ABSTRACT

Title of Thesis: DETECTION AND CHARACTERIZATION OF HARMFUL ALGAE BY BIOLUMINESCENT STRESS FINGERPRINTING

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Harmful algal blooms (HABs) pose serious health and economic problems due to biotoxins produced by algae species. A biosensing method employing luminous bacteria was used to detect and characterize the response generated when encountering four critical harmful algae, *Karlodinium micrum*, *Pfiesteria piscicida*, *Chattonella marina*, and *Prorocentrum minimum*. This sensing system includes six *Escherichia coli* strains containing different stress-responsive promoters fused to the *Photohabdus luminescens* *luxCDABE* reporter. At the concentration of approximatly 6,000 cells/ml, these algal species induced stress responses of the biosensing strains higher than did the control, a non-toxic dinoflagellate *Akashiwo sanguinea*. The stress responses induced by harmful species showed unique patterns for each of the algae investigated, suggesting that characteristic fingerprints could be generated based on such stress responses. Moreover, dose dependency was observed between the bioluminescence from the sensing strains

and the level of algae concentrations, indicating possible quantification of harmful algal species using specific stress response.

DETECTION AND CHARACTERIZATION OF HARMFUL ALGAE BY BIOLUMINESCENT STRESS FINGERPRINTING

By

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Chapter 1: Introduction

Occurring through recorded history, harmful algal blooms (HABs) are completely natural phenomena. But, in the past two decades, such events have dramatically increased in frequency, intensity and geographic distribution, which cause adverse impacts on public health and economy. Toxins produced by harmful algae can affect and even kill the higher forms of life such as zooplankton, shellfish, fish, birds, marine mammals, and even humans that feed either directly or indirectly on them. On a global scale, close to 2000 cases of human poisoning (15% mortality) through fish or shellfish consumption have been reported each year and, if not controlled, the economic damage through reduced local consumption and reduced export of seafood products can be considerable (Hallegraeff, 1993). In Maryland coastal water areas, the most interest is in four harmful algal species: *Chattonella marina*, *Karlodinium micrum*, *Prorocentrum minimum*, and *Pfiesteria piscicida*.

The development of advanced detection methods becomes more and more important to deal with many health and economic problems caused by HABs. Harmful algal toxins have been detected and quantified using one or a combination of several techniques. Generally, biological methods(Amzil and Pouchus, 1992, Yasumoto and Underdal, 1990), chemical analysis (Bates and Rapoport, 1975, Sullivan and Jonas-Davies, 1985, Lee *et al.*, 1987, Subba Rao *et al.*, 1988) and immunological methods are applied in many toxic algae studies. The major drawback of bioassays is the necessity to maintain or to purchase frequently a large number of animals with almost same weights (Lee, Yanagi, Kema and Yasumoto, 1987). In addition, high cost and complicated

operation process limit the wide application of chemical analysis and immunological methods (Andersen, 1996). In recent years, an advanced cell-based assay has been successfully conducted to detect harmful algal toxins, such as brevetoxins, saxitoxins and ciguatoxins, using a stably expressed c-fos-luciferase reporter gene (Fairey *et al.*, 1997). The work evaluated the sensitivity of this reporter gene assay to those algal toxins that activate or inhibit sodium channels. Although this assay presented the potential to enhance the sensitivity of existing bioassays for sodium channel active algal toxins, it could not identify and further quantify harmful algal species with high specificity.

A set of six *Escherichia coli* strains was assembled in Microbial Genetics Lab (DuPont Company, Wilmington, DE) with different selected stress-responsive promoter that was fused to the *Photohabdus luminescens luxCDABE* reporter. These fusions were found to be responsive to oxidative damage, internal acidification, DNA damage, protein damage, "super-stationary phase" and sigma S stress response (Van Dyk, 1998). Thus, six selected stress responsive fusions can be used to characterize a variety of chemically-induced stresses, which provides a feasible way to apply these biosensing strains in detecting and quantifying harmful algae species based on the stress fingerprints induced by the toxins.

Chapter 2: Literature Review

2.1 Harmful Algal Blooms (HABs)

Harmful algal blooms (HABs) can be defined as events where the concentration of one or several harmful algae reach levels which can cause harm to other organisms in the sea or cause accumulation of algal toxins in marine organisms which eventually harm other organisms who will eat the toxic species (Andersen, 1996).

Harmful algae are microscopic, single-celled plants living in the sea. Most species of algae or phytoplankton are not harmful and serve as the energy producers at the base of marine food chain. Occasionally, the algae grow very fast and accumulate into dense, visible patches near the surface of the water. Therefore, the algal species make their presence known sometimes as a massive “bloom” of cells that can discolor the water (Turgeon *et al.*, 1998). For example, "Red Tide" is such a common term for a phenomenon where certain phytoplankton species contain reddish pigments and "bloom" such that the water appears to be colored red. Usually, the “red tide” is not harmful to the environment and human beings.

Unfortunately, there are a few dozen that produce toxins among thousands of species of microscopic algae. The potent algal toxins can be transferred through the food web where they affect and even kill the higher forms of life such as zooplankton, shellfish, fish, birds, marine mammals, and even humans that feed either directly or indirectly on them. Typically, shellfish are only marginally affected, even though a single clam can sometimes accumulate sufficient toxin to kill a human.

At the same time, the term “harmful algal blooms” applies not only to toxic microscopic algae but also to nontoxic microscopic algae and macroalgae (seaweeds). A large amount of non-toxic algae can also accumulate out of control and cause such ecological impacts as displacing indigenous species, altering habitat suitability, and depleting oxygen (Turgeon, Sellner and Scavia, 1998). For example, during the Labor Day weekend of 2003, the thick blue-green algal bloom had been detected in the Potomac River upstream of Aquia Creek, VA. The water was dominated by *Microcystis aeruginosa* at the concentration of 2 million cells/ml. The single cells of this species can join together in groups as colonies that have the potential to result in fish and shellfish kills by causing low oxygen level in the water.

2.1.1 Harmful Algal Toxins

Many researchers view HAB toxins as secondary metabolites. Plant physiologists defined the term “second metabolite” about 30 years ago to identify compounds that do not fulfill a role in intermediary metabolism (Vining *et al.*, 1990). Microbiologists often consider a secondary metabolite to be chemical substances produced by an organism for purposes other than primary physiological functions such as respiration, genetic definition and transcription, energy transfer and storage, and other such life-sustaining processes. The synthesis of a given secondary metabolite is generally limited, occurring only in a small group of organisms, frequently only in one species (Hashimoto and Yamada, 1994). Even though secondary metabolites are not essential to cell survival, as viewed by plant physiologists, their role may be intrinsic or extrinsic. They perform specific functions, for example, degrading food sources or fighting off other organisms.

HAB toxins easily fit these criteria and each may have evolved to play an active role in one or more intrinsic and (or) extrinsic functions. For instance, saxitoxins, the etiological agent of PSP, may play an intrinsic role in DNA metabolism, or N storage, and (or) an extrinsic role as an antipredation compound (Plumley, 1997)

The potent toxins produced by the harmful algae species can find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses. Table 2.1 lists major categories of harmful algal toxins involved in fish and shellfish poisoning when consumed by humans. Each of these syndromes results from different species of toxic algae occurring in a variety of coastal waters of the world.

Generally, Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), and Neurotoxic Shellfish Poisoning (NSP) are caused by biotoxins synthesized by marine dinoflagellates. PSP includes mild and extreme symptoms. The most serious case is human death caused by respiratory paralysis. But, there is no lasting effect after the recovery due to this kind of toxin. The phenomenon of DSP was first documented in 1976 from Japan where it caused major problems for the scallop fishery (Yasumoto *et al*, 1978). Some polyether toxins involved, such as okadaic acid, can promote stomach tumours and cause chronic problems in shellfish consumers (Suganuma *et al.*, 1988). However, no human fatalities have been reported and patients usually can recover in three days (Hallegraeff, 1993). With the ingestion of contaminated shellfish, NSP presents as a milder gastroenteritis with neurological symptoms than PSP. NSP produces an intoxication syndrome nearly identical to that of ciguatera. No deaths have been reported and the syndrome is less severe than ciguatera, but nevertheless debilitating. The

Table 2.1 Families of toxins involved in human food poisonings (Anderson *et al.*, 1993)

TOXIN FAMILY	SYNDROME	SOLUBILITY	ACTION ON
Brevetoxin	NSP (Neurotoxic Shellfish Poisoning)	Fat	Nerve, muscle, lung, brain
Saxitoxin	PSP (Paralytic Shellfish Poisoning)	Water	Nerve, brain
Okadaic Acid	DSP (Diarrhetic Shellfish Poisoning)	Fat	Enzymes
Domoic Acid	ASP (Amnesic Shellfish Poisoning)	Water	Brain
Ciguatoxin	CFP (Ciguatera Fish Poisoning)	Fat	Nerve, muscle, heart, brain

recovery is generally complete in a few days (Ishida *et al*, 1996) .

Amnesic Shellfish Poisoning (ASP) is produced by diatoms that, until recently, were all thought to be free of toxins and generally harmless. However, ASP can be a life-threatening syndrome. It is characterized by both gastrointestinal and neurological disorders (Bates *et al.*, 1989). The excitatory amino acid domoic acid can cause the symptoms including abdominal cramps, vomiting, disorientation and memory loss (amnesia). Shellfish containing more than 20 μg domoic acid per gram of shellfish meat are considered unfit for human consumption (Hallegraeff, 1993).

Ciguatera Fish Poisoning (CFP) is caused by dinoflagellates that grow on seaweeds and other surfaces in coral reef communities. The causative dinoflagellate, such as *Gambierdiscus toxicus*, produces ciguatoxin throughout tropical regions of the world. As a result, Ciguatera is the most commonly reported marine toxin disease in the world associated with consumption of contaminated reef fish such as barracuda, grouper, and snapper. Especially in the Caribbean, it produces neurological symptoms, including profound weakness, temperature sensation changes, pain, and numbness in the extremities.

In a word, humans suffer from the naturally-occurring toxins synthesized by harmful algae through the consumption of contaminated seafood products. The development of advanced detection methods and treatment becomes more and more important to deal with many health and economic problems caused by harmful algal blooms.

2.1.2 Occurrence

One of the first fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins was recorded in 1793, when Captain George Vancouver and his crew landed in British Columbia in an area now known as Poison Cove (Hallegraeff, 1993).

Occurring through recorded history, harmful algal blooms are completely natural phenomena. But, in the past two decades, such events have increased in frequency, intensity and geographic distribution, which cause lots of public health and economic impacts. The phenomena are worldwide with expanding problems in Scandinavia, western Europe, the Mediterranean, South America, Asia-Pacific islands, and other coastal nations. On a global scale, close to 2000 cases of human poisoning (15% mortality) though fish or shellfish consumption are reported each year and, if not controlled, the economic damage through reduced local consumption and reduced export of seafood products can be considerable (Hallegraeff, 1993). For instance, dinoflagellate blooms related to PSP were only detected from the temperate waters of Europe, North America and Japan until 1970 (Dale and Yentsch, 1978). However, by 1990, PSP was well recorded from throughout the Southern Hemisphere, in South Africa, Australia, India, Thailand, Brunci, Sabah, the Philippines and Papua New Guinea (Hallegraeff, 1993).

Typically, in the United States, there are only few coastal water areas that are unaffected by harmful algal blooms. Documented episodes of PSP human intoxication on the West Coast extend back to 1903 in California. Over the last several decades, the United States coastal waters have periodically experienced extensive blooms of algae that

impact living resources, local economies, and public health. Figure 2.1 indicates the scale of HABs problems have expanded significantly after 1972. On the East Coast, observations of PSP events prior to 1972 were limited to eastern Maine. Now, PSP cases have increased throughout the rest of New England and to Georges Bank (Turgeon, Sellner and Scavia, 1998). Five explanations for this dramatic increase of harmful algal blooms have been discussed by the researchers. (1) Transport of toxic algae species has been caused by ship ballast water or the movement of shellfish stocks from one area to another. (2) More coastal waters for aquaculture have been utilized. (3) Increased nutrients that human load to coastal waters may stimulate cultural eutrophication on microscopic and macroscopic algae and fertilize them into blooms. (4) Better detection methods and more scientific awareness of toxic algae species have been involved to monitor this kind of events. (5) The plankton blooms have been initiated by unusual climatological conditions, such as a severe hurricane which occurred immediately prior to the 1972 bloom.

Especially in the Chesapeake Bay area, the harmful algal blooms are becoming a more and more significant problem due to nutrient over-enrichment and subsequent blooms of numerous species of algae. For example, since 1996, three fish kill events associated with blooms of *Karlodinium micrum* have occurred at HyRock Fish Farm, an estuarine pond aquaculture facility raising hybrid striped bass on the Chesapeake Bay

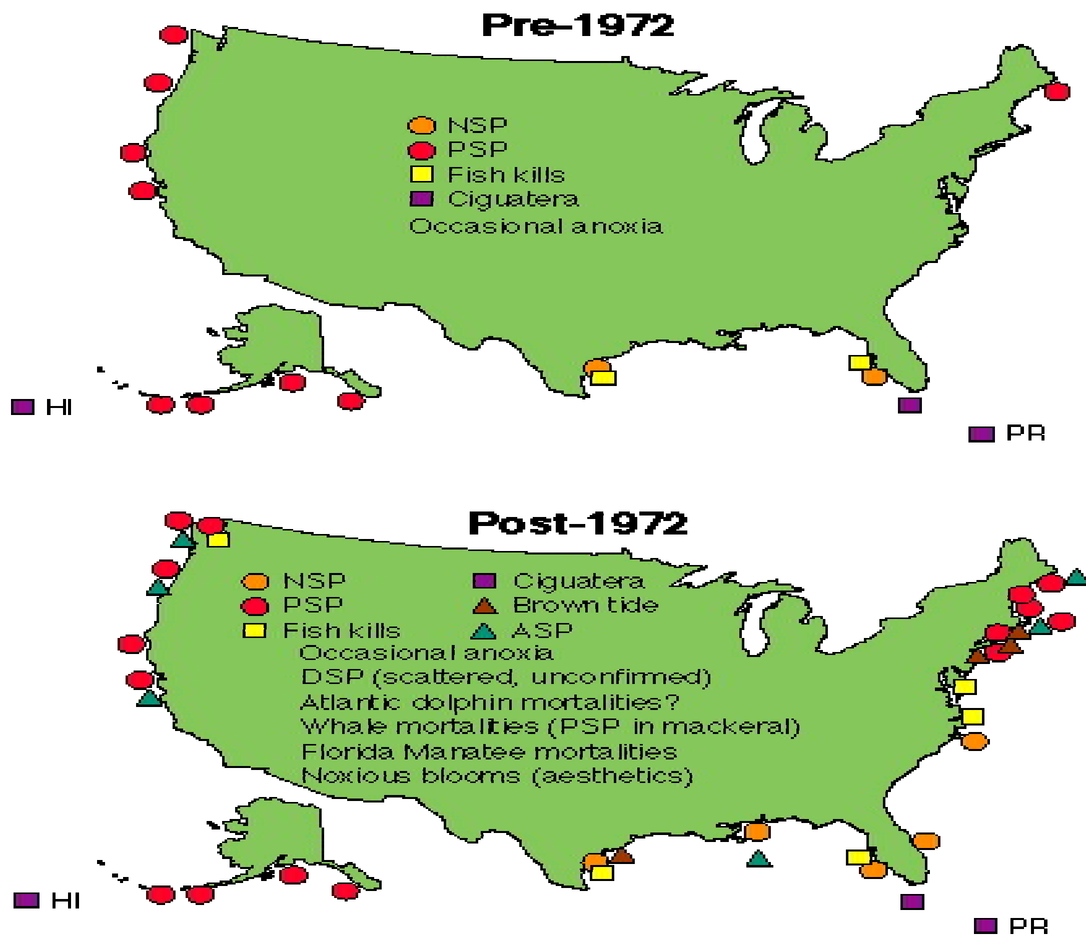


Figure 2.1 Since 1972, U.S. HAB distribution appears to be expanding and events occurring more commonly (Turgeon, Sellner and Scavia, 1998)

(Deeds and Terlizzi, 2002). In order to control those problems, Maryland Department of Natural Resources operates a longstanding comprehensive Chesapeake Bay Monitoring Program that includes the regular measurement of algae.

2.1.3 Consequences

The rapidly increasing frequency of harmful algal blooms causes a large quantity of adverse effects on worldwide coastal water areas. There are five major consequences following the dramatic occurrences of HABs:

(1) Threaten coastal living resources. Overabundant harmful algae species have been related to thousands of fish kills in the world. Not only fish, but also whales, porpoises, manatees and seabirds are victims of harmful algae toxins via contaminated zooplankton or fish.

(2) Restrict local harvests of fish and shellfish. During 1997 in Maryland across all seafood industry segments combined sales had declined by over 10% (Maryland Sea Grant). $43 million was lost due to *Pfiesteria* panic compared with estimated sales without *Pfiesteria* panic of $253 million in the State.

(3) Divert public funds to monitor programs. As one of the greatest threats to U.S. coastal areas, increasingly frequent outbreaks of harmful algal blooms have been efficiently and effectively addressed by the federal government. Only in 1998, federal agencies including NOAA, CDC, FDA, EPA, NSF, NIEHS, USDA and DOI totally offered $25,740,000 funding to support harmful algal blooms research, monitoring and assessment activities in U.S. coastal waters(Turgeon, Sellner and Scavia, 1998).

(4) Depress local recreational and service industries. For example, the significant blue-green algae *Microcystis aeruginosa* resulted in temporary and precautionary beach closure during the summer of 2000 in the upper Chesapeake Bay. Betterton Beach (Sassafras River, Chesapeake Bay) closed due to blue-green algal bloom again on August 7, 2003.

(5) Burden medical facilities. With the increase in interstate and international transport of seafood, as well as international travel by seafood consumers, human are entirely exposed to the poisoning risk caused by toxic algae species. Scientists have input a lot of efforts to develop effective treatments to recover the patients suffered from the algal toxins.

2.1.4 Four critical harmful algal species

Especially in Maryland coastal water areas, there is the most interest in four critical harmful algal species, *Chattonella marina, Karlodinium micrum*, *Prorocentrum minimum*, and *Pfiesteria piscicida*, that have caused very serious problems. Based on the data source from Chesapeake Bay Monitoring Program operated by Maryland Department of Natural Resources in 2001-2003, the occurring frequencies and toxicity characters of these four algal species are compared in Table 2.2.

Table 2.2 Summary of reported incidences on four major harmful algae species during 2001 to 2003 in Maryland coastal water areas (MDNR, 2003)

Species	Concentration associated with fish kills (cells/ml)	No. of events	Highest concentration reported (cells/ml)	Location of highest concentration
Chattonella spp.	≤10,000	6	11,448 to 70,000	Marshall Creek (Massey Branch)
Karlodinium micrum	≤10,000 to 30,000	12	1,500,000	St. Leonard's Creek
Prorocentrum minimum	≤3,000	21	243,800	Lower Patuxent River and tributaries
Pfiesteria piscicida	≤100 to 300	17	217	Chicamacomico River

2.1.4.1 *Chattonella marina*

Chattonella marina is a raphidophycean flagellate with high toxicity that causes severe damage to fish farming. Recent studies demonstrated that *Chattonella marina* could continuously produce reactive oxygen species (ROS) such as superoxide anion (O^{2-}) and H_2O_2 under normal growth conditions, which is partially controlled by electrons donated through photosynthetic electron transfer (Oda *et al.*, 1998). The drop of arterial oxygen partial pressure (Pa02) of the yellowtail (*Seriola quinqueradiate*) appeared after it was exposed to this kind of harmful red tide toxin (Lee *et al.*, 2003). At densities above 10,000 cells / ml *Chattonella marina* begins to put impacts on fish health. The resulting 9-anthrylmethyl esters products of *Heterosigma akashiwo*, *Chattonella antiqua* and *Chattonella marina* reacted with 9-anthyryldiazomethane were analyzed without any purification by RP-HPLC on a highly efficient C18 column (Terasaki *et al.*, 2002). The reactive oxygen species (ROS) released by *Chattonella marina* could be reduced using the photosynthesis blocking herbicide DCMU (Marshall *et al*, 2002) .

2.1.4.2 *Karlodinium micrum*

Like other dinoflagellates, *Karlodinium micrum* appears to accumulate in nutrient-rich condition. In 2002, a dinoflagellate bloom of *Karlodinium micrum* was found associated with a fish kill event in a South Carolina brakish water retention pond. Toxicity of filtrate from bloom samples was tested by a hemolytic assay using rainbow trout (*Oncorhynchus mykis*) erythrocytes and an ichthyotoxicity assay using larval zebrafish (*Danio rerio*). The filtrate demonstrated high hemolytic activity (80% hymolysis) and high ichthyotoxic activity (Kempton *et al*, 2002).

The Chesapeake Bay also becomes a good place for algae species to thrive and surveys indicate *Karlodinium micrum* is more and more common in recent decades. High concentrations (1,322,500 cells / ml water) of this species were observed on Sept. 9, 1982 in Bodkin Creek. Since 1996, *Karlodinium micrum* has been involved in three fish kill events at HyRock Fish Farm, an estuarine pond aquaculture facility raising hybrid striped bass on the Chesapeake Bay, Maryland (Deeds *et al.*, 2002). Using an assay based on the lysis of rainbow trout erythrocytes, cultures of a Chesapeake Bay isolate of *Karlodinium micrum* have been shown hemolytic and ichthyotoxic activity, which represents the first direct evidence that the toxicity of *Karlodinium micrum* isolated from the Chesapeake Bay are released upon cell disturbance or damage (Deeds *et al.*, 2002).

Karlodinium micrum is 10-16 μm long and 6.5-12 μm wide. It can give the water a coffee color in large numbers. Its dominance season is the summer. Normally, it produces sufficient toxin (ichthyotoxicity) to kill fish with concentrations of at least 10,000 to 30,000 cells/ml, which means it is apparently not highly toxic. *Karlodinium micrum* contains chlorophylls c1 + c2 and 19'-hexanoyloxy-fucoxanthin and/or 19'-butanoyloxy-fucoxanthin as the major carotenoid but lacks peridinin (Yoon *et al.*, 2002).

2.1.4.3 *Prorocentrum minimum*

The cell shape of *Prorocentrum minimum* varies form triangular to oval-round including intermediate forms in valve view. The thecal surface is covered by evenly distributed short spines, while the circular thecal pores are mainly situated near the valve margin (Pertola *et al*, 2003) .

High concentrations of *Prorocentrum minimum*, above 3000 cells/ml**,** may cause water discolored a red-brown and form Mahogany tides that can reduce the amount of oxygen available to other living organisms at localized bloom sites. Usually, harmful algal blooms due to *Prorocentrum minimum* occur every spring in the mid-salinity waters of the Chesapeake Bay at varying intensities. Blooms of *Prorocentrum minimum* induced the stress on eastern oysters and *Crassostrea virginica* including an increase in hemocyte number, especially granulocytes and small granulocytes, and an increase in phagocytosis associated with a decrease in aggregation and mortality of the hemocytes, as compared with oysters in pre-bloom analyses (Hégaret and Wikfors, 2004). The rare cases of Venerupin Shellfish Poisoning (VSP) produced by *Prorocentrum minimum* were recorded in the last century, though there were no such cases reported from Maryland waters. In 1998 and 1999, the blooms of *Prorocentrum minimum* and *Aureococcus anophagefferens* were not toxic in the Chesapeake Bay and coastal bays of Maryland, but reached sufficiently high densities to have ecological consequences (Glibert *et al.*, 2001).

In 2001, scientists developed and tested a single-cell immunoassay for identifying phosphate stress in the model dinoflagellate *Prorocentrum minimum* (Dyhrman and Palenik, 2001). In 2002, monitored salinity distributions and optical properties were continuously used to study the spring bloom of the red tide caused by *Prorocentrum minimum* in the Rhode River, Maryland, a tributary embayment of upper Chesapeake Bay (Gallegos and Jordan, 2002).

2.1.4.4 *Pfiesteria piscicida*

Pfiesteria piscicida is an armored dinoflagellate that has been identified only in the last decade in estuaries in North Carolina and Delaware and in the Chesapeake Bay. It became a subject of national algae research and caused lots of confusion and speculation since the questions about how it affects and impacts are still not very clear. The most remarkable properties of *Pfiesteria piscicida* are its multiple life stages and unique toxin production. Differing from other known harmful algal species, it does not produce a pigment so that there is no visual evidence of its activity. Scientists only can use fish with deep sores and fish kills as the indicators of the algal toxins produced by it. Some research suggests that *Pfiesteria piscicida* toxin can result in health problems by skin contact and inhalation. It can generate fish kills with relatively low densities of only 100-300 cells/milliliter. *Pfiesteria* and related toxic species produce as yet unidentified toxins that have been implicated in temporary short-term losses of neurocognitive abilities (short-term memory) in Maryland residents exposed to water or aerosol containing the organism (Turgeon *et al*, 1998).

Pfiesteria piscicida was successfully identified using internal transcribed spacer-specific PCR assays with a minimum sensitivity of 1 cell/ml (Litaker *et al.*, 2003). The ichthyocidal activity of *Pfiesteria piscicida* was detected by the bioassay accomplished with sheephead minnows(Quesenberry *et al.*, 2002).

2.1.5 Detection and quantification of HABs toxins

Harmful algal toxins have been detected and quantified using one or a combination of several techniques. Generally, biological methods, chemical analysis and immunological methods are applied in many toxic algae studies. For the detection and quantification of five general types of algal toxins, biological, chemical and immunological methods have been properly used as listed in Table 2.3. Furthermore, Table 2.4 shows the advantages and disadvantages of these three methods.

2.1.5.1 Chemical methods

Most chemical methods for toxin analysis are based on detection of the toxins or their derivatives accomplished by high pressure liquid chromatography (HPLC) or autoanalyzer (Andersen, 1996).

The analysis of saxitoxin, the paralytic shellfish poisoning (PSP), was based on the technique involving alkaline hydrogen peroxide oxidation of saxitoxin to fluorescent derivatives, which used a fluorescent detector as an autoanalyzer (Bates and Rapoport, 1975). Alternatively, PSP-toxins was analyzed using HPLC where each toxic components could be detected after the oxidation of toxins to fluorescent products (Sullivan and Jonas-Davies, 1985). One of Amnesic Shellfish Poisoning (ASP) related toxins, domoic acid, was isolated from two red algae, *Chondria armata* and *Alsidium corallinum*. Determination of domoic acid directly in extracts of the whole culture and in filtrates of the culture was fulfilled by a high sensitive (nanograms per milliliter) modification of the 9-fluorenylmethoxycarbonyl chloride (FMOC) precolumn derivatization method for amino acids followed by reversed-phase HPLC with fluorescence detection (Subba Rao,

Quilliam and Pocklington, 1988). Two principal toxins of diarrhetic shellfish poisoning (DSP), okadaic acid and dinophysistoxin-1, were esterified with 9-anthryldiazomethane in methanol. The fluorescent esters of two toxins were detected on a Develosil ODS column with MeCN-MeOH-H_2O (8:1:1) after cleaning with a Sep-pak silica cartridge column (Lee, Yanagi, Kema and Yasumoto, 1987).

Chemical methods can discriminate different kinds of toxins in most cases. However, the high cost prevents them to be used in a very large scale.

2.1.5.2 Immunological methods

There are several types of immunological methods, such as Enzyme Linked Immunosorbant Assay (ELISA), Radioimmunoassay (RIA), Competitive Enzyme Immunoassay (EIA) and Solid-Phase Immunobead Assay (S-PIA). The algal toxins, as a functional antigen, can provocate the animal to produce antibodies. The extraction of antibodies from the animal serum is marked with either radioactive or fluorescent label. The extracted algal toxins are exposed to the marked antibodies followed by detection of the amount of radioactivity or fluorescence of the antiserum-antigen complex. Therefore, the amount of toxins in the sample can be measured as well (Andersen, 1996). Immunological methods can detect specific algal toxins with high precision. But, the complicated techniques involved cost too much money and process time for routine use.

2.1.5.3 Biological methods

The biological methods are called bio tests or bioassays as well. These techniques are based on the exposure of living organisms, such as mice, rats or flies, to extraction of

Table 2.3 Biological, chemical and immunological methods used for the detection and quantification of harmful algal toxins

Approaches	Methods	Target Toxins					Advantages	Limitations	References
		PSP	DSP	ASP	NSP	CFP			
Chemical methods	HPLC	+	+	+			Discriminate the different toxins in most cases	The high cost of chemical methods prevents them to be used in a large scale	Bates and Rapoport, 1975 Subba *et al.*, 1988 Lee *et al.*, 1987
Immunological methods	ELISA		+		+		Precise Specific	High cost	Andersen, 1996
	RIA					+			
	EIA					+			
	S-PIA					+			
Biological methods	Animal	+	+	+	+	+	Low cost Simple procedure Fast response	The use of living animals is not very precise and not very specific	Lee *et al.*, 1987 Amzil and Pouchus, 1992 Yasumoto and Underdal, 1990 Fairey *et al.*, 1997
	Cytotoxicity		+						
	Reporter gene	+			+	+			

harmful algal toxins. Normally, the living animals are intraperitoneal injected or fed the toxins. The reaction of the animals to the exposure followed in time is estimated corresponding to a particular kind of toxin. The mouse bioassay is the most popularly used bioassay to detect algal toxins. The major drawback of bioassays is the necessity to maintain or to purchase frequently a large number of animals with almost same weights (Lee, Yanagi, Kema and Yasumoto, 1987).

Without using living animals, the method based on cytotoxicity could be more convenient instead of biological tests. This alternative method has the same advantage of quick response as bioassays.

The cytotoxicity of okadain acid was detected based on the induced changes of KB cell cultures in cell morphology (Amzil and Pouchus, 1992). The toxicity of *Chrysochromylina polylepis* and *Gyrodinium aureolum* was tested using hemolysis test with rat blood cells (Yasumoto and Underdal, 1990).

Recently, a novel cell-based assay was applied to detect Brevetoxins (NSP-toxins), Saxitoxins (PSP-toxins), and Ciguatoxins (CFP-toxins) using a stably expressed *c-fos*-Luciferase reporter gene (Fairey, Edmunds and Ramsdell, 1997). The work evaluated the sensitivity of this reporter gene assay to those algal toxins that activate or inhibit sodium channels. Although this assay presented the potential to enhance the sensitivity of existing bioassays for sodium channel active algal toxins, it could not identify and further quantify harmful algal species with high specificity.

2.2 Bioluminescence

Bioluminescent cells have been used more and more widely used in whole-cell based biosensors as biological recognition element. The high sensitivity of light detection makes bioluminescent and chemiluminescent reactions particularly attractive for the measurement of compounds for which classical analytical procedures do not provide enough sensitivity. Compared with enzyme-based biosensor, this kind of biosensor might be simpler and less expensive to develop for some applications with longer sensing time (Buerk, 1993).

2.2.1 Bioluminescent bacteria

Bioluminescent organisms are widely distributed in nature and remarkably comprise diverse set of species (Campbell, 1989). Among them, luminous bacteria are the most abundant and are found in marine, freshwater and terrestrial environment (Meighen, 1991). These bacteria are all gram-negative motile rods and can function as facultative anaerobes (Baumann *et al*, 1983) .

Generally, there are three major genera of bioluminescent cells: *Vibrio*, *Photobacterium* and *Photorhabdus* (formerly called *Xenorhabdus*). Except *Photorhabdus* species, almost all luminous bacteria are free-living organisms, saprophytes, gut symbionts or animal parasites in the ocean (Chatterjee and Meighen, 1995) . They can be isolated as specific light organ symbionts of fishes (Hastings *et al.*, 1987) or in other marine habitats. Differing form other luminous cells, *Photohabdus* species only infect terrestrial organisms (Thomas and Poinar, 1979). It has even been isolated from human wounds (Farmer and Jorgensea, 1989).

The most widely studied of these luminous bacteria are *Vibrio harveyi*, *Photobacterium fischeri*, *Photobacterium leiognathi*, *Photobacterium phosphoreum* and *Photorhabdus luminescence*(Chatterjee and Meighen, 1995). The *lux* gene products from *Photorhabdus luminescens* are thermostable up to 45 °C, allowing more applications over a larger temperature range than other marine luminous bacteria (Szittner and Meighen, 1990). This character makes it feasible to grow and test *E. coli* strains bearing *lux* gene fusion at 37 °C that is the optimal growth temperature for bacteria (Van Dyk, 1998). *E. coli* normally grows very slowly at the temperature less than 37 °C.

2.2.2 Bacterial bioluminescence pathway

Figure 2.2 shows the bioluminescence pathway catalyzed by the luciferase and other enzymes. Those enzymes involved in bacterial luminescent system are generated from five structural genes, *luxA*, *luxB*, *luxC*, *luxD* and *luxE* respectively found in the *lux* operons of all luminescent bacteria (Meighen, 1991).

Acylated acyl-carrier protein (Acyl-ACP) is hydrolyzed from fatty acid biosynthesis to a fourteen-carbon fatty acid, tetradecanoic acid. This process is generated by the luxD product, transferase.

The reduction of tetradecanoic acid to tetradecanal, a aldehyde, is catalyzed by fatty acid reductase complex, reductase and synthetase. The synthetase originated from the luxE activates the fatty acid to form a fatty acyl-AMP intermediate that is tightly bound to the enzyme. The acyl group is first transferred to the synthetase and

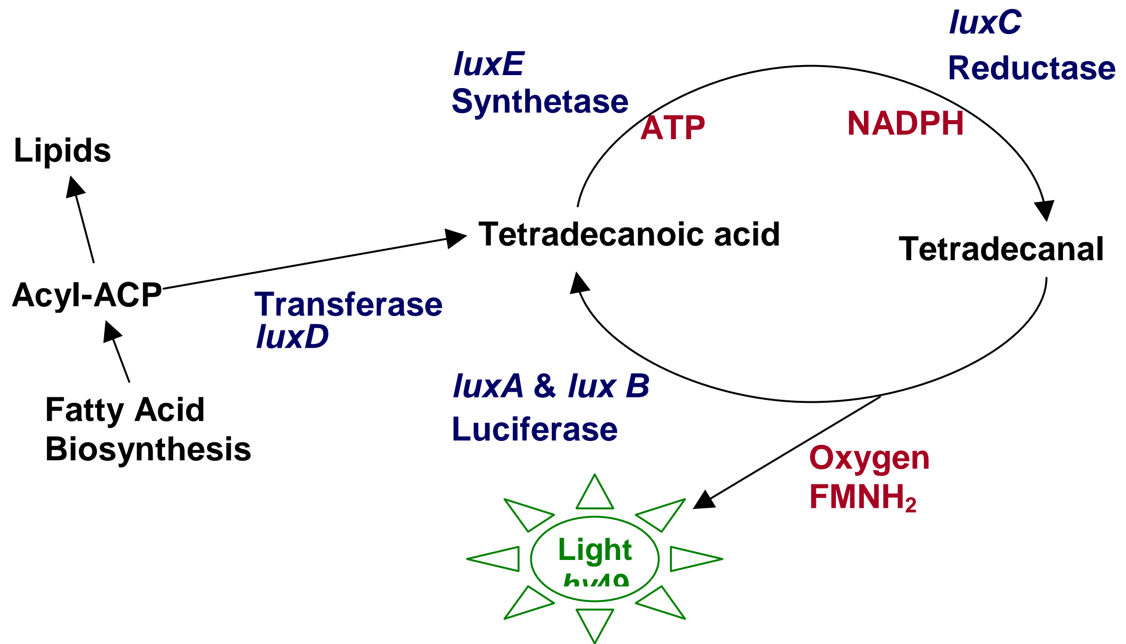


Figure 2.2 Bacterial bioluminescence pathway (Van Dyk, 1998)

then to the reductase produced by luxC before being reduced to aldehyde by NADPH (Wall *et al*, 1986) .

The light-emitting reaction involves the oxidation of reduced riboflavin phosphate (FMNH2) and tetradecanal with the emission of blue-green light. The luciferase is generated from luxA and luxB to catalyze this light production reaction (Meighen, 1991). The bioluminescent pathway depends on the cellular production of ATP providing the energy. Therefore, living cells are necessary for the occurrence of bioluminescence phenomena (Van Dyk, 1998).

Because of the difficulties in incubating natural luminous bacteria, the *luxCDABE* gene can be constructed and introduced into a variety of microorganisms, such as *Pseudomonas putida* (Applegate *et al.*, 1998), *Escherichia coli* (Gil *et al.*, 2000), *Pseudomonas fluorescens* (Burlace *et al.*, 1990). They are more convenient to be applied as a reporter corresponding to different stresses.

2.2.3 Bioluminescent bacteria applications and limitations

The biosensors based on luminous bacteria are becoming more and more popular in several fields, including medicine, pharmacology, biochemistry, bioprocessing and environmental engineering, because the light production is easily and rapidly analyzed (Konstantinov *et al*, 1993) .

Depending on the changes in light emission responded to environmental stresses, the bioluminescent biosensors can be categorized into two types: “light out” and “light on” (Van Dyk, 1998). The applications of these two kinds of biosensors are listed in Table 2.4.

“Light out” biosensors only indicate the loss of bioluminescent activities due to impaired metabolism or reduced cellular viability that is caused by the toxicity of the sample. They cannot imply much expression of bioluminescent reporter gene. Microtox^{TM}, the most popular commercialized bacteria tool, is one good example of this kind of biosensors (Bulich and Isenberg, 1981).

Oppositely, “light on” biosensors test the increase of the bioluminescence from bacteria after introducing the stress. The increasing amounts of light signal are different due to specific stress responses from luminescent bacteria. For example, the bioluminescent reporter bacterium *Pseudomonas fluorescens* HK44, which contains a *nahG-luxCDABE* fusion for naphthalene catabolism and bioavailability was applied for environmental samples and mixed contaminants containing a large number of aliphatic and aromatic hydrocarbons, with the naphthalene fractions in the range of 0.1 to 0.5 weight percent (Heitzer, 1998). The strain showed a linear correlation between bioluminescence and the amount of JP-4 jet fuel present in an aqueous solution, representing a mixture of compounds including naphthalene. However, the bioluminescent response averaged ~30-fold greater than if exposed to these naphthalene concentrations alone. Exposure of HK44 to some non-inducing organic solvents such as toluene, p-xylene and acetone also resulted in a significant bioluminescence increase. Further analysis of *nah-lux* mRNA from cells exposed to toluene revealed that the bioluminescence response was not due to increased *nah-luxCDABE* gene expression, whereas increased *lux* mRNA levels were found with exposure to naphthalene or JP-4 jet fuel. The addition of n-decanal, a substrate for luciferase, showed the same relative response pattern as the *lux* mRNA transcript level. Generally, the addition of n-decanal to

either non-induced or induced cultures resulted in a significant increase in culture bioluminescence indicating an aldehyde-limited light reaction. If aldehyde was added, only the presence of naphthalene caused a significantly increased bioluminescence response over the control. It was postulated that the increase in bioluminescence after exposure to solvents was due to changed fatty acid synthesis patterns affecting the aldehyde supply for the bioluminescence reaction.

Limited by the difficulties of getting and growing natural bioluminescent bacteria, *Escherichia coli* has been usually used to carry the luminescent gene. This kind of whole-cell assay is capable of detecting a large variety of chemical compounds with high sensitivity and low cost. However, it has not been widely applied because of the lack of high specificity and stability using only one cell strain. The environmental stress could not be exactly identified and quantified.

Table 2.4 Applications of bioluminescent bacteria based biosensors

Category	Stresses	Bacteria	References
Light Out	Phenol	*E. coli*	(Kim *et al*, 2003)
	Benzene	*E. coli*	(Gil, Mitchell, Chang and Gu, 2000)
	Urea	*Photobacterium phosphoreum*	(Bulich and Isenberg, 1981)
Light On	Octane	*E. coli*	(Sticher *et al*, 1997)
	Hg(II)	*E. coli*	(Selifnova *et al.*, 1993)
	Ethanol	*E. coli*	(Gu and Dhurjati, 1996)
	Naphthalene	*Pseudomonas fluorescens*	(Burlace, Sayler and Larimer, 1990)
	Nitrate	*E. coli*	(Prest *et al.*, 1997)
	Nickel	*E. coli*	(Guzzo and DuBow, 1994)
	Pentachlorophenol	*E. coli*	(Van Dyk *et al.*, 1994)
	Benzene	*Pseudomonas putida*	(Applegate, Kehrmeyer and Sayler, 1998)

Chapter 3: Objectives

The ultimate goal of this project is to detect and characterize four critical harmful algae species using a panel of six bioluminescent *E. coli* strains. Each *E. coli* strain contains different selected stress-responsive promoter fused to the *Photorhabdus luminescens luxCDABE* reporter. Activated by the key harmful algal cultures in the Chesapeake Bay and Maryland Coastal waters, the light generated from bioluminescent *E. coli* strains increases without cell lysis. The amounts of increasing light from six strains may be different due to different bacteria promoters. This character of the panel of six strains of bioluminescent *E. coli* makes it feasible to identify the stress fingerprints induced by specific harmful algae species. Therefore, the four key harmful algal cultures can be detected and quantified by this robust and novel biosensor that consists of ideal biosensing elements, the six bioluminescent *Escherichia coli* strains.

In order to achieve the ultimate goal, the proposed research project includes two stages:

First, to identify the stress fingerprints activated by the harmful algae species using bioluminescent *E. coli* strains. Containing different stress-responsive promoters, a panel of six bioluminescent *E. coli* strains will generate stress fingerprints due to specific algal cultures. The patterns of induced *luxCDABE* gene expression can be indicated by the increasing light signals after adding the harmful algae.

Second, to quantify the amount of the harmful algae species indicating by the light changes generated from six bioluminescent E. coli strains. A model simulating the changes of light signals to different concentrations of algal cultures will be developed.

Chapter 4: Materials and Methods

4.1 Bacteria strains

A panel of twelve *E. coli* strains obtained from DuPont Microbial Genetics Lab (DuPont Company, Wilmington, DE) was employed in this study. Each of them contains different selected stress-responsive promoter that is fused to the *Photorhabdus luminescens luxCDABE* reporter. Table 4.1 summarizes the fusions that are responsive to oxidative damage, internal acidification, DNA damage, protein damage, “super-stationary phase” and sigma S stress response respectively (Van Dyk, 1998). Based on the character that six bioluminescent strains can respond with increased light production in the presence of environmental stresses, such as ethanol, acrylamide and some toxins, the genetically engineered *E. coli* panel was used in stress responsive experiments.

Two versions of the panel of stress-responsive *E. coli* strains are listed in Table 4.1. One set introduces an outer membrane mutation, *tolC*, enhancing highly sensitive detection of a variety of organic molecules. The other set is *tolC+* as the control. The set of *tolC-* was used the most in order to detect the stresses more sensitively. These E. coli strains contain either a plasmid-borne *lux* fusion or a chromosomal integrant of the *lux* fusion. Furthermore, two of the strains with plasmid-borne *lux* fusions carry a chromosomal mutation in the *pcnB* gene resulting in a reduced copy number of the plasmid (Van Dyk, 1998).

Table 4.1 Stress-responsive *E. coli lux* fusion strains (adapted from (Van Dyk, 1998)

Stress Response	Regulatory Circuit	Promoter Fused to *lux*	Strain Name	*tolC* allele	Plasmid-containing
Oxidative damage	OxyR & σ^s	*katG*	DPD2227	+	Yes
			DPD2238	-	Yes
Internal acidification	Mar/Sox/Rob	*inaA*	DPD2226	+	Yes
			DPD2240	-	Yes
DNA damage	SOS	*recA*	DPD1710	+	No
			DPD2222	-	No
Protein damage	Heatshock (σ^{32})	*grpE*	DPD3084	+	No
			DPD2234	-	No
"Super-stationary phase"	?	*o513*	DPD2173	+	Yes
			DPD2232	-	Yes
Sigma S stress response	Stationary phase (σ^s)	*yciG*	DPD2161	+	Yes
			DPD2233	-	Yes

4.2 Algae species and cell count

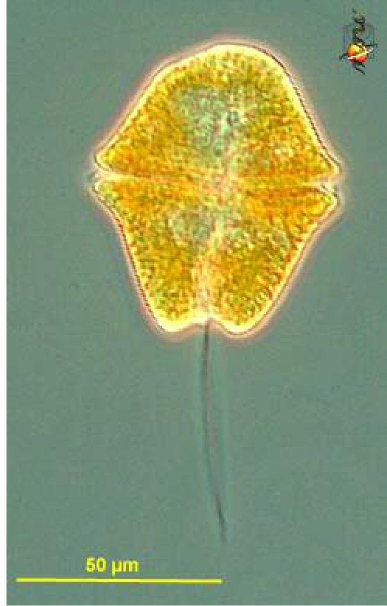
One non-toxic algal species *Akashiwo sanguinea* (isolated by Dr. Aishao Li, Choptank River, MD, 1998) and four key algae cultures commonly found in the coastal water of Maryland and the Mid-Atlantic area including *Chattonella marina* CCMP 2049 (isolated by T. Aramaki, Kagoshima Bay, Japan, 1982), *Karlodinium micrum* GE (isolated by Dr. Aishao Li, Choptank River, MD, 1996), *Prorocentrum minimum* D-5 (isolated by Matt Johnson, Choptank River, MD, 2002), and *Pfiesteria piscicida* MDFDEPMDR23 (provided by Dr. Karen A. Steidinger, Chicamacomico River, MD, 1998). *Pfiesteria piscicida* strain MDFDEPMDR23 has not been shown to be toxic in bioassays with fish and is considered non-inducible to toxicity (Stoecker *et al.*, 2000). Figure 4.1 shows the pictures of five algae species. For each algae culture, 950 μl sample was fixed and stained in the 15 ml plastic tube by 5% acid Lugol's solution (containing per liter: 40 g Iodine, 80 g KI, 80 g Acetic acid). Palmer-Maloney counting cell was washed initially with mild detergent and water, and then dried with clean tissue paper. 100 μl algal solution after the fixation was picked into Palmer-Maloney chamber evenly without any bubble. The chamber was covered with a square coverslip that contacted securely with the stainless steel chamber ring. Labophot Nikon microscope (10×10) was used to count how many organisms per field. The original cell concentration was calculated in the following way:

$$cell\ density\ d = \frac{cells}{field} \times \frac{area\ of\ disc}{transect} \times \frac{1\,ml}{volume\ of\ chamber}$$

Area of disc = $\pi \times (\text{diameter} / 2)^2 = \pi \times (17.9\ \text{mm} / 2)^2 = 251.52\ \text{mm}^2$

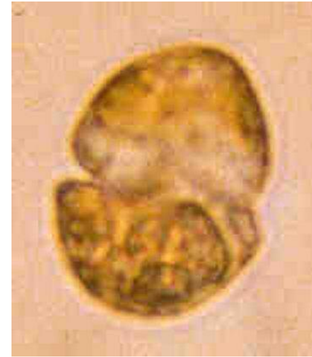
Transect = whipple width × diameter = 0.8 mm × 17.9 mm = 14.32 mm^2

Volume of chamber = 0.1 ml



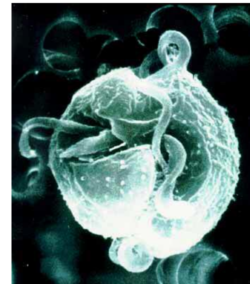
Akashiwo sanguinea

Chattonella marina



Karlodinium micrum

Prorocentrum minimum



Pfiesteria piscicida

Figure 4.1 Microscopic images of algal species investigated. The size of the algae follows: *Akashiwo sanguinea* (50–80 µm long), *Chattonella marina* (30–50 µm long), *Prorocentrum minimum* (13–25 µm long and 12-22 µm wide), *Karlodinium micrum* (10–16 µm long and 6.5-12 µm wide), and *Pfiesteria piscicida* (5-18 µm long).

4.3 E. coli cell growth and bioluminescence activity

4.3.1 Growth condition and calibration curve

All six *tolC- E. coli* strains were maintained in glycerol at –78 ˚C before use. Luria-Bertani (LB) medium (containing per liter: 10g NaCl, 10g Tryptone, 5g Yeast Extract) was used for culturing all bacteria strains. 100 μg/ml of ampicillin was added into LB medium for the plasmid-containing strains to keep the plasmid (Korpimaki *et al.*, 2003). 100 μl stock culture of each strain was inoculated into 250 ml flask containing 50 ml sterilized LB medium with initial pH adjusted to 7.4. The cells were grown aerobically overnight in the incubator at 37 ˚C in a model 1575 orbital shaking incubator (VWR Scientific, Cornelius, Oregon) at 300 rpm. The cell solution was diluted at serial ratios: 1:5, 1:7.5, 1:10, 1:12.5, and 1:15. From each level of dilution, 1 ml solution was put into a 1.5 ml microtube with a 5415e centrifuge (Eppendorf, Hamburg, Germany) at 12,000 rpm for 10 minutes. After the suspending water was removed, the cells were dried in an oven at 60 ˚C to measure the dry cell weight. The optical density of diluted solution was measured in a Heλios β spectrophotometer (Thermo spectronic Rochester, New York).

4.3.2 Bioluminescent activity tests

The six *E. coli* strains were cultured under the same growth condition as mentioned before. After 4 growth hours, 1 ml of each strain was taken out to measure the light emission with a 20e luminometer (Tuner Design, Sunnyvale, CA) in every hour. A 1:10 dilution was made to the cell solution prior to the light detection to avoid that the

light signals emitted from the cells might be too strong to be read by the luminometer. The samples were collected until the cells had been grown 10 hours. In order to get more details of cell bioluminescent activity, the tests were conducted from 6.5 growth hours to 12.5 growth hours in every 30 minutes. The growth time of *E. coli* strains for the following studies of testing the harmful algae species was determined based on the time during which the E. coli cells yielded relatively high and the most stable light signals.

4.4 E. coli stress fingerprints

These *E. coli* cultures grown shortly after reaching the desirable growth time were diluted at a ratio of 1:10. 1 ml culture solution was put in a transparent glass cuvette. Relative Light Unit (RLU) was measured from the bioluminescent bacteria in the luminometer by correction after comparison with the light reading from an internal light-emitting diode. The new RLU value was recorded again after separately adding four key algal toxic cultures and one non-toxic algae species as the control at almost the same concentration 6,000 ± 1,000 cells/ml. All experiments were triplicately conducted. The difference and ratio of two RLU values were both calculated to indicate the stress responses from the bioluminescent *E. coli* strains. The resultant stress fingerprints were displayed as a series of RLU ratios. If the RLU ratios are greater than 1.0, the *lux* gene fusion will be expressed and thus the biotoxins from harmful algal cultures as specific stresses can be indicated. If the RLU ratios are less than 1.0, the bioluminescent E. coli strains can be killed by the harmful algal toxins at relative high concentrations.

The stress responsive signals emitted by the panel of six *E. coli* strains could be expressed as ΔRLU, the signal changes upon contact with toxic algae, or RLU ratio, a

ratio comparing the RLU after and before contacting with toxic algae. For the purpose of analyzing the stress responses from bioluminescent *E. coli* strains upon exposure to various algae species, ΔRLU is a direct way to show the stress effect. But, this kind of data expression is limited by the background noise of *E. coli* cell concentrations. The ratio of RLU could get rid of the factor of original cell densities to compare the light changes based on the effects of environmental stresses. The only drawback of using RLU ratio is the lack of expressing the real levels of RLU values. If the reading number of RLU from the machine is below 1, it may imply a low accuracy, which enlarges the experimental error. Therefore, not only ΔRLU but also RLU ratio has been used to analyze the data. One of them was considered more than the other depending on the particular research need.

Data were analyzed using the Univariate and one-way Mixed Procedures of the Statistical Analysis System version 6.02 (SAS Institute Inc., Cary, NC). Pairwise mean comparisons were conducted using the Tukey's test ($\alpha = 0.05$) and LSD (least significant difference) test.

4.5 Quantification of harmful algae species

To elucidate the relationships between the light signals emitted by the panel *of E. coli* cells and the harmful algae in question, samples of algae at various concentrations around the level resulting in fish kills were subjected to the panel. Every test was triplicate to reduce the experimental error.

Karlodinium micrum was tested by six *E. coli* strains at five concentrations 7,000 cells/ml, 8,000 cells/ml, 9,000 cells/ml, 10,000 cells/ml and 11,000 cells/ml. *Pfiesteria piscicida* was tested by six *E. coli* strains at five concentrations 100 cells/ml, 200 cells/ml, 300 cells/ml, 400 cells/ml and 500 cells/ml. *Chattonella marina* was tested by six *E. coli* strains at five concentrations 5,000 cells/ml, 7,500 cells/ml, 10,000 cells/ml, 12,500 cells/ml and 15,000 cells/ml. *Prorocentrum minimum* was tested by six *E. coli* strains at five concentrations 1,000 cells/ml, 2,000 cells/ml, 3,000 cells/ml, 4,000 cells/ml and 5,000 cells/ml.

The light changes from some *E. coli* strains of the panel might be more sensitive to harmful algae cultures at different concentrations. These sensitive *E. coli* strains could be chosen as major subjects for analysis. The changes of light signals from significant *E. coli* strains could be related to different concentrations of algal cultures in a linear relationship.

Chapter 5: Results and Discussion

5.1 Bioluminescence and E. coli *cell growth*

Since all the requirements for bioluminescence are present in the cell, the five gene *luxCDABE* reporter is capable of giving out light signals even without adding any environmental stresses (Van Dyk, 1998). In addition, emission of bioluminescence by the six *E. coli* strains employed in the present study has been shown to be growth dependent (Chu, 2001). Therefore, it is essential to characterize the respective optimal growth-time window during which relatively high and stable light is emitted by the strains. In the present study, the cell density of *E. coli* cells, calibrated based on the linear dependency of cell density (g/ml) on the optical density of evenly suspended cells at 600 nm (OD_{600}) under the same growth conditions (Figure 5.1), was used to monitor the growth of each strain as well as to standardize the number of cells used in detecting toxic algae. It was found that all six strains reached about the same level of cell growth (0.0035 g/ml) after overnight incubation.

The baseline bioluminescence profile of the *E. coli* strains over 12.5 hours of growth time is shown in Figure 5.2. No significant light signals were detected during the first four hours (lag phase). As the cells entered the exponential phase, the light intensity also increased. Despite of the unique bioluminescence profile of each strain, the signals emitted by all six strains reached a quasi-plateau period between 10 and 12 hours, in which cells were in the exponential phase, giving a two-hour window with high intensity yet stable bioluminescence for background noise standardization. Moreover, use of *E. coli* cells during exponential growth phase is desirable because it enables observation of

"super-stationary phase" and sigma S stress responses, if any, that are constructed in *E. coli* strains DPD2232 and DPD2233, respectively.

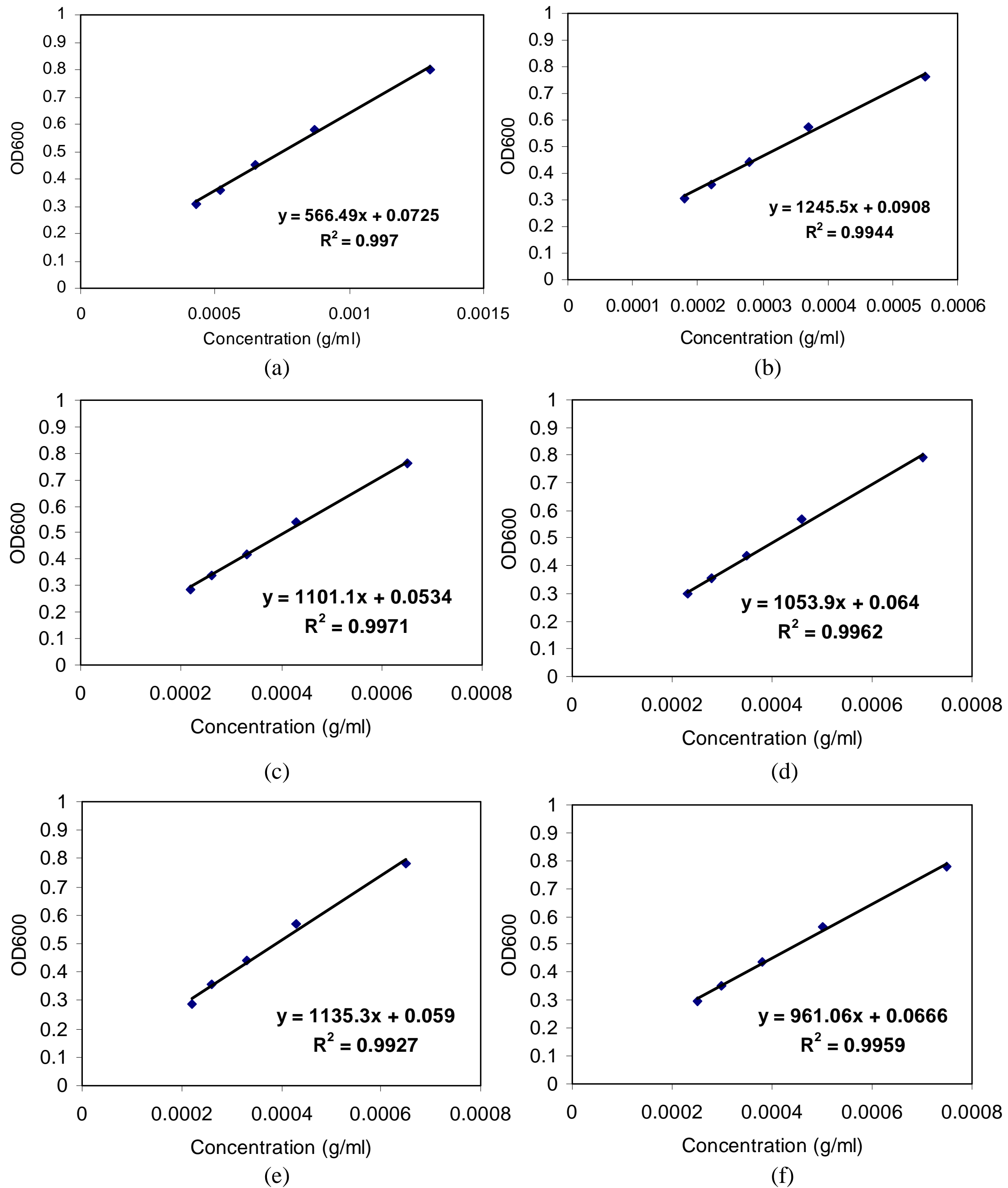
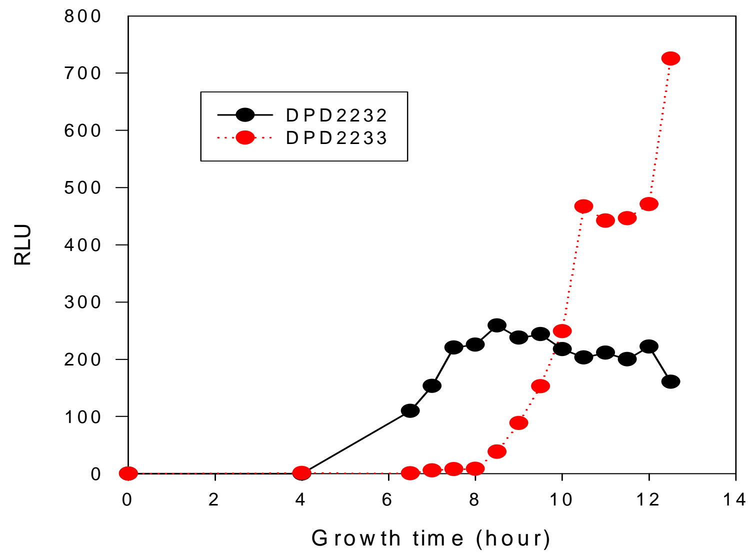
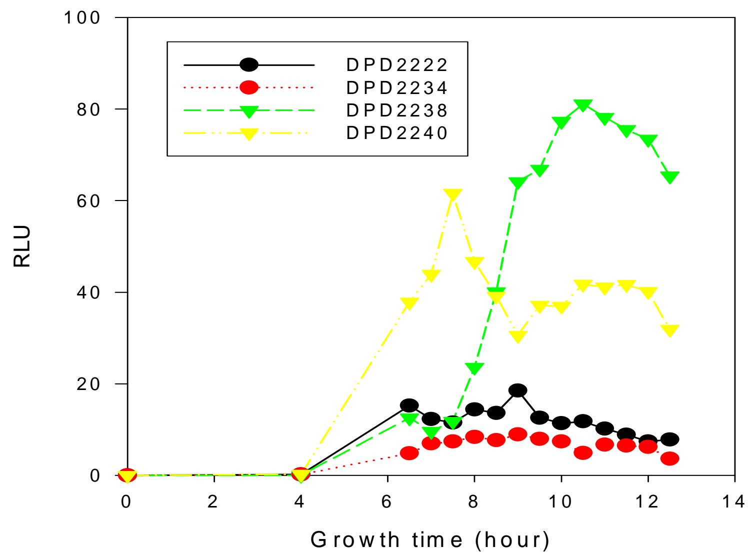


Figure 5.1 Calibration curves of optical density at 600 nm versus concentration (g/ml) of bioluminescent *E. coli* strains (a) DPD2238, (b) DPD 2240, (c) DPD 2232, (d) DPD2233, (e) DPD 2222, and (f) DPD 2234



(a)



(b)

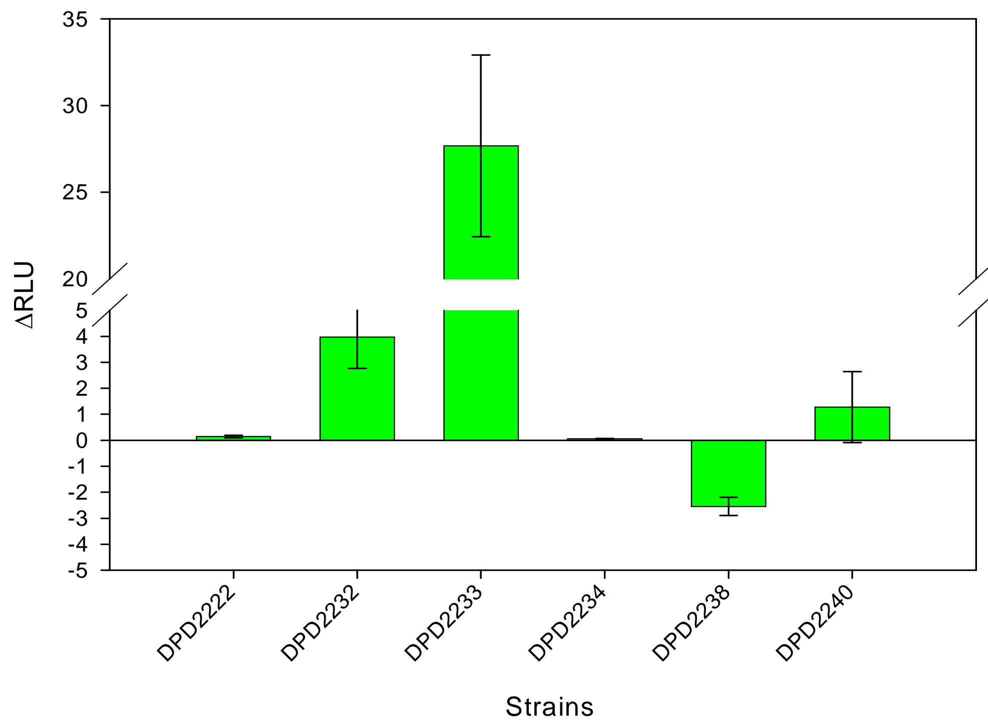
Figure 5.2 The profile of light emission (RLU) from six bioluminescent *E. coli* strains: (a) DPD2232 and DPD2233; (b) DPD2222, DPD2234, DPD2238, and DPD2240.

5.2 Bioluminescence in response to non-toxic algal culture

Akashiwo sanguinea, a red-tide dinoflagellate with no harmful effects known in the Chesapeake Bay (Armstrong and Coats, 2002), was employed to evaluate the bioluminescence generated by the six *E. coli* strains when in contact with a non-toxic algal culture. The bioluminescence induced by *A. sanguinea* was relatively weak, as indicated by the increases in relative light unit (ΔRLU) of strains DPD2222 and DPD2234 (fused with *recA-lux* and *grpE-lux*, respectively) approached zero (Figure 5.3). This indicates that there was no DNA and protein damage at cellular level when *A. sanguinea* was present.

Strain DPD2233 showed high response to *A. sanguinea* at the concentration of 6,000 cells/ml. Strain DPD2233 is constructed to contain the plasmid in which the *E. coli* *yciG* promoter is fused to *luxCDABE* genes. As expression of *yciG* gene is under control of the stationary phase sigma factor σ^s, the *yciG-lux* fusion is expected to report on the activation of the σ^s-dependent stress response (Van Dyk, 1998). With the large cell size, *A. sanguinea* might pose a stress to the *E. coli* cells because its presence could take up a large space and rapidly consume nutrients in the media (Leong *et al.*, 2004).

Conversely, the ΔRLU from *katG*-fused DPD2238 strain showed significant reduction in bioluminescence when exposed to *A. sanguinea*. The strain DPD2238 is constructed to contain the plasmid in which the *E. coli katG* (catalase) promoter is fused to *luxCDABE* genes. An *E. coli* strain harboring this plasmid is known to exhibit low basal levels of luminescence, which increased up to 1,000-fold in the presence of oxidative stress such as hydrogen peroxide, organic peroxides, alcohols, and cigarette



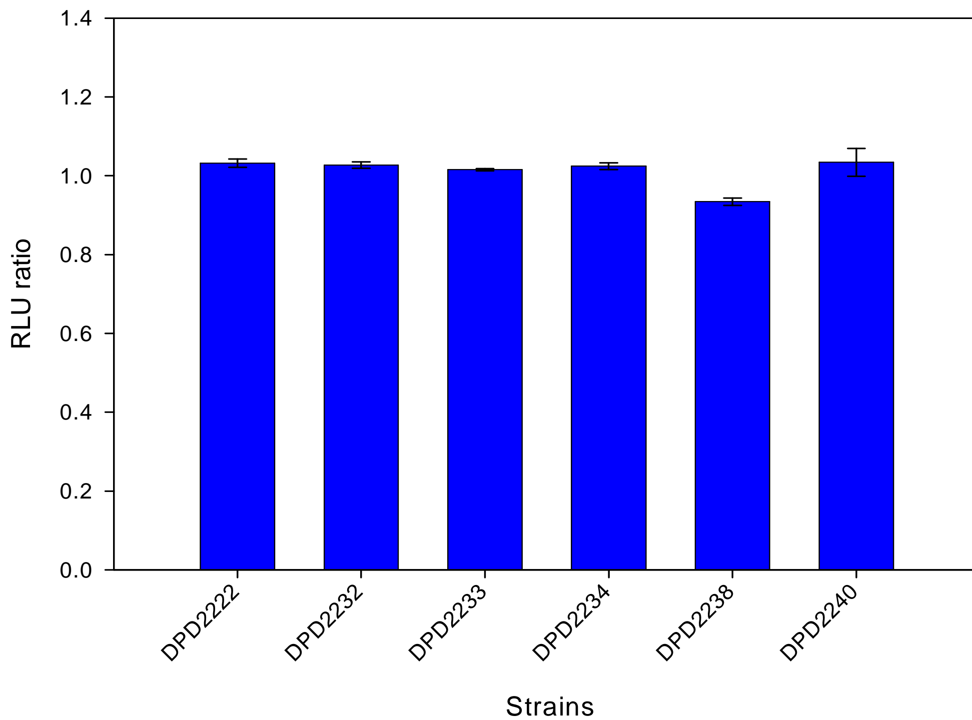


Figure 5.3 Stress responses of six *E. coli* strains to *Akashiwo sanguinea*(6,000 cells/ml), a non-toxic red-tide dinoflagellate. The error bars in the figure represent standard error of mean of three replicates.

smoke (Belkin *et al*., 1996). In the case of the non-toxic *A. sanguinea*, it appeared that no oxidative stress response was triggered, since there is no evidence of radical reactions similar to those caused by peroxides. Since a living cell is a steady-state ensemble of hundreds of interacting biochemical pathways, it is possible that certain physiological responses, for example global changes to the rates of gene transcription and protein synthesis, and degradation rates of proteins and DNA, may non-specifically modulate signal output (Wood and Gruber, 1996). However, the specific factors contributing to the reduction in bioluminescence remain to be studied.

5.3 Stress fingerprints of harmful algal species

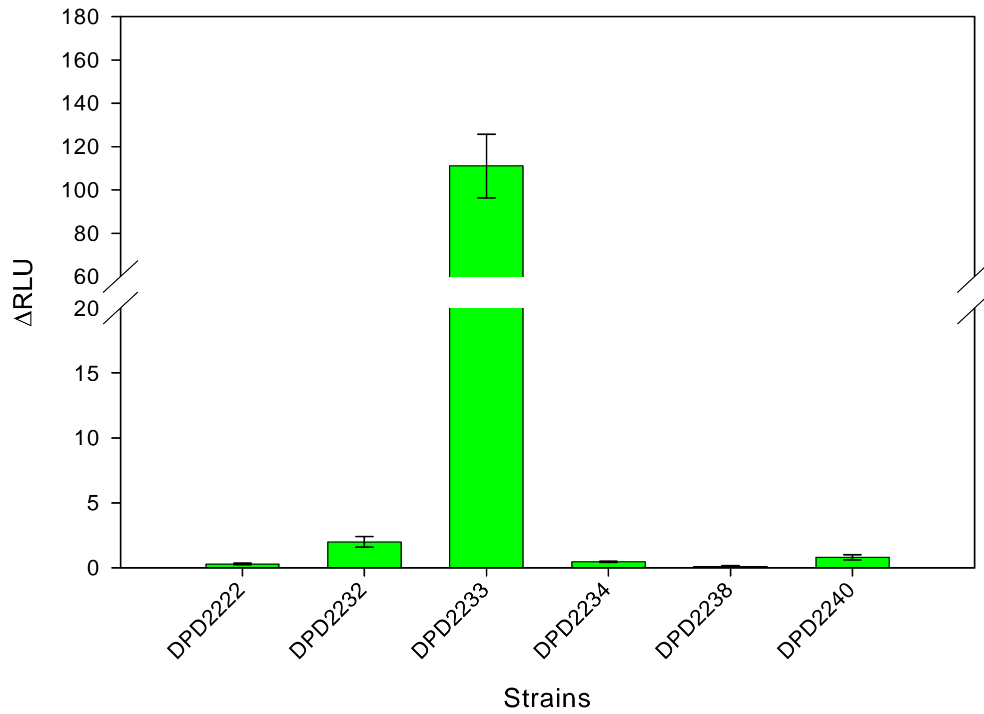
Bioluminescent reactions are considered an extremely useful bioreporter system in that they produce a physical signal, as opposed to a chemical signal, which may or may not accumulate, possibly lead to toxicity, diffuse, or be unstable (Billard and DuBow, 1998)(Billard and DuBow, 1998). To evaluate the sensing capacity of the six *E. coli* strains designed to produce light under defined conditions, four toxic algae species recognized in the Chesapeake Bay were investigated, including *C. marina* (10,000 cells/ml), *K. micrum* (10,000 cells/ml), *P. minimum* (3,000 cells/ml), and *P. piscicida* (300 cells/ml). The numbers in parenthesis following each species represent the respective minimum concentration associated with fish kills.

Assays based on the bacterial SOS-response have been shown to offer the possibility of screening genotoxicity and other toxic effects (Billard and DuBow, 1998, Dreier *et al.*, 2002). In the present study, since the toxins generated by each alga are known to be more than one type, it was anticipated that the luminescence generated by

the six stress-responsive *E. coli* strains might produce characteristic fingerprints specific to each algal species. Both ΔRLU and RLU ratio were employed for signal analysis. When there is no significant difference between the RLU values before and after the *E. coli* strains were subjected to the algae, RLU ratio becomes a valuable tool to determine the level of gene expression, as it could magnify subtle differences between RLU while indicating either the strain undergoes a "light-on" or "light-out" mechanism.

5.3.1 Stress fingerprint of *Chattonella marina*

Upon contacting the *E. coli* strains, *C. marina* only induced a prominent increase of bioluminescence (ΔRLU ≅ 110) in strain DPD2233, which contains the *yciG-luxCDABE* fusion (Figure 5.4). The ΔRLU values of the other strains were less than 2, indicating lack of stress responses in those strains. The value of RLU ratio was relatively high for DPD2233, indicating that the general stress sigma factor σ^s, a sigma subunit of RNA polymerase that determines stationary phase physiology and cell morphology (Hengge-Aronis, 2002), was a major stress response induced by *C. marina*. This result is in support of the findings by Oda (1998) that *C. marina* could continuously produce reactive oxygen species (ROS) such as superoxide anion (O^{2-}) and H_2O_2 under normal growth conditions, which is controlled by available electrons donated through photosynthetic electron transfer. However, stress response exists when bacteria grow and divide slowly or not at all in stationary phase as a consequence to nutrient limitation and other stressful condition. On the molecular level, the multiple regulatory circuits are a member of the σ^S-dependent stress response regulon and are responsible for the induction of HPI catalase activity in the stationary phase (Ivanova *et al*, 1994) .



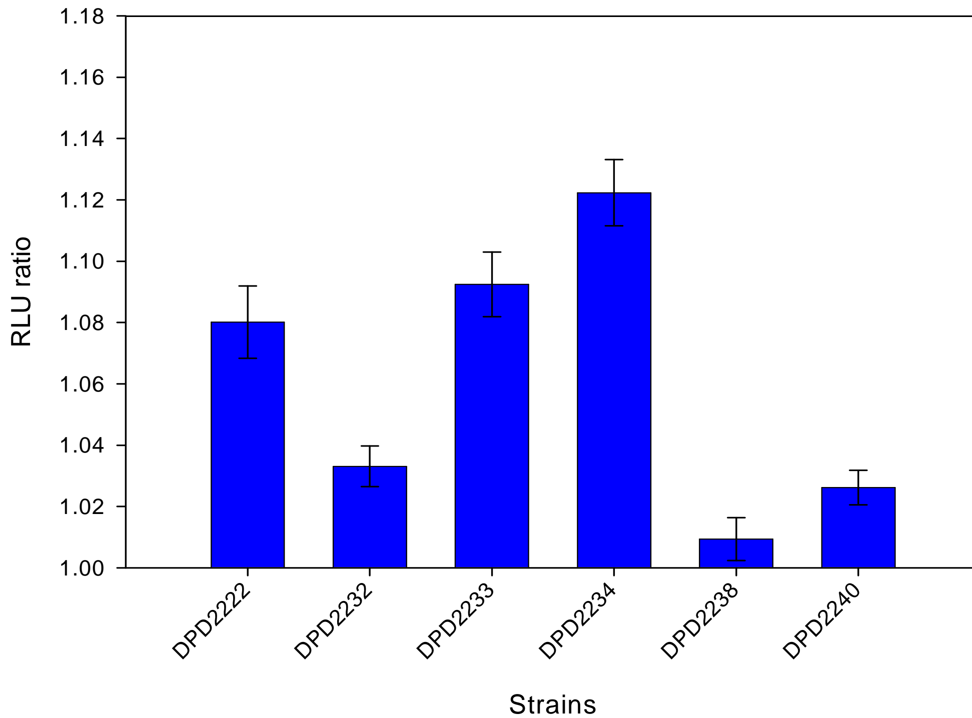
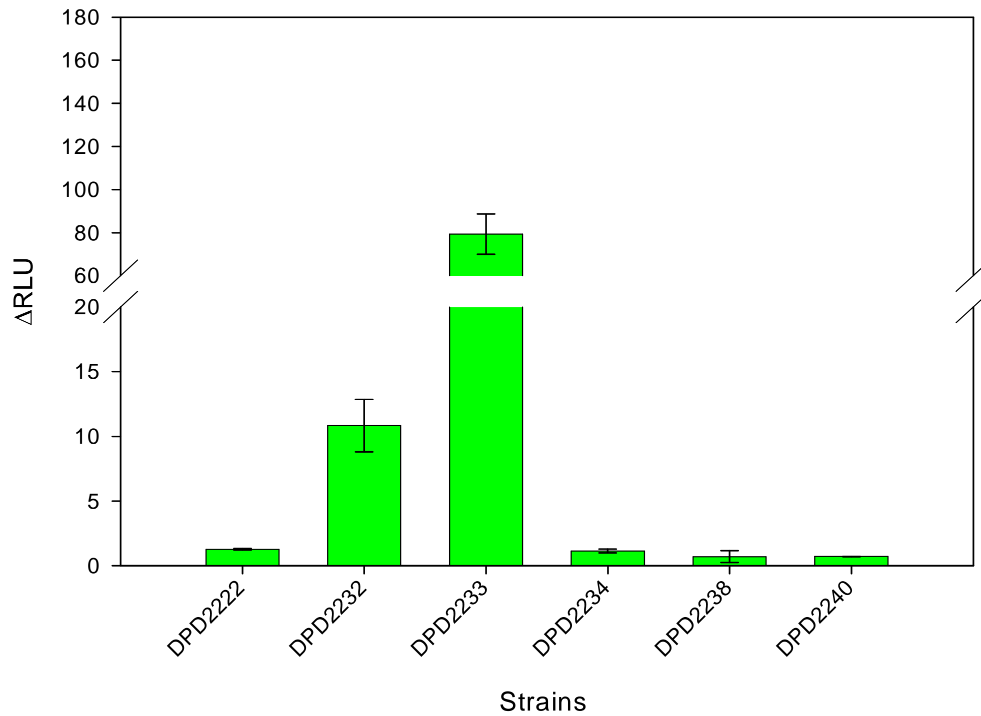


Figure 5.4 Stress responses of six *E. coli* strains to *Chattonella marina* (6,000 cells/ml). The error bars in the figure represent standard error of mean of three replicates.

Also seen in Figure 5.4 is that, similar to strain DPD2233, the RLU ratios of strains DPD2222 and DPD2234 were also prominent; however, their correspondent ΔRLU values were very small. The high RLU ratios in DPD2222 and DPD2234 were due to the extremely low bioluminescence both before and after the strains were exposed to *C. marina.* On the contrary, DPD2238 showed relatively low RLU ratio with its low ΔRLU, mainly due to the high bioluminescence before and after *C. marina* exposure. The ability of these six *E. coli* strains to generate different RLU ratios even with similar ΔRLU values offers an opportunity to produce characteristic fingerprints in response to exposure of different harmful algal species.

5.3.2 Stress fingerprint of *Karlodinium micrum*

Bioluminescent signal patterns from six *E. coli* strains upon contact with *K. micrum* are in support of Deeds's study that *K. micrum* produces at least one substance which is hemolytic, ichthyotoxic, and cytotoxic (Deeds *et al.*, 2002). Similar to the stress responses to *C. marina, E. coli* strain DPD2233 showed a prominent luminescent peak (Figure 5.5), indicating the activation of the σ^s-dependent stress response upon contact with *K. micrum* (Van Dyk, 1998). Converse to the response to *C. marina*, a significant increase of bioluminescence was also observed in strain DPD2232, which contains the *o513-luxCDABE* fusion. Although the regulation of *o513* has not been well characterized, it was reported that expression of a *lux* fusion to an open reading frame *o513* is highly induced as the culture ages, suggesting that stationary phase induces the expression of *o513*; however such expression is not controlled by σ^s (Van Dyk, 1998).



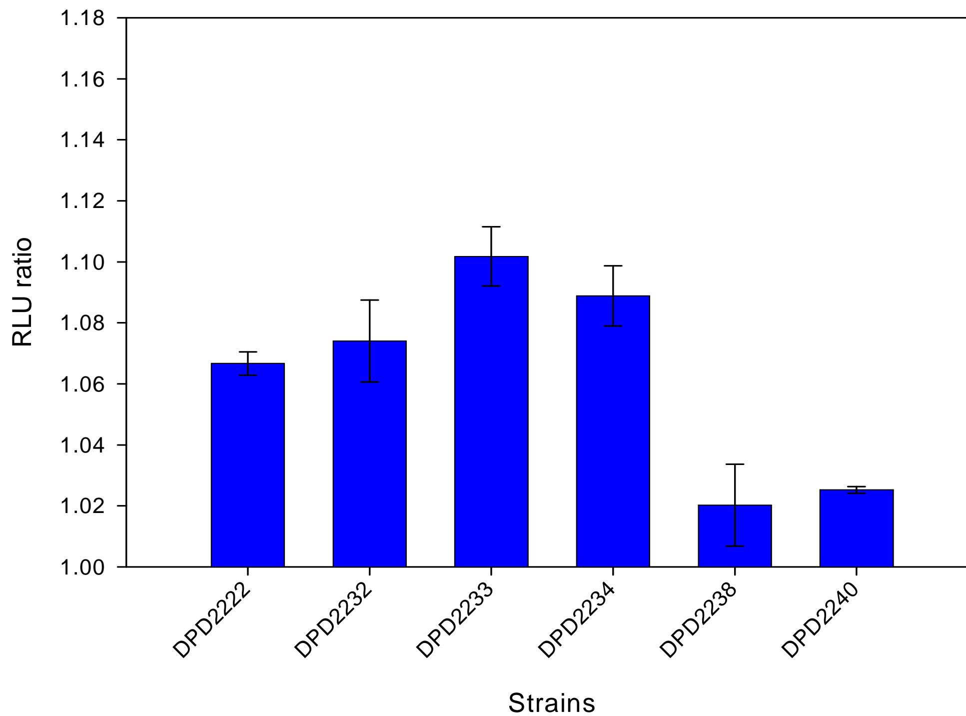


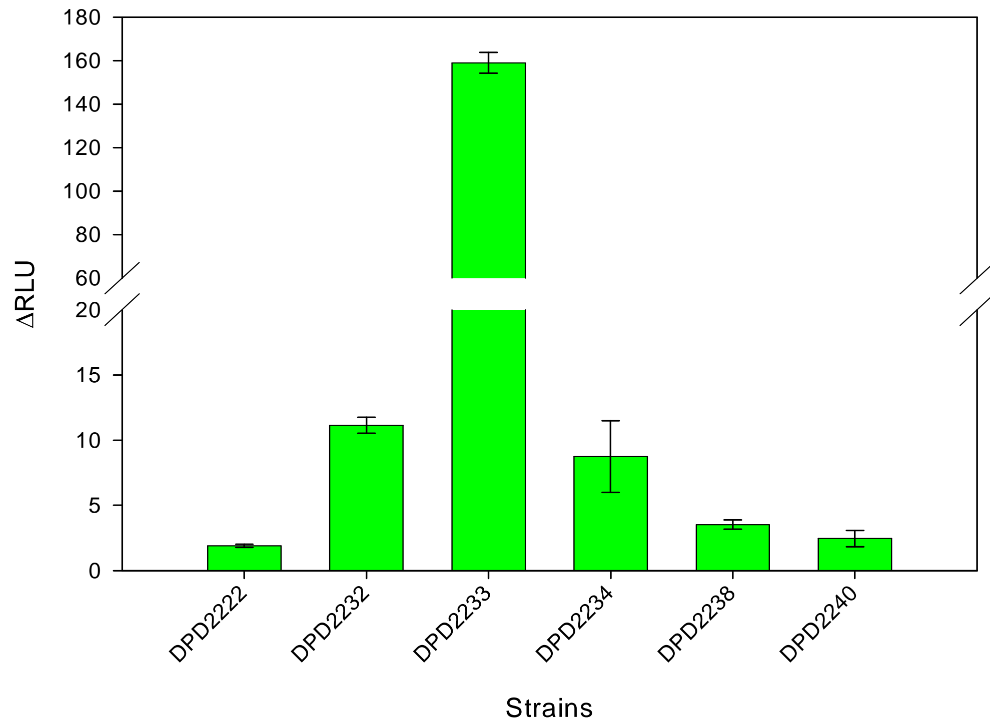
Figure 5.5 Stress responses of six *E. coli* strains to *Karlodinium micrum* **(6,000 cells/ml). The error bars in the figure represent standard error of mean of three replicates.**

Van Dyk (1998) also observed that *o513-lux* fusion did not yield increased bioluminescence to the wide range of chemicals (e.g., hydrogen peroxide, nalidixic acid, ethanol, sodium salicylate, and paraquat). Rather, it gave a response ratio of less than 1.0 ("light out"). Therefore, Van Dyk proposed to use the strain containing *o513-lux* fusion as a general indicator of toxicity. However, in the present study, *K. micrum* induced significant bioluminescence of the *o513-lux* fusion strain (DPD2232), and the "light out"response was not observed under the conditions studied. This result suggests possible induction of the super-stationery phase stress response, in which *o513* promoter was activated in response to the stress caused by the toxins or metabolites produced by *K. micrum*.

Moreover, *K. micrum* induced very low bioluminescence in strains DPD2238 and DPD2240, indicating weak expression of *katG-lux* and *inaA-lux* fusion, respectively, which suggests that *K. micrum* did not cause oxidative damage and internal acidification at cellular level. Therefore, compared with the bioluminescent signal pattern of *C. marina*, the six *E. coli* strains exhibited different bioluminescent signal patterns when exposed to *K. micrum*.

5.3.3 Stress fingerprint of *Prorocentrum minimum*

In addition to inducing stress responses in *E. coli* strains DPD2233 and DPD2232, *P. minimum* induced a significant luminescent signal (both in ΔRLU and RLU ratio) in strain DPD2234 that contains chromosomal insertion of a *grpE* promoter fused to the *P. luminescens luxCDABE* (Figure 5.6). Since *grpE* gene is in the heat shock regulon controlled by σ^{32}, the *grpE-lux* fusion responds to stresses that induce this protein-



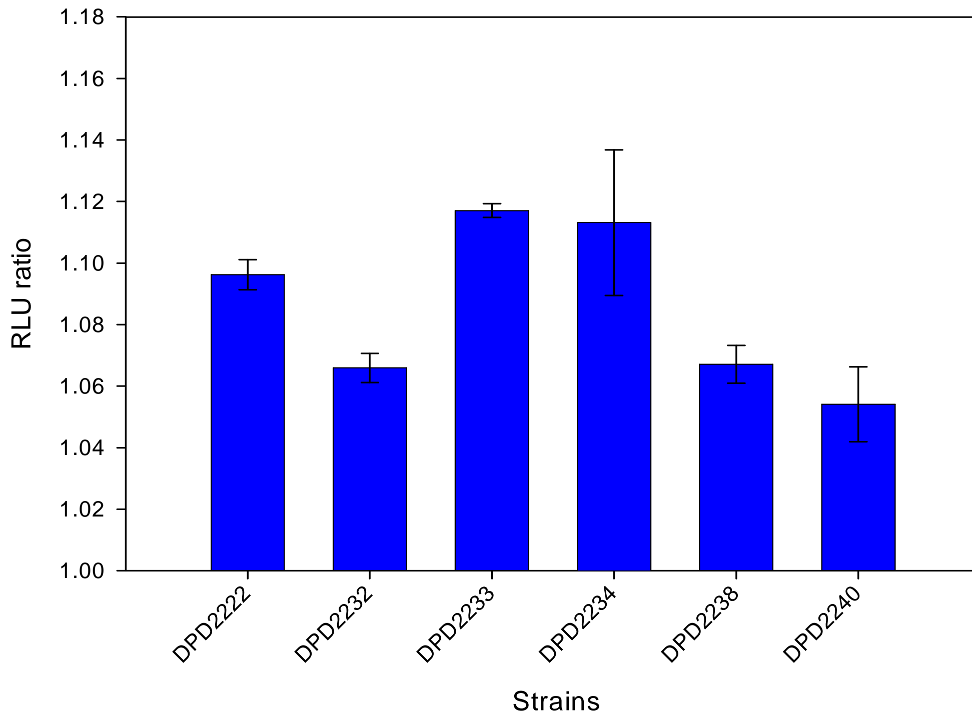


Figure 5.6 Stress responses of six *E. coli* strains to *Prorocentrum minimum* (6,000 cells/ml). The error bars in the figure represent standard error of mean of three replicates.

damage responsive regulon (Van Dyk, 1998), and it is also known to respond to elevation in temperature, the presence of abnormal proteins, and a variety of stresses and chemicals, including organics (Srivastava *et al.*, 2001), heavy metals (Van Dyk, Majarian, Konstantinov, Young, Dhurjati and Larossa, 1994), and oxidative agents (Van Dyk, 1995). Increased ΔRLU and RLU ratio of the strain DPD2234 indicate that *P. minimum* might be a potent inducer of the protein damage sensing heat shock response.

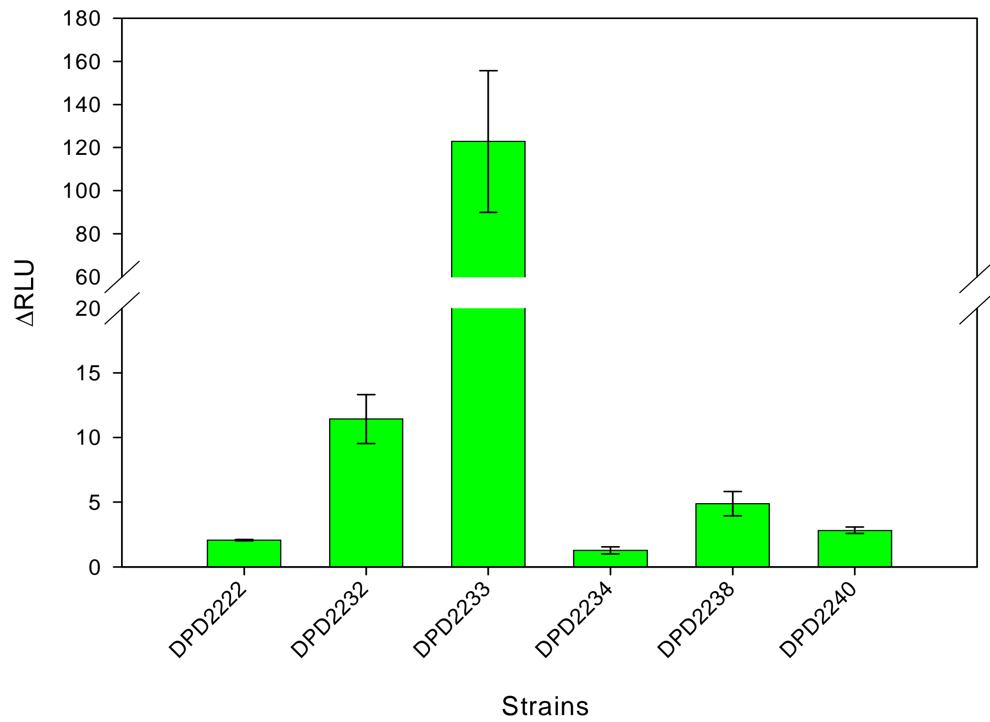
Based on their specific roles in metabolism, heat-shock proteins can be divided into three categories: (1) as chaperones, assisting in protein folding processes; (2) as proteases, taking part in native and foreign protein denaturation; and (3) as effector proteins, activating the heat shock protein synthesis (Daunert, 2000). In *E. coli* strain DPD2234, *P. minimum* may play a role in inducing the *dnaK-dnaJ-grpE* chaperone team, regulating the activity and stability of the σ^{32} regulon (Srivastava *et al*., 2001).

P. minimum induced luminescent signals in strain DPD2240 (*inaA-lux* fusion) at a low level but relatively higher than those of *C. marina* and *K. micrum*. Known as an acid-inducible gene, *inaA* has been shown to give strong response to salicylate, a membrane-permeant weak acid which results in cytoplasmic acidification, when fused with *luxCDABE* (Van Dyk et al., 1998). This result suggests that *P. minimum* may cause weak internal cytoplasmic acidification to the *E. coli* cells. Furthermore, stress response to *P. minimum* was also observed in strain DPD2238, which is sensitive to different types of oxidative agents (Belkin *et al*., 1996), as indicated by the increase in ΔRLU and RLU ratio when compared with *C. marina* and *K. micrum*.

5.3.4 Stress fingerprint of *Pfiesteria piscicida*

P. piscicida strain MDFDEPMDR23 employed in this study, although considered non-toxic, induced a stress fingerprint (Figure 5.7) similar to that of *P. minimum* (Figure 5.6). Strains DPD2233 and DPD2232 remained the most prominent peaks when exposed to *P. piscicida*. However, two distinct differences in bioluminescence emission were found in strains DPD2234 (*grpE-lux* fusion) and DPD2238 (*katG-lux* fusion). *P. piscicida* induced less bioluminescence than *P. minimum* did in DPD2234, which indicates the protein damage caused by *P. piscicida* was less than *P. minimum*. On the other hand, more increased bioluminescence was detected when DPD2238 was exposed to *P. piscicida* as compared to *P. minimum*. This suggests that *P. piscicida* resulted in more severe oxidative damage than *P. minimum* did.

An armored dinoflagellate identified only in the last decade in estuaries in North Carolina and Delaware as well as in the Chesapeake Bay, *P. piscicida* has been shown to possess multiple life stages, which are commonly recognized to associate with its unique toxin production (Litaker *et al*, 2003). In addition, different from other known harmful algal species, *P. piscicida* does not produce any pigment such that there is no visual evidence of its activity. In fact, the non-toxic zoospores (NTZ) of *P. piscicida* strain MDFDEPMDR23 has not been shown to be toxic in bioassays with fish (Stoecker *et al.*, 2000). However, the panel of six *E. coli* strains still showed distinguished stress responses to this particular strain, suggesting certain levels of cell damages on the *E. coli* strains. It is possible that the *E. coli* cells were more stressed than fish in the presence of *P. piscicida* strain MDFDEPMDR23.



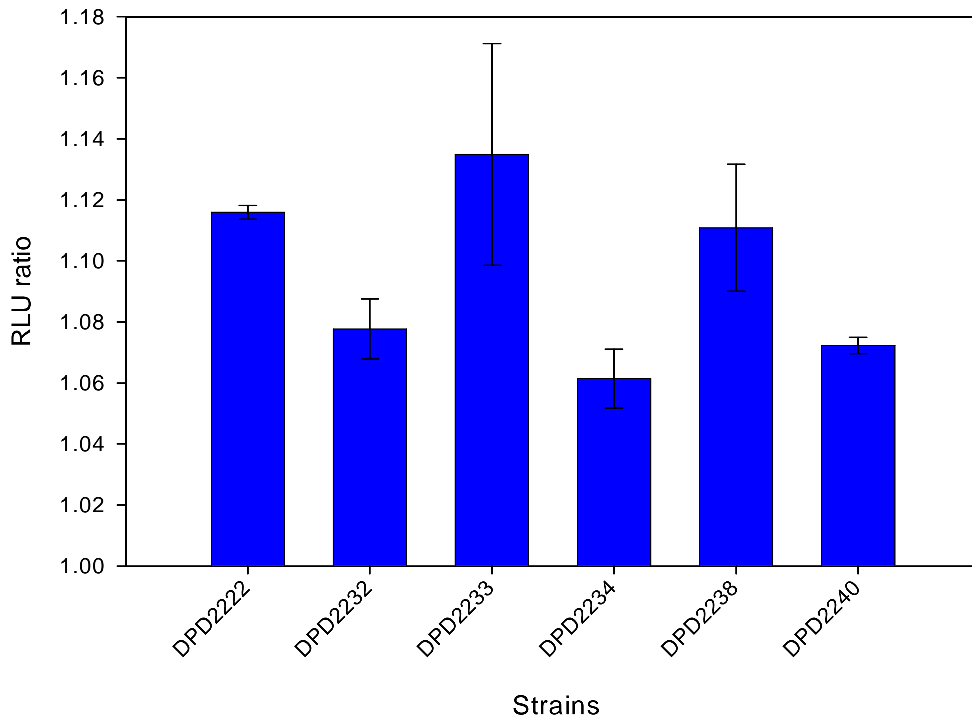


Figure 5.7 Stress responses of six *E. coli* strains to *Pfiesteria piscicida* (6,000 cells/ml). The error bars in the figure represent standard error of mean of three replicates.

5.3.5 Signal and fingerprint comparison

In summary, the stress responses of the six *E. coli* strains to all four harmful algal species showed significant yet characteristic increase in bioluminescence (Table 5.1). The differences among the values of ΔRLU were more obvious than those of RLU ratios. The signal characteristics were distinctive among the four species, suggesting that temporal exposure (30 seconds of contacting time) of the six *E. coli* strains to toxin-generating algae generates stress responses that could serve as fingerprints of respective species.

In addition, exposure of *E. coli* DPD2233 containing *yciG-lux* fusion to the toxic species *C. marina*, *K. micrum*, *P. minimum*, and *P. piscicida* resulted in a 4.0-fold, 2.9-fold, 5.7-fold, and 4.4-fold respective increase in the values of ΔRLU as compared to the signal responding to the non-toxic *A. sanguinea*. The RLU ratios in response to the four harmful algae were also significantly higher than that to the non-toxic algae.

The increased expression of the *yciG-lux* fusion in response to all four harmful algal species suggests a general response to sigma S stress, which occurs in nature when bacteria cells are subjected to starvation or nutrient limitation (Hengge-Aronis, 2002). This indicates that the harmful species resulted in a faster nutrient limitation than non-toxic species on the *E. coli* cells. As discussed above, expression of the other types of fusions constructed within different *E. coli* strains used in the present study were effective in distinguishing among species while suggesting respective damage mechanism at cellular level.

Table 5.1 The fingerprints from six *E.coli* strains responding to four harmful algal species

E. coli strains	Light Parameters*	HAB species *Chattonella marina*	*Karlodinium micrum*	*Prorocentrum minimum*	*Pfiesteria piscicida*
DPD2238	ΔRLU	0.09 ± 0.068^{a}	0.7 ± 0.46^{a}	3.5 ± 0.35^{b}	4.9 ± 0.94^{b}
	RLU ratio	1.009 ± 0.0070^{a}	1.02 ± 0.013^{a}	1.067 ± 0.0061^{b}	1.11 ± 0.021^{b}
DPD2240	ΔRLU	0.8 ± 0.20^{a}	0.703 ± 0.0088^{a}	2.4 ± 0.63^{ab}	2.8 ± 0.25^{b}
	RLU ratio	1.026 ± 0.0056^{a}	1.025 ± 0.0011^{a}	1.05 ± 0.012^{ab}	1.072 ± 0.0027^{b}
DPD2232	ΔRLU	2.0 ± 0.40^{b}	10.8 ± 2.04^{a}	11.1 ± 0.62^{a}	11.4 ± 1.89^{a}
	RLU ratio	1.033 ± 0.0066^{b}	1.07 ± 0.013^{ab}	1.066 ± 0.0048^{a}	1.078 ± 0.0099^{a}
DPD2233	ΔRLU	111 ± 14.7^{ab}	79 ± 9.4^{a}	159 ± 4.7^{b}	123 ± 32.8^{ab}
	RLU ratio	1.09 ± 0.011^{a}	1.102 ± 0.0096^{a}	1.117 ± 0.0022^{a}	1.13 ± 0.036^{a}
DPD2222	ΔRLU	0.30 ± 0.063^{c}	1.26 ± 0.061^{a}	1.9 ± 0.13^{b}	2.06 ± 0.055^{b}
	RLU ratio	1.08 ± 0.012^{ab}	1.067 ± 0.0038^{a}	1.096 ± 0.0049^{a}	1.116 ± 0.0023^{b}
DPD2234	ΔRLU	0.48 ± 0.050^{b}	1.1 ± 0.15^{a}	8.7 ± 2.8^{c}	1.3 ± 0.28^{a}
	RLU ratio	1.12 ± 0.011^{a}	1.089 ± 0.0098^{ab}	1.11 ± 0.023^{ab}	1.061 ± 0.0096^{b}

*Means ± SEM
Replicates n = 3
Values in the same row with the same letter superscripts are not significantly different (P<0.05)

5.4 Quantification of harmful algal species

While qualitative indication of the algal species employed in the present study was successful, stress responses of the *E. coli* strains might also provide insights on the dose dependency to each of the species. Should dose-responsive be established, these *E. coli* strain(s) could also serve as a tool for quantification of harmful algae. As mentioned earlier, the level of cell concentration that causes fish-kill is different in the four harmful species. To evaluate the dose dependency of the E. coli strains on each of the algal species, five concentrations adjacent to the respective fish-kill levels were investigated.

5.4.1 Quantification of *Chattonella marina*

A dose-dependent induction of bioluminescence was found in both ΔRLU and RLU ratio when most of the *E. coli* strains were exposed to *C. marina* (7,500 to 12,500 cells/ml) (Figure 5.8). At 15,000 cells/ml, the stress responses in strains DPD2233 and DPD2238 appeared to decline, possibly due to the toxicity of *C. marina* was too strong for both strains. The expression of *yciG-lux* (DPD2233) and *katG-lux* (DPD2238) fusions was therefore hindered, resulting in reduced bioluminescence. A linear relationship between the RLU ratio and the concentration of *C. marina* was found in strain DPD2222. This finding suggests that the *recA-lux* fusion, which responds to DNA damage, might be a sensitive indicator of concentration changes for *C. marina*. Such relationships could be attributed to activation of the SOS repair network in response to the DNA damage caused by

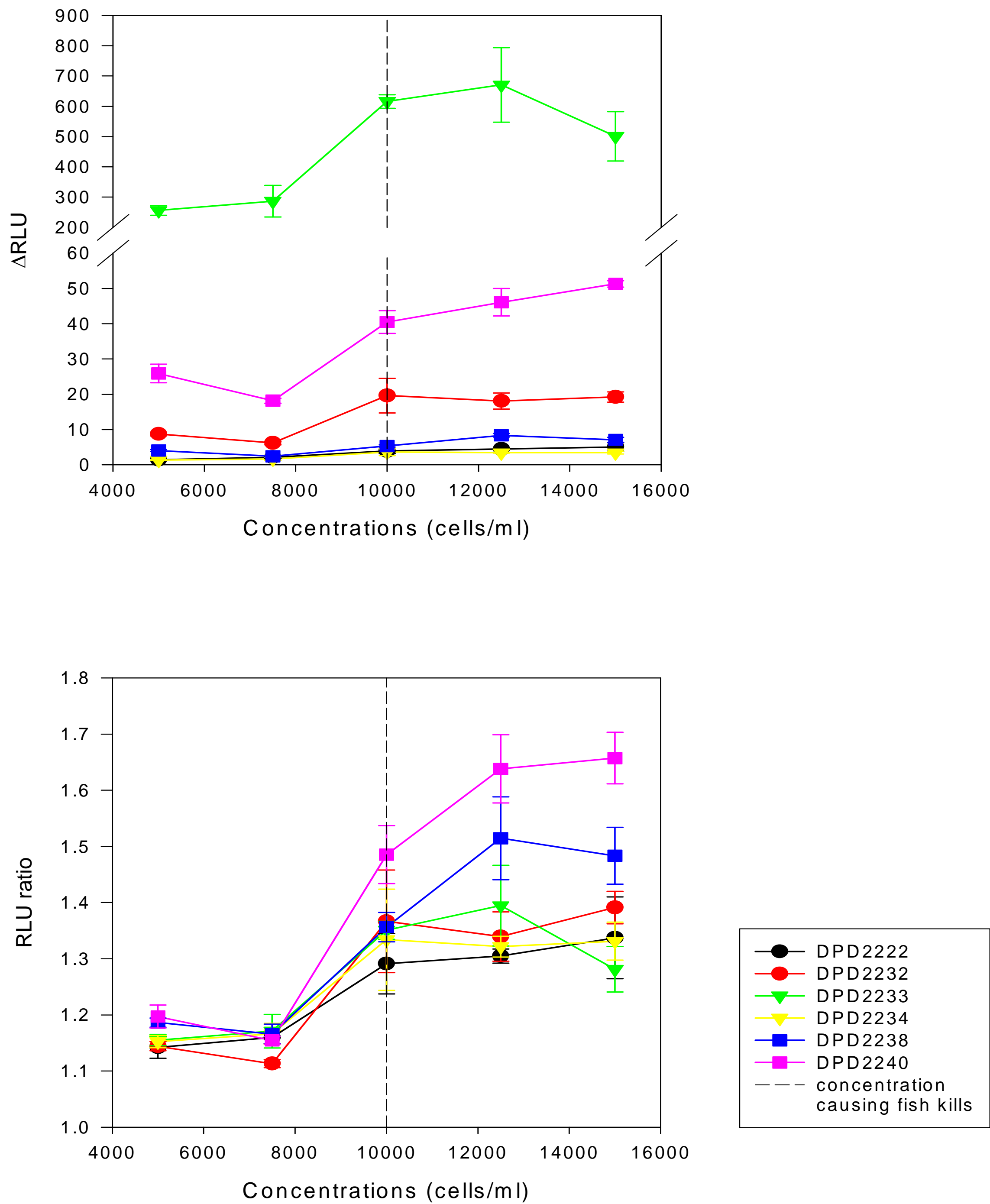


Figure 5.8 Effects of *Chattonella marina* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

C. marina (Daunert *et al.*, 2000). As the concentration of *C. marina* increases, more RecA proteins controlling SOS response can be produced as a DNA recombinase to induce more light signals.

5.4.2 Quantification of *Karlodinium micrum*

The RLU ratios of strain DPD2233 and DPD2240 exhibited linear dependency on the concentration of *K. micrum* up to 10,000 cells/ml (Figure 5.9), indicating that *K. micrum* toxins stressed *E. coli* cells via stationary phase sigma S factor and internal acidification, respectively. The significant decrease in RLU ratio at concentration higher than 10,000 cells/ml could possibly be attributed to lysis of *E. coli* cells, since the toxin produced by *K. micrum* might have exceeded the level of tolerance for the cells. It could also be due to damages caused by the toxins beyond the mechanism designed by the fusion, since the concentration 10,000 cells/ml exceeds the fish-kill dosage (MDNR, 2003).

E. coli DPD2232 (*o513-lux* fusion) showed similar responses and sensitivity to the concentration changes of *K. micrum*. However, the increase in bioluminescence, as indicated by both ΔRLU and RLU ratio, started to decline once the concentration exceeded 9,000 cells/ml. The decline in bioluminescence at a lower concentration than that in the two former strains suggests that DPD2232 might have less tolerance to the toxin, which resulted in more cell lysis at such concentrations.

Compared to the strains discussed above, the RLU ratios of *E. coli* DPD2222 and DPD2234 started to decrease at lower concentration (8,000 cells/ml), which suggests that the DNA and protein damages caused by *K. micrum* could not be fully

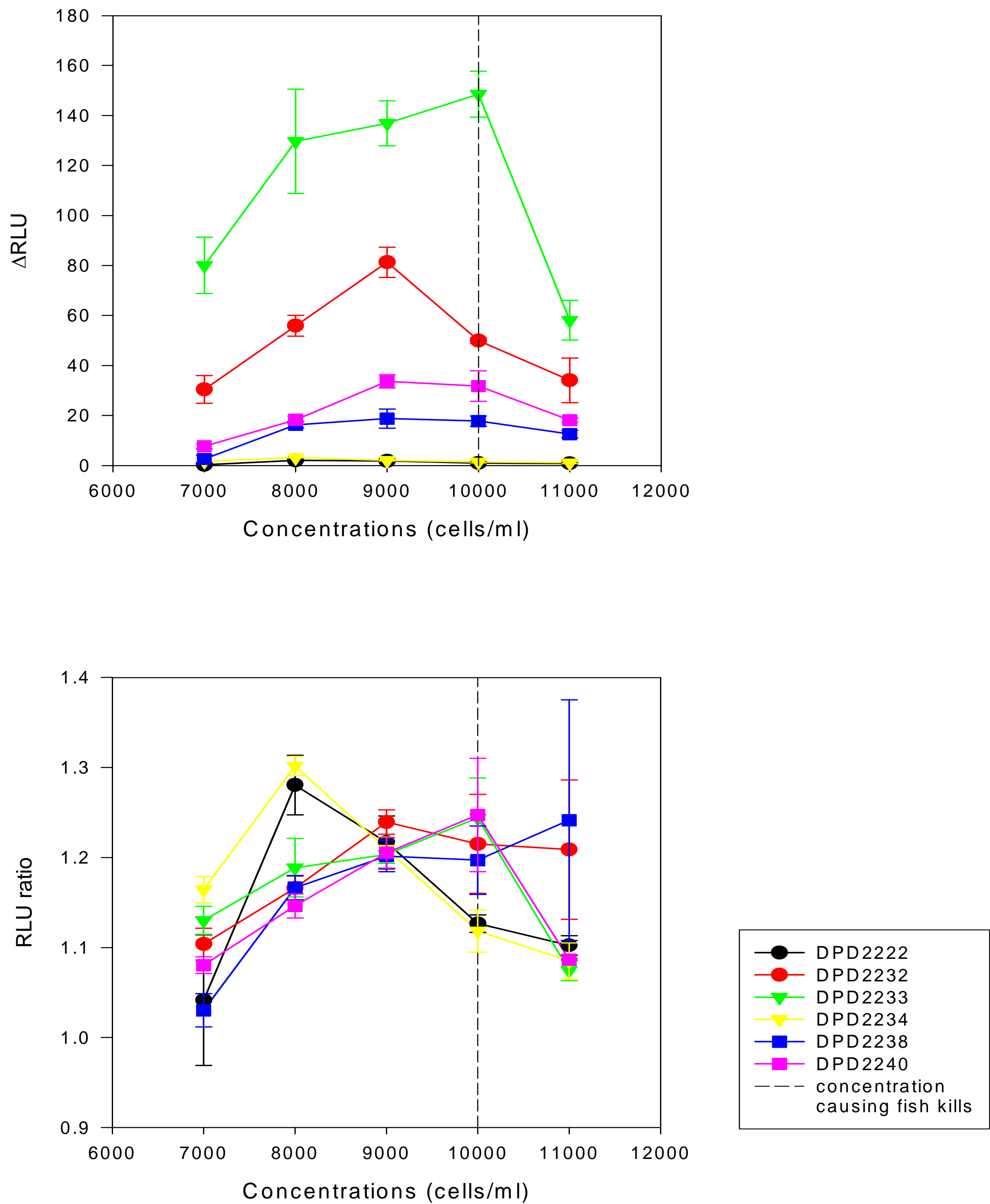


Figure 5.9 Effects of *Karlodinium micrum* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

repaired due to the decreased production of RecA protein. Furthermore, among all the strains studied, only DPD2238 showed linear relationship between bioluminescence (in RLU ratio) and *K. micrum* concentrations. The dose-dependent expression of the *katG-lux* fusion specific to oxidative damage might be a good tool for quantification of *K. micrum*.

5.4.3 Quantification of *Prorocentrum minimum*

The RLU ratios of strains DPD2232, DPD2238, and DPD2240 increased linearly as a function of *P. minimum* concentrations up to 4,000 cells/ml (Figure 5.10). The dramatic decline in stress responses at higher concentrations such as 5,000 cells/ml suggests that the toxic *P. minimum* might reduce the number of active *E. coli* cells, and subsequently influence the expressions of *o513-lux*, *katG-lux*, and *inaA-lux* fusions. Strain DPD2234 showed similar pattern of stress responses as the concentration of *P. minimum* increased, except that the RLU ratio started to decrease when the algal concentration was higher than 3,000 cells/ml.

Interestingly, the RLU ratios of strain DPD2233 showed a linear decrease as the concentrations of *P. minimum* increased with a little hump occurring at 4,000 cells/ml. This unique phenomenon could be attributed to the low tolerance of *yciG-lux* fusion to the highly toxic algal solution containing *P. minimum*, in particular sigma S stress response have been shown to be relatively more sensitive compared to other stress responses (Wosten, 1998).

Similar to the dose responses to *C. marina*, *E. coli* DPD2222 was the only strain showing linear RLU-ratio increase in response to increasing *P. minimum*

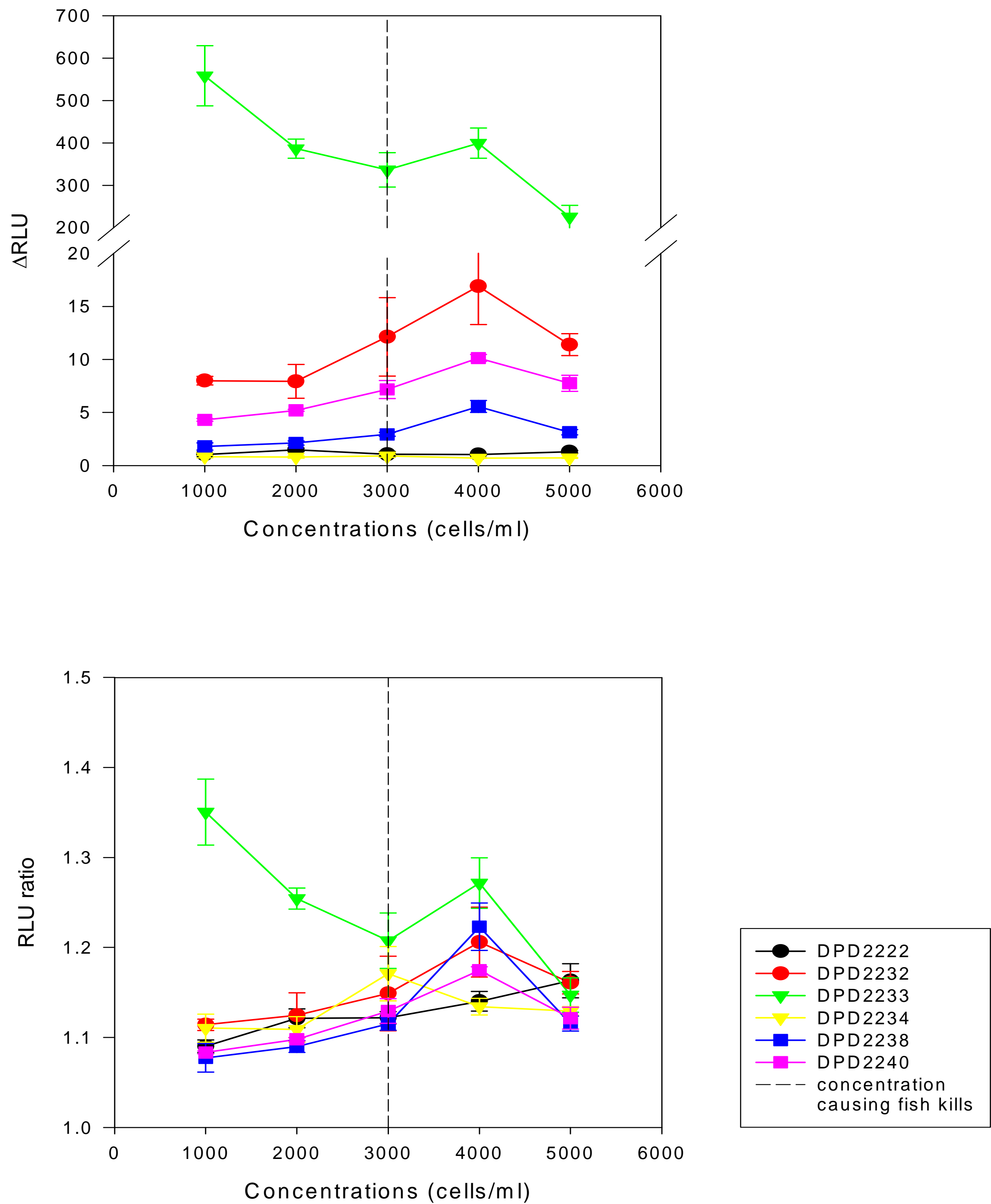


Figure 5.10 Effects of *Prorocentrum minimum* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

concentration. Therefore, the *recA-lux* fusion that responds to DNA damage could serve as a sensitive indicator of *P. minimum* concentration changes. While such relationships could also be attributed to activation of the SOS repair network in response to the DNA damage caused by *P. minimum* (Daunert, Barrett, Feliciano, Shetty, Shrestha and Smith-Spencer, 2000), in which more RecA proteins controlling SOS response can be produced as a DNA recombinase to induce more luminescence in response to the increase of *P. minimum* concentration.

5.4.4. Quantification of *Pfiesteria piscicida*

Unlike other harmful algae species studied, the RLU ratios of *E. coli* strains DPD2222, DPD2232, and DPD2234 were found linearly dependent on the increase of *P. piscicida* concentrations as shown in Figure 5.11. One possible reason is because of the low concentration level of *P. piscicida* employed, whereas other algal culture concentrations were at least 1-2 orders of magnitude higher than *P. piscicida*. The slight changes of concentrations, therefore, induced respective expressions of *recA-lux*, *o513-lux*, and *grpE-lux* fusions that are more stable, which increases the reliability of RLU ratios for quantification (Van Dyk, 1998).

On the other hand, strains DPD2233, DPD2238, and DPD2240 showed linear increase in RLU ratios up to concentration of 400 *P. piscicida* cells/ml. The bioluminescence of these three strains then declined when the *P. piscicida* concentration was at 500 cells/ml. This finding suggests that the stress responses to

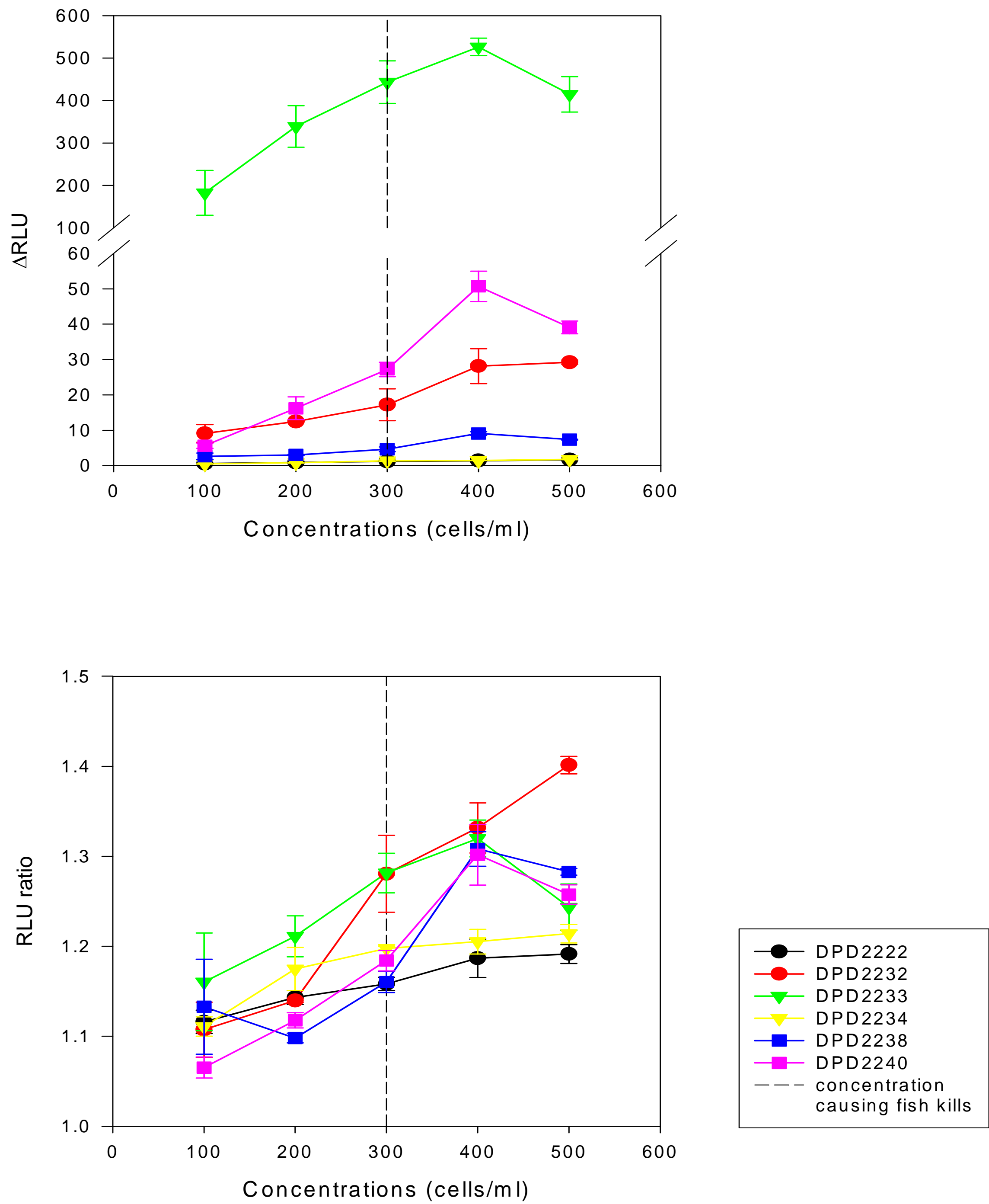


Figure 5.11 Effects of *Pfiesteria piscicida* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

sigma S stress, oxidative damage, and internal acidification might be reduced as a result of the extremely high toxicity of *P. piscicida* toxins.

In summary, when subjected to different HAB species at different concentrations, the increased bioluminescence emitted from almost all *E. coli* strains was quantitatively indicative of stress concentrations, as shown in Figures 5.8 through 5.11. Most ΔRLU or RLU ratio increased linearly with the concentrations of harmful algae species until the concentration or the corresponding toxicity exceeds the cell's tolerance.

In addition, strains DPD2222 and DPD2234 showed much weaker stress response (ΔRLU) to all the algal cultures compared to the other four strains. This could be attributed to the fact that DPD2222 and DPD2234 were constructed in the host strain MM28 using plasmids pDEW14 and pDEW107, respectively, whereas the parental strain of other four strains is GC4468 (Van Dyk, 1998). In addition, both DPD2222 and DPD2234 contain a chromosomal integrant of the *lux* fusion while the other four strains carry a plasmid-borne *lux* fusion. The location of lux fusion inside the cells might affect the sensitivity in detecting harmful algae species.

Chapter 6: Conclusions

Despite of much research focused on the ecophysiology of four major harmful algae species, *Karlodinium micrum*, *Pfiesteria piscicida*, *Chattonella marina* and *Prorocentrum minimum*, the toxicity mechanism of these cultures has not been fully understood yet. In the present study, four algae species could be identified and quantified using six bioluminescent *E. coli* strains.

Containing different stress-responsive promoters, a panel of six bioluminescent *E. coli* strains generated different stress fingerprints due to specific algal cultures. The patterns of induced *luxCDABE* gene expression could be distinguished by the increasing light signals clearly after adding certain harmful algae. At about the same concentration 6,000 cells/ml, four harmful algae species induced much higher stress responses from biosensing strains than non-toxic dinoflagellate *Akashiwo sanguinea* did. Strain DPD2233 (containing an *yciG::luxCDABE* fusion) produced the highest increased bioluminescence responding to all toxic algal cultures among six *E.coli* strains. Increased expression of the *yciG-lux* fusion demonstrated the general nature of sigma S stress response induced by the toxicity of harmful algae species.

Furthermore, dose-dependent characterization of bioluminescence enabled quantification of stress fingerprints specific to the toxic algae in the range of concentrations causing fish kills most likely, suggesting the potential of detecting the presence and severity of harmful algae in coastal water. Stress responses from most strains increased linearly as a function of concentrations of toxic algal cultures and decreased slightly at the highest concentration. Nervertheless, strain DPD2222 showed

increased bioluminescence linearly related to all the five concentrations of *Chattonella marina*, *Prorocentrum minimum*, and *Pfiesteria piscicida*, which implies consistently increasing DNA damages caused by these three toxic algal cultures. The RLU ratios of strain DPD2238 had a linear relationship with increasing concentrations of *Karlodinium micrum*, suggesting the intensity of oxidative damage could be a good indicator to the cell numbers of this harmful algae species. Testing at more concentrations beyond highly toxic level is needed to obtain more quantitative information.

Appendices
Experimental Data from Figures

Figure 5.1 Calibration curves of optical density at 600 nm versus concentration (g/ml) of bioluminescent *E. coli* strains (a) DPD2238, (b) DPD 2240, (c) DPD 2232, (d) DPD2233, (e) DPD2222, and (f) DPD 2234

***E. coli* strains**	**Cell Concentration (cells/ml)**	**OD_{600}**
DPD2238	0.00043	0.308
	0.00052	0.361
	0.00065	0.449
	0.00087	0.58
	0.0013	0.8
DPD 2240	0.00018	0.306
	0.00022	0.36
	0.00028	0.443
	0.00037	0.574
	0.00055	0.764
DPD 2232	0.00022	0.285
	0.00026	0.341
	0.00033	0.419
	0.00043	0.542
	0.00065	0.761
DPD2233	0.00023	0.3
	0.00028	0.353
	0.00035	0.435
	0.00046	0.569
	0.0007	0.792
DPD2222	0.00022	0.289
	0.00026	0.357
	0.00033	0.442
	0.00043	0.569
	0.00065	0.784
DPD2234	0.00025	0.295
	0.0003	0.353
	0.00038	0.438
	0.0005	0.565
	0.00075	0.777

Figure 5.2 The profile of light emission (RLU) from six bioluminescent *E. coli* strains: (a) DPD2232 and DPD2233; (b) DPD2222, DPD2234, DPD2238, and DPD2240.

Growth Time (hour)	RLU					
	DPD2232	DPD2233	DPD2222	DPD2234	DPD2238	DPD2240
0	0	0	0	0	0	0
4	0.103	1.124	0.153	0.161	0.018	0.227
6.5	109.7	0.233	15.19	4.777	12.53	37.85
7	153.3	5.459	12.27	6.934	9.58	43.91
7.5	220.2	7.75	11.49	7.363	11.75	61.59
8	225.5	8.27	14.4	8.379	23.61	46.7
8.5	258.9	38.36	13.62	7.643	40.1	39.14
9	237.3	88.3	18.47	8.89	64.17	30.6
9.5	243.5	152.8	12.55	7.94	66.86	37.13
10	217.8	248.6	11.39	7.393	77.3	36.95
10.5	203	467.1	11.76	4.861	81.1	41.73
11	211.1	442	10.16	6.676	78.2	41.11
11.5	199.8	446.4	8.83	6.437	75.5	41.66
12	222	470.7	7.355	6.168	73.4	40.15
12.5	160.4	725.1	7.792	3.622	65.37	31.87

Figure 5.3 Stress responses of six *E. coli* strains to *Akashiwo sanguinea* (6,000 cells/ml), a non-toxic red-tide dinoflagellate. The error bars in the figure represent standard error of mean of three replicates.

***E. coli* strains**	**ΔRLU**	**RLU ratio**
DPD2222	0.14 ± 0.050	1.03 ± 0.011
DPD2232	4.0 ± 1.2	1.027 ± 0.0082
DPD2233	27.7 ± 5.2	1.015 ± 0.0029
DPD2234	0.05 ± 0.017	1.024 ± 0.0083
DPD2238	-2.5 ± 0.35	0.934 ± 0.0093
DPD2240	1.3 ± 1.4	1.03 ± 0.035

Figure 5.8 Effects of *Chattonella marina* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

ΔRLU:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	**DPD2232**	**DPD2233**	**DPD2234**	**DPD2238**	**DPD2240**
5000	1.5 ± 0.21	8.7 ± 0.56	256 ± 16.1	1.3 ± 0.10	4.1 ± 0.29	26 ± 2.7
7500	2.2 ± 0.31	6.2 ± 0.49	286 ± 52.3	1.7 ± 0.40	2.4 ± 0.19	18.2 ± 0.71
10000	3.9 ± 0.71	19.6 ± 4.9	616 ± 22.6	3.6 ± 1.0	5.3 ± 0.32	40 ± 3.2
12500	4.5 ± 0.26	18 ± 2.3	670 ± 123.4	3.4 ± 1.13	8.3 ± 0.51	46 ± 3.8
15000	5.0 ± 1.2	19 ± 1.5	501 ± 81.4	3.4 ± 0.33	7.1 ± 0.73	51.2 ± 0.9

RLU ratio:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	**DPD2232**	**DPD2233**	**DPD2234**	**DPD2238**	**DPD2240**
5000	1.14 ± 0.019	1.144 ± 0.0082	1.16 ± 0.010	1.15 ± 0.011	1.186 ± 0.0082	1.20 ± 0.021
7500	1.16 ± 0.011	1.113 ± 0.0071	1.17 ± 0.030	1.17 ± 0.019	1.16 ± 0.018	1.155 ± 0.0065
10000	1.29 ± 0.054	1.37 ± 0.091	1.35 ± 0.016	1.33 ± 0.090	1.35 ± 0.026	1.48 ± 0.052
12500	1.30 ± 0.013	1.34 ± 0.044	1.39 ± 0.072	1.32 ± 0.019	1.51 ± 0.074	1.64 ± 0.061
15000	1.34 ± 0.073	1.39 ± 0.029	1.28 ± 0.040	1.33 ± 0.034	1.48 ± 0.051	1.66 ± 0.046

Figure 5.9 Effects of *Karlodinium micrum* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

ΔRLU:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	**DPD2232**	**DPD2233**	**DPD2234**	**DPD2238**	**DPD2240**
7000	0.2 ± 0.55	30 ± 5.5	80 ± 11.2	1.7 ± 0.15	2.6 ± 1.61	7.7 ± 0.93
8000	2.0 ± 0.26	56 ± 4.1	130 ± 20.9	3.1 ± 0.12	16 ± 1.2	18 ± 1.6
9000	1.8 ± 0.29	81 ± 6.0	137 ± 8.9	2.0 ± 0.25	19 ± 3.8	34 ± 2.7
10000	0.97 ± 0.057	50 ± 1.1	149 ± 9.2	1.4 ± 0.38	18 ± 2.1	32 ± 6.1
11000	0.86 ± 0.098	34 ± 8.9	58 ± 7.9	1.1 ± 0.41	12 ± 1.5	18 ± 0.87

RLU ratio:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	**DPD2232**	**DPD2233**	**DPD2234**	**DPD2238**	**DPD2240**
7000	1.04 ± 0.073	1.10 ± 0.018	1.13 ± 0.016	1.16 ± 0.015	1.03 ± 0.018	1.080 ± 0.0093
8000	1.28 ± 0.033	1.17 ± 0.013	1.19 ± 0.033	1.30 ± 0.011	1.17 ± 0.013	1.14 ± 0.013
9000	1.22 ± 0.029	1.24 ± 0.013	1.203 ± 0.0096	1.21 ± 0.013	1.20 ± 0.017	1.21 ± 0.017
10000	1.126 ± 0.0097	1.22 ± 0.055	1.24 ± 0.044	1.12 ± 0.023	1.20 ± 0.038	1.24 ± 0.063
11000	1.10 ± 0.011	1.21 ± 0.077	1.07 ± 0.012	1.08 ± 0.020	1.2 ± 0.13	1.087 ± 0.0011

Figure 5.10 Effects of *Prorocentrum minimum* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

ΔRLU:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	**DPD2232**	**DPD2233**	**DPD2234**	**DPD2238**	**DPD2240**
1000	1.03 ± 0.073	8 ± 0.40	558 ± 70.7	0.8 ± 0.12	1.8 ± 0.34	4.3 ± 0.12
2000	1.5 ± 0.15	7.9 ± 1.6	386 ± 22.7	0.80 ± 0.098	2.1 ± 0.20	5.2 ± 0.10
3000	1.0 ± 0.26	12 ± 3.7	336 ± 40.6	0.9 ± 0.13	2.9 ± 0.18	7.2 ± 0.85
4000	1.03 ± 0.063	17 ± 3.6	399 ± 35.6	0.70 ± 0.045	5.6 ± 0.56	10.1 ± 0.33
5000	1.3 ± 0.13	11 ± 1.0	225 ± 28.0	0.71 ± 0.039	3.1 ± 0.24	7.8 ± 0.76

RLU ratio:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	**DPD2232**	**DPD2233**	**DPD2234**	**DPD2238**	**DPD2240**
1000	1.090 ± 0.0071	1.114 ± 0.0063	1.35 ± 0.037	1.11 ± 0.016	1.08 ± 0.016	1.083 ± 0.0015
2000	1.12 ± 0.010	1.12 ± 0.025	1.25 ± 0.012	1.11 ± 0.014	1.090 ± 0.0066	1.098 ± 0.0020
3000	1.122 ± 0.0068	1.15 ± 0.041	1.21 ± 0.031	1.17 ± 0.030	1.115 ± 0.0072	1.13 ± 0.014
4000	1.14 ± 0.011	1.21 ± 0.039	1.27 ± 0.028	1.134 ± 0.0093	1.22 ± 0.026	1.175 ± 0.0039
5000	1.16 ± 0.019	1.16 ± 0.012	1.15 ± 0.019	1.129 ± 0.0051	1.115 ± 0.0082	1.12 ± 0.012

Figure 5.11 Effects of *Pfiesteria piscicida* concentration on the increased bioluminescence of six *E. coli* strains. The error bars in the figure represent standard error of mean of three replicates.

ΔRLU:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	DPD2232	DPD2233	DPD2234	DPD2238	DPD2240
100	0.58 ± 0.044	9 ± 2.5	182 ± 53.1	0.45 ± 0.055	2.6 ± 0.93	5.6 ± 0.73
200	0.8 ± 0.12	12.4 ± 0.12	339 ± 48.7	0.77 ± 0.062	3.0 ± 0.14	16 ± 3.2
300	1.20 ± 0.044	17 ± 4.5	443 ± 50.2	1.36 ± 0.17	4.6 ± 0.63	27 ± 2.1
400	1.4 ± 0.11	28 ± 5.0	526 ± 20.6	1.45 ± 0.057	9.1 ± 0.53	51 ± 4.3
500	1.6 ± 0.15	29.2 ± 0.58	415 ± 41.7	1.8 ± 0.12	7.3 ± 0.11	39 ± 1.8

RLU ratio:

Concentrations (cells/ml)	*E. coli* strains					
	DPD2222	DPD2232	DPD2233	DPD2234	DPD2238	DPD2240
100	1.12 ± 0.013	1.11 ± 0.031	1.16 ± 0.054	1.11 ± 0.011	1.13 ± 0.053	1.06 ± 0.012
200	1.143 ± 0.0078	1.140 ± 0.0020	1.21 ± 0.023	1.17 ± 0.024	1.098 ± 0.0053	1.118 ± 0.0085
300	1.158 ± 0.0074	1.28 ± 0.043	1.28 ± 0.022	1.198 ± 0.0024	1.16 ± 0.012	1.18 ± 0.011
400	1.19 ± 0.021	1.33 ± 0.028	1.32 ± 0.021	1.21 ± 0.014	1.31 ± 0.019	1.30 ± 0.034
500	1.19 ± 0.010	1.401 ± 0.0098	1.24 ± 0.026	1.21 ± 0.010	1.283 ± 0.0037	1.26 ± 0.011

References

Amzil, Z. and Y.F. Pouchus. "Short time cytotoxicity of mussel extracts;a new bioassay for okadaic acid detection." Toxicon 30(1992): 1419-1425.

Andersen, Per. Design and implementation of some harmful algal monitoring systems. Paris: Unesco, 1996.

Anderson, D. M., S. B. Galloway and J. D. Joseph. *Marine biotoxins and Harmful Algae: A national plan*. Woods Hole Oceanog. Inst. Tech. Rept., 1993.

Applegate, B. M., S. R. Kehrmeyer and G. S. Sayler. "A chromosomally based *tod-luxCDABE* whole-cell reporter for benzene, toluene, ethybenzene and xylene (BTEX) sensing." *Applied and Environmental Microbiology* 64(1998): 2730-2735.

Armstrong, T. N., D. W. Coats. Influence of suspended solids and host diversity on dinoflagellate parasitism. 10th East Coast Protozoology Conference, June 8-10, 2002.

Bates, H. A. and H. Rapoport. "A chemical assay for saxitoxin, the paralytic shellfish poison." *J. Agric. Food Chem.* 23(1975): 237-239.

Bates, S. S., C. J. Bird and A. S. W. DeFreitas. "Pennate diatom *Nitzshia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada." *Canadian Journal of Fisheries and Aquatic Sciences* 46(1989): 1203-1215.

Baumann, P., L. Baumann, M. Woolkalis and S. Bang. "Evolutionary relationships in *Vibrio* and *Photobacterium*. A basis for a natural classification." *Annu. Rev. Microbiol.* 37(1983): 369-398.

Billard, P. and M. S. DuBow. "Bioluminescence-based assays for detection and characterization of bacteria and chemicals in clinical laboratories." *Clinical Biochemistry* 31(1998): 1-14.

Buerk, Donald G. *Biosensors Theory and Applications*. Lancaster, PA: Technomic Publishing Company, Inc., 1993.

Bulich, A. A. and D. L. Isenberg. "Use of the luminescent bacterial system for the rapid assessment of aquatic toxicity." *ISA Transactions* 20(1981): 29-33.

Burlace, Robert S., Gary S. Sayler and Frank Larimer. "Monitoring of Naphthalene Catabolism by Bioluminescence with *nah-lux* Transcription Fusions." *Journal of Bacteriology* 172(1990): 4749-4757.

Campbell, A. K. "Living light: biochemistry, function and biomedical applications." *Essays Biochem.* 24(1989): 41-76.

Chance, B. "Optical Method." *Annu. Rev. Biophys. Chem.* 20(1991): 1-28.

Chatterjee, Jaidip and Edward A. Meighen. "Biotechnological applications of bacterial bioluminescence (*lux*) genes." *Photochemistry and Photobiology* 62(1995): 641-650.

Chu, Y. F. Effects of surface characteristics of cells and matrices on the immobilization of bioluminescent E. coli. Mater thesis. Newark, Delaware: University of Delaware, Department of Animal and Food Science, 2001.

Clark, L. C. and C. Lyons. "Electrode systems for continuous monitoring in cardiovascular surgery." *Ann. NY Acad. Sci* 102(1962): 29-45.

Coulet, Pierre R. *Biosensor Principles and Applications*. New York, New York: Marcel Dekker Inc., 1991.

Dale, B. and C. M. Yentsch. "Red tide and paralytic shellfish poisoning." *Oceanus* 21(1978): 41-49.

Daunert, S., G. Barrett, J. S. Feliciano, R. S. Shetty, S. Shrestha, W. Smith-Spencer. “Genetically engineered whole-cell sensing sustems: coupling biological recognition with reporter genes.” Chem Rev 100 (2000): 2705-2738.

Deeds, Jonathan R. and Daniel E. Terlizzi, J. E. Adolf, D. K. Stoecker, and Allen R. Place. "Toxic activity from cultures of *Karlodinium micrum* (=*Gyrodinium galatheanum*) (Dinophyceae) - a dinoflagellate associated with fish mortalities in an estuarine aquaculture facilities." *Harmful Algae* 1(2002): 169-189.

Dyhrman, S. T. and B. Palenik. "A single-cell immunoassay for phosphate stress in the dinoflagellate Prorocentrum minimum (Dinophyceae)." *Journal of Phycology* 37(2001): 400-410.

Ebersole, R. C., R. P. Foss and M. D. Ward. "Piezoelectric Cell Growth Sensor." *Bio. Technol.* 9(1991): 450-454.

Fairey, E. R., J. S. G. Edmunds and J. S. Ramsdell. "A cell-based assay for brevetoxins, saxitoxins, and ciguatoxins using a stably expressed c-fos-luciferase reporter gene." *Analytical Biochemistry* 251(1997): 129-132.

Farmer, J. J. and J. H. Jorgensea. "*Xenorhabdus luminescens* (DNA hybridization group 5) from human clinical specimens." *J. Clin. Microbiol.* 27(1989): 1594-1600.

Gallegos, C. L. and T. E. Jordan. "Impact of the Spring 2000 phytoplankton bloom in Chesapeake Bay on optical properties and light penetration in the Rhode River, Maryland." *Estuaries* 25(2002): 508-518.

Gil, Geun Cheol, Robert J. Mitchell, Suk Tai Chang and Man Bock Gu. "A biosensor for the detection of gas toxicity using a recombiant bioluminescent bacterium." *Biosensors and Bioelectronics* 15(2000): 23-30.

Glibert, P. M., R. Magnien, M. W. Lomas, J. Alexander, C. K. Fan, E. Haramoto, M. Trice and T. M. Kana. "Harmful algal blooms in the Chesapeake and coastal bays of Maryland, USA: Comparison of 1997, 1998, and 1999 events." *Estuaries* 24(2001): 875-883.

Gu, Man Bock and Prasad S. Dhurjati. "A miniature bioreactor for sensing toxicity using recombiant bioluminescent *Escherichia coli* cells." *Biotechnol. Prog.* 12(1996): 393-397.

Guilbault, G. G. and F. R. Shu. "Enzyme electrodes based on the use of a carbon dioxide sensor: Urea and L-tyrosine electrodes." *Anal. Chem.* 44(1972): 2161.

Guibault, G. "Enzyme glucose electrodes." *Diabetes Care* 5(1982): 181-183.

Guzzo, Angelina and Michael S. DuBow. "A *luxAB* transcriptional fusion to the cryptic *celF* gene of *Escherichia coli* displays increased luminescence in the presence of nickel." *Mol. Gen. Genet.* 242(1994): 455-460.

Hallegraeff, G. M. "A review of harmful algal blooms and their apparent global increase." Phycologia 32(1993): 79-99.

Hashimoto, T. and Y. Yamada. "Alkaloid biogenesis: Molecular aspects." Annu. Rev. Plant Physiol. Plant. Mol. Biol. 45(1994): 257-285.

Hastings, J. W., J. Makemson and P.V. Dunlap. "How are growth and luminescence regulated independently in light organ symbionts." *Symbiosis*4(1987): 3 -24.

Hégaret, H. and G. H. Wikfors. "Effects of natural and field-simulated blooms of the dinoflagellate *Prorocentrum minimum* upon hemocytes of eastern oysters, *Crassostrea virginica*, from two different populations." *Harmful Algae* in press (2004).

Hengge-Aronis, R. "Stationary phase gene regulation: what makes an Escherichia coli promoter sigma S selective." *Current opinion in Microbiology* 5(2002): 591-595.

Ishida, H., N. Muramatsu and H. Nukay. "Study on neurotoxic shellfish poisoning involving the oyster, Crassostrea gigas, in New Zealand." *Toxicon.* 34(1996): 1050-1053.

Ivanova, A., C. Miller, G. Glinsky, A. Eisenstarck. "Role of rpoS (katF) in oxyR-independent regulation of hydroperoxidase I in Escherichia coli." *Mol. Microbiol.* 12(1994): 571-578.

Kempton, J. W., A. J. Lewitus, J. R. Deeds, J. M. Law and A. R. Place. "Toxicity of *Karlodinium micrum* (Dinophyceae) associatied with a fish kill in a South Carolina brackish retention pond." *Harmful Algae* 1(2002): 233-241.

Kim, Byoung Chan, Kyeong Seo Park, Sang Don Kim and Man Bock Gu. "Evaluation of a high throughput toxicity biosensor and comparison with a *Daphnia magna* bioassay." *Biosensors and Bioelectronics* 00(2003): 1-6.

Konstantinov, Konstantin B., Prasad Dhurjati, Tina Van Dyk, William Majarian and LaRossa Robert. "Real-Time Compensation of the Inner Filter Effect in High-

Density Bioluminescent Cultures." *Biotechnology and Bioengineering* 42(1993): 1190-1198.

Korpimaki, T., J. Kurittu and M. Karp. "Surprisingly fast diappearance of belta-lactam selection pressure in cultivation as detected with novel biosensing approaches." *Journal of Microbiological Methods* 53(2003): 37-42.

Lee, J. S., T. Yanagi, R. Kema and T. Yasumoto. "Fluorometric determination of diarrhetic shellfish toxins by high pressure liquid chromatography." *Agric. Biol. Chem.* 51(1987): 877-881.

Lee, K. S., A. Ishimatsu, H. Sakaguchi and T. Oda. "Cardiac output during exposure to *Chattonella marina* and environmental hypoxia in yellowtail (Seriola quinqueradiata)." *Marine Biology* 142(2003): 391-397.

Leong, S. C. Y., A. Murata, Y. Nagashima and S. Taguchi. “Variability in toxicity of the dinoflagellate Alexandrium tamarense in response to different nitrogen sources and concentrations." *Toxicon* 43(2004): 407-415.

Litaker, R. W., M. W. Vandersea, S. R. Kibler, K. S. Reece, N. A. Stokes, K. A. Steidinger, D. F. Millie, B. J. Bendis, R. J. Pigg and P. A. Tester. "Identification of Pfiesteria piscicida (Dinophyceae) and Pfiesteria-like organisms using internal transcribed spacer-specific PCR assays." *Journal of Phycology* 39(2003): 754-761.

Marshall, J. A., M. Hovenden, T. Oda and G. M. Hallegraeff. "Photosynthesis does influence superoxide production in the ichthyotoxic alga Chattonella marina (Raphidophyceae)." *Journal of Plankton Research* 24(2002): 1231-1236.

Meighen, Edward A. "Molecular Biology of Bacteria Bioluminescence." *Microbiological Reviews* 55(1991): 123-142.

Oda, T., A. Nakamura, T. Okamoto, A. Ishimatsu and N. Muramatsu. "Lectin-induced enhancement of superoxide anion production by red tide phytoplankton." *Marine Bology* 131(1998): 383-390.

Pertola, S., M. A. Faust, H. Kuosa and G. Hallfors. "Morphology of Prorocentrum minimum (Dinophyceae) in the Baltic Sea and in Chesapeake Bay: Comparison of cell shapes and thecal ornamentation." *Botanica Marina* 46(2003): 477-486.

Plumley, F. Gerald. "Marine algal toxins: Biochemistry, genetics, and molecular biology." *Limnol. Oceanogr.* 42(1997): 1252-1264.

Prest, A. G., M. K. Winson, J. R. M. Hammond and G. S. A. B. Stewart. "The construction and application of a *lux*-based nitrate biosensor." *Letters in Applied Microbiology* 24(1997): 355-360.

Quesenberry, M. S., K. Saito, D. N. Krupatkina, J. A. F. Robledo, T. Drgon, W. T. Pecher, N. O'Leary, M. Alavi, T. Miller, R. E. Schneider, R. Belas, J. R. Deeds, A. R. Place, Y. Zohar and G. R. Vasta. "Bioassay for ichthyocidal activity of Pfiesteria piscicida: Characterization of a culture flask assay format." *Journal of Applied Phycology* 14(2002): 241-254.

Rechnitz, G.A. and M.Y. Ho. "Biosensors based on cell and tissue material." *Journal of Biotechnology* 15(1990): 201-218.

Selifnova, Olga, Robert Burlage and Tamar Barkay. "Bioluminescent sensors for detection of bioavailable Hg(II) in the environment." *Applied and Environmental Microbiology* 59(1993): 3083-3090.

Srivastava, R., M. S. Peterson, W. E. Bentley. “Stochastic kinetic analysis of the *Escherichia coli* stress circuit using sigma 32 targeted antisense.” *Biotechnology and Bioengineering* 75(2001): 120-129.

Sticher, Patrick, Marco C. M. Jaspers, Konrad Stemmler and Hauke Harms. "Development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples." *Applied and Environmental Microbiology* 63(1997): 4053-4060.

Stoecker, D. K., K. Stevens, and D. E. Gustafson. “Grazing on *Pfiesteria piscicida* by microzooplankton.” *Aquatic Microbial Ecology* 22(2000): 261-270.

Subba Rao, D. V., M. A. Quilliam and R. Pocklington. "Domoic acid - A neurotoxic amino acid produced by the marine diatom *Nitzschia pungens* in culture." *Can. J. Fish. Aquatic. Sci.* 45(1988): 2076-2079.

Suganuma, M., H. Fujiki and H. Suguri. "Okadaic acid: an additional non-phorbol-12-teradecanoate-13-acetate-type tumor promoter." *Proceeding of the National Academy Sciences USA* 85(1988): 1768-1771.

Sullivan, J. J. and J. Jonas-Davies. *Toxic Dinoflagellates*. New York: Elsevier, 1985.

Szittner, R. and E. Meighen. "Nucleotide sequence, expression and properties of luciferase coded by *lux* genes from a terrestrial bacterium." *J. Biol. Chem.* 265(1990): 16581-16587.

Terasaki, M., Y. Itabashi, T. Suzuki and K. Nishimura. "An improved method for determining the composition of FFA in red tide flagellates by RP-HPLC with fluorescence detection." *Journal of the American Oil Chemists Society* 79(2002): 1181-1186.

Thomas, G. M. and G. O. Poinar. "*Xenorhabdus gen. nov.*, a genus of entomopathogenic, nematophilic bacteria of the family *Enterobacteriaceae*." *Int. J. Syst. Bacteriol.* 29(1979): 352-360.

Turgeon, Donna D., Kevin G. Sellner and Donald Scavia. *Status of U.S. harmful algal blooms : progress toward a national program : harmful algal blooms kill coastal marine wildlife and poison humans*. Washington, D.C.: National Oceanic and Atmospheric Administration, 1998.

Van Dyk, Tina K, William Majarian, Konstantin B. Konstantinov, Roslyn M. Young, Prasad S. Dhurjati and Robert A. Larossa. "Rapid and sensitive pollutant detection by induction of heat shock gene-bioluminescence gene fusion." *Applied and Environmental Microbiology* 60(1994): 1414-1420.

Van Dyk, Tina K. "Bioluminescent gene fusions for characterization of Xenobiotic-induced gene expression changes in *Escherichia coli*." PhD diss. Newark, Delaware: University of Delaware, Department of Animal and Food Science, 1998.

Vining, K., J. Schell and G. Kahl. "Functions of secondary metabolites." *Annu. Rev. Microbiol.* 44(1990): 395-427.

Wall, L., A. Rodriguez and E. Meighen. "Intersubunit transfer of fatty acyl groups during fatty acid reduction." *J. Biol. Chem.* 261(1986): 16018-16025.

Wood, K. V. and M. G. Gruber. "Transduction in microbial biosensors using multiplexed bioluminescence." *Biosensors and Bioelectronics* 11(1996): 207-214.

Wosten, M. M. S. M. "Eubacterial sigma-factors." *FEMS Microbiology Reviews* 22(1998): 127-150.

Yasumoto, T., Y. Oshima and Sugawara W. "Identification of *Dinophysis fortii* as the causative organisn of diarrhetic shellfish poisoning." *Bulletin of the Japanese Society for Scientific Fisheries* 46(1978): 1405-1411.

Yasumoto, T. and T. Underdal. Screening for hemolytic and ichtyotoxic components of *Chrysochromylina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In *Toxic Marine Phytoplankton*, 436-440. New York: Elsevier Science Publishing Co., 1990.

Yoon, H. S., J. D. Hackett and D. Bhattacharya. "A single origin of the preidinin- and fucoxanthin containing plastids in dinoflagellates through teritiary endosymbiosis." *Proceeding of the National Academy Sciences of the United States of America* 99(2002): 11724-11729