)
Zn	0.092 (Liu et al., 1997), 3.185 (Gao et al., 2003), 7.684 (Deng et al., 2007), 64.503 (Song et al., 2008), 0.80 (Ma and Wang, 2013)
Cd	0.298 (Liu et al., 1997), 4.361 (Gao et al., 2003), 5.021 (Deng et al., 2007), 8.887 (Song et al., 2008), 2.587 (Huangfu et al., 2010), 0.40 (Ma and Wang, 2013)
Hg	0.495 (Liu et al., 1997), 0.465 (Gao et al., 2003), 0.103 (Huangfu et al., 2010), 0.59 (Ma and Wang, 2013)
Ni	12.503 (Gao et al., 2003), 102.009 (Deng et al., 2007), 126.078 (Song et al., 2008)
Cr	211.838 (Cr(III)) (Deng et al., 2007), 5.835 (Cr(VI)) (Huangfu et al., 2010), 2.71 (Ma and Wang, 2013)
Co	48.991 (Deng et al., 2007), 82.436 (Song et al., 2008)
Pb	6.731 (Huangfu et al., 2010), 1.09 (Ma and Wang, 2013)
As	5.801 (Huangfu et al., 2010)
Fe	25.506 (Deng et al., 2007)
Mn	223.65 (Deng et al., 2007)
Se	37.812 (Deng et al., 2007)

Because the Q67 toxicity tests are usually conducted under freshwater environment, the disturbance of coexisting salts on the tests is a large obstacle for precise evaluation of the ecotoxicity of inorganic toxicants. NaCl and $MgCl_2$, as available nutrients for Q67 bacteria, can often result in a stimulating effect on Q67 even at a normal concentration level. Although passive sampling may be a method for concentrating target ionic metals for eliminating the influence from nutrient salts (Roig et al., 2011), it can only be applicable in very special cases but not a common pretreatment procedure. The stimulating effect of nutrient salts is almost impossible to eliminate for complex samples, such as soil sample extracts because of the difficulty in preparing the blank control with exactly the same components of salts. How to isolate the target inorganic substances and remove the interference substances is still a topic of study for obtaining more reliable Q67 toxicity testing results.

3.3 Toxicity assays for practical water samples

Domestic wastewater is one of the major sources of pollutants in urban regions. Therefore, the toxic effect of effluent discharge on receiving water bodies often draws wide attention from the viewpoint of ecological safeguard of water environment. Regarding this, a study was conducted on the variation of ecotoxicity of organic pollutants at different stages of an oxidation ditch process which is commonly applied for domestic wastewater treatment in China. By a bioassay using Q67 associated with solid-phase extraction (SPE) for isolating organic substances from water samples, the toxicity impact index (TII_{50}) was found to decrease from 50.51% in the influent to 25.84% after the grid chamber and then to 1.38% for the secondary effluent, but the final chlorination stage resulted in a slight TII_{50} increase. A good linear relationship was found on the log-log plot of the total COD concentration versus the TII_{50} value (Ma et al., 2011). The significant toxicity reduction in the biological treatment unit well agreed with the finding of Katsoyiannis and Smara (2007) who conducted toxicity tests using *Vibrio fischeri* and pointed out that the water toxicity was mainly contributed by the biodegradable fraction of the organic substances in the wastewater. The Q67 test was thus proved to be as equally effective as the *Vibrio fischeri* test for toxicity assessment of domestic wastewaters.

The toxicity tests could also assist an optimization of the biological wastewater treatment process. Huang et al. (2010) used luminescent bacterium inhibition rate (LBIR) as the toxicity parameter and investigated how water toxicity might be influenced by the operational parameters, such as hydraulic retention time, sludge retention time and internal recycle ratio, of an anaerobic-anoxic-oxic (A^2O) process. They found that under the optimum operation condition, 82.2% toxicity reduction could be achieved by the A^2O treatment.

By Q67 bioassay, the ecotoxicities of gas-field, oil-field, dyeing, electroplating, and coking wastewaters were studied. By comparison with the organic and heavy metal contents of each wastewater, it was identified that the highest ecotoxicity of the coking wastewater would be mainly caused by organic contaminants, while the ecotoxicity of the electroplating wastewater that was also very high would be mainly caused by heavy metals (Ma and Wang, 2013).

Toxicity evaluation of practical water samples has often been conducted by bioassays not only using Q67 bacteria but also other fauna or flora. In a comparative study of domestic wastewater and pharmaceutical wastewater treated by conventional biological

processes with toxicity bioassays using Q67, prawn, and fish, it was illuminated that after the treatment processes the toxicities of the domestic wastewater on all these organisms could be effectively reduced while the reduction of toxicities of the pharmaceutical wastewater on prawn and fish still needed improvement. The Q67 toxicity test seemed to be less sensitive in the case of the domestic wastewater than the pharmaceutical wastewater (Gerhardt et al., 2002). For surface water quality evaluation, Q67 and *Vicia faba* root tip tests were carried out regarding 3 rivers, two lakes and two streams with effluent flows from domestic wastewater treatment plants. Although no correlative relation was found between COD and TII_{50} obtained by the Q67 tests or RMCN (relative frequency of micronucleus) obtained by the *Vicia faba* root tip tests, there was a linear correlative relation between TII_{50} and RMCN for most water samples. The TII_{50} and RMCN were also found to correlate to the dissolved oxygen (DO) concentration of the water, indicating that sufficient DO in surface water would be an indicator of a healthy water environmental condition (Ma et al., 2012).

Q67 and another luminescent bacteria *Photobacterium phosphoreum* (T3) were used for the toxicity study of an explosive wastewater. The results indicated that Q67 was more sensitive than T3 for acute toxicity evaluation. As the high toxicity of the explosive wastewater was identified to be caused by a group of non-biodegradable substances, resin adsorption was recommended as an effective method for toxicity reduction and improvement of the biodegradability to facilitate further treatment by a biological process (Ye et al., 2011).

In order to identify the sources and intensities of heavy metal pollution from originating from mining, smelting and panning activities and their impacts on the river water, a series of ecotoxicity tests using Q67, T3 and *Daphnia magna* were integrated with chemical analyses and ecological response using algae, rotozoa and zooplankton for a overall evaluation of the adverse effects. In addition to the proposal of options for the metal source control, it was illustrated that any of the chemical, toxicological and ecological assessments might have its disciplinary limitation, and the integration of all these methods could be very advantageous for better understanding the impacts of heavy metal pollution on aquatic ecosystems (He et al., 1998; Liu et al., 2003).

CONCLUSIONS

Luminescent bacteria test using Q67 is a method developed in China. As the mechanisms of luminescent light emission and inhibitory effect evaluation for Q67 are similar to that for the marine luminescent bacterium *vibrio fischeri*, the method can be equally used for ecotoxicity assessment of aquatic systems. An additional advantage of using Q67 for ecotoxicity tests is the simplification of the procedures because as a freshwater bacterium Q67 can be directly used under ordinary environment without salt addition that is an unavoidable step when *vibrio fischeri* is used for a freshwater sample. In this article, the characteristics of Q67 for the toxicity assessment of organic substances, inorganic substances, and practical water samples were reviewed based on the literatures mostly contributed by Chinese researchers but published in international journals. The information provided may assist better understanding the advantage and limitation of this method. Because the method of Q67 toxicity test has not been standardized, further study is still needed for its improvement.

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