

# **Literature Review of the Microalga *Prymnesium parvum* and its Associated Toxicity**

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## **Introduction**

Recent large-scale fish kills associated with the golden-alga, *Prymnesium parvum*, have imposed monetary and ecological losses on the state of Texas. This phytoflagellate has been implicated in fish kills around the world since the 1930's (Reichenbach-Klinke 1973). Kills due to *P. parvum* blooms are normally accompanied by water with a golden-yellow coloration that foams in riffles (Rhodes and Hubbs 1992). The factors responsible for the appearance of toxic *P. parvum* blooms have yet to be determined.

The purpose of this paper is to present a review of the work by those around the globe whom have worked with *Prymnesium parvum* in an attempt to better understand the biology and ecology of this organism as well as its associated toxicity. I will concentrate on the relevant biology important in the ecology and identification of this organism, its occurrence, nutritional requirements, factors governing its toxicity, and methods used to control toxic blooms with which it is associated.

## **Background Biology and Diagnostic Features**

*Prymnesium parvum* is a microalga in the class Prymnesiophyceae, order Prymnesiales and family Prymnesiaceae, and is a common member of the marine phytoplankton (Bold and Wynne 1985, Larsen 1999, Lee 1980). It is a uninucleate, unicellular flagellate with an ellipsoid or narrowly oval cell shape (Lee 1980, Prescott 1968). Green, Hibberd and Pienaar (1982) reported that the cells range from 8-11 micrometers long and 4-6 micrometers wide. The authors also noted that the cells are

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sometimes slightly compressed with the posterior end rounded or tapered and the anterior end obliquely truncate. An individual *P. parvum* cell has two equal flagella and a well-developed haptonema (Lee 1980). The flagella are used for motility and the haptonema may be involved in attachment and/or phagotrophy (McLaughlin 1958, Prescott 1968). Green, Hibberd and Pienaar (1982) found that the flagella range from 12-15 micrometers long and the flexible, non-coiling haptonema ranges from 3-5 micrometers long. These authors noticed that each cell has body scales of two types found in two layers with scales of the outer layer having narrow inflexed rims and those of the inner layer having wide, strongly inflexed rims. The scales are an important diagnostic feature invaluable in distinguishing *P. parvum* from closely related algal species, and the flagella-to-cell length ratio and the haptonema-to-cell length ratio are also important diagnostic features that aid in identifying this organism, especially when collected in mixed algal blooms (Chang and Ryan 1985).

In *P. parvum*, the nucleus is located centrally between two chloroplasts, one being lateral and the other parietal, that are usually yellow-green to olive in color (Green et al. 1982). Lee (1980) noted that a two-membrane chloroplast endoplasmic reticulum is present with the outer membrane of the chloroplast ER being continuous with the outer membrane of the nuclear envelope. The author also found a large Golgi apparatus located at the anterior end of the cell. This single polarized Golgi apparatus is always located between the bases of the two flagella and the nucleus (Bold and Wynne 1985). A contractile vacuole is also sometimes found at the anterior end of *P. parvum* cells (Lee

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1980). The reserve metabolite chrysolaminarin is found in posterior vesicles (Green et al. 1982, Lee 1980). Peripheral muciferous bodies and lipoidal globules may also be present, and the cysts formed by *P. parvum* have been reported as having an oval shape (Green et al. 1982).

Bold and Wynne (1985) described the microalga *P. parvum* as photosynthetic with possible heterotrophic growth (phagotrophy) when cells sink below the euphotic zone. They also found that it is a euryhaline and eurythermal organism tolerating a broad range of salinities and temperatures.

### **Global Occurrence of Fish Kills**

*Prymnesium parvum* was first identified as the culprit of mass fish mortalities in the brackish waters of Denmark and Holland (McLaughlin 1958, Shilo and Aschner 1953). According to records of these mortalities, thousands of pike, perch, roach, eels, bream, and tench were killed in 1938 in the Ketting Nor off the coast of Jutland, and again in 1939 in the Selso So located on a peninsula of Sjælland Island (Reichenbach-Klinke 1973). In 1947, Israel reported mass mortalities in carp ponds, and it has been a reoccurring problem (Shilo and Shilo 1953). *P. parvum* has been implicated in fish kills in Palestine, in rock pools of Scotland, Germany, Spain, Bulgaria and in South Africa as well (Comin and Ferrer 1978, Dietrich and Hesse 1990, Johnsen and Lein 1989, Linam et al. 1991, Rahat and Jahn 1965, Reichenbach-Klinke 1973).

Bales, Moss, Phillips, Irvine and Stansfield (1993) noted that well-documented accounts of multiple fish mortalities associated with *P. parvum* were recorded in the

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River Thurne system (Norfolk Broads, England) starting in 1969 and becoming less severe until 1975. They stated that large kills occurred in mid-August 1969 at Horsey Mere and Hickling Broad, in early September at Heigham Sound, Candle Dyke and the River Thurne, and another large kill occurred in April 1970 with smaller kills in 1973 and 1975. The authors believed that *P. parvum* was stimulated by gull-guano from the large number of black-headed gulls (*Larus ridibundus* L.) nesting in the area. They remarked that guanotrophication may have lead to an abundance of *P. parvum* due to supply of associated organic nutrients and noted that a decline in gull numbers was followed by a decline in *P. parvum* numbers. Anecdotal records described brown-colored water (possibly *P. parvum*) associated with fish kills in 1894, 1911, 1914, 1925 (kill comparable to the 1969 kill), 1934, 1954, 1966, and 1967 in this same area (Holdway et al. 1978).

In July-August 1989, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) died in aquaculture enclosures in the Sandsfjord system (southwest Norway) with fewer of the free-living fish in the brackish water fjord system affected (Kaartvedt et al. 1991). From 1989-1996, mixed blooms of *P. parvum* have occurred every summer in the Sandsfjord system (Larsen and Bryant 1998).

Hallegraeff (1992) noted that since the 1970's, *P. parvum* blooms have been related to recurrent fish kills in Vasse-Wonnerup estuary (W. A.) of Australia with kills most common in January-March. The author remarked that these fish kills, like those of

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the Sandsfjord system in Norway, show that wild fish stocks are less vulnerable to the *P. parvum* toxins than caged fish since they can swim away from toxic areas. Fish kills in Oued Mellah Reservoir in Morocco occurred in November-December 1998 and again in September-October 1999 (Sabour et al. 2000). Recurrent kills in carp ponds due to *P. parvum* in the People's Republic of China have also been reported since 1963 (Guo et al. 1996).

### **Occurrence of Fish Kills in Texas**

Mass fish mortalities have occurred in Texas in recent history. In October 1982 in the Brazos River Basin, an estimated 2,300 fish were killed in California Creek with the suspected killer being *Prymnesium parvum* (Glass et al. 1991). The first confirmed fish kill due to *P. parvum* occurred in October and November 1985 on the Pecos River with approximately 110,000 fish dying during this time in the stretch of river between Iraan, Pecos Co., to the mouth of Independence Creek (James and De La Cruz 1989, Rhodes and Hubbs 1992). Additional kills occurred in November and December of 1986 where an estimated 500,000 fish died in the same stretch of the Pecos River, and in November and December of 1988, a number of fish kills resulted in more than a million and a half fish dying in the reach of the Pecos River from Malaga, New Mexico to below the town of Imperial in Pecos County, Texas, as well as in the segment between Iraan and Sheffield (James and De La Cruz 1989, Rhodes and Hubbs 1992). In November of 1988, another 48,000 fish were killed in the Paint Creek tributary of the Brazos River, in Throckmorton and Haskell counties near Abilene, Texas (James and De L Cruz 1989). In

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April 1989, another fish kill on Paint Creek claimed another 15,000 fish, and an estimated 180,000 fish were also killed in August through October 1989 in a stretch of the Colorado River below Spence Reservoir (Glass et al. 1991).

According to a TPW news release, on January 11, 2001, *Prymnesium parvum* blooms were responsible for the death of approximately 175,000 fish in the Brazos River basin at Possum Kingdom Lake, 261,000 fish at Lake Grandbury since January 26, 2001, and this microalga has been implicated in recent kills at Lake Whitney (Cisneros 2001a and Cisneros 2001b). *P. parvum* has also wiped out the striped and hybrid bass production at Dundee State Fish Hatchery near Wichita Falls recently (Lightfoot 2001).

James and De La Cruz (1989) noted that, during the 1986 Pecos River fish kill, cell densities of 150 million cells per liter were recorded. They also reported that all species of fish in the fish kill areas were affected. The authors noted that some of the species of fish affected include *Cyprinus carpio*, *Etheostoma grahami*, *Gambusia affinis*, *Lepisosteus osseus*, *Micropterus salmoides*, and *Pylodictus olivaris*. They also remarked that the bivalves of the Unionidae family and the Asiatic clam, *Corbicula fluminea*, were also adversely affected. The authors discerned that *C. fluminea* was once common to the Pecos river with densities in the past as high as 100 per square foot, and that no live *C. fluminea* have been observed since the 1985 kill on the Pecos River. The authors remarked that this suggests a recent introduction of *Prymnesium parvum* to the Pecos River, and noted that results from recent data suggests that *P. parvum* is expanding its range in Texas.

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## Environmental Requirements

### *Salinity*

A study by McLaughlin (1958) showed that optimal NaCl concentrations for the growth of one Scottish and two Israeli strains *P. parvum* occurred at 0.3%-6% with growth possible at 0.1%-10%. Padilla (1970) observed that low salinities (less than 10%) increased the doubling time of *P. parvum* cells and induced high levels of protein and nucleic acid. Paster (1973) noted 0.3%-5% NaCl as optimal for growth of *P. parvum*. *P. parvum* germinated in the low-salinity environment (4-5%) of the fjord branch Hylsfjorden in the Sandsfjord system of southwest Norway (Kaartvedt et al. 1991). A 1993 study reported an optimal salinity range of 8-25% for a *P. parvum* strain from Denmark (Larsen et al. 1993). Larsen and Bryant (1998) reported that the Norwegian, Danish and English *P. parvum* strains they tested grew over a wide range of salinities each with different optimum growth concentrations, and that all three strains survived salinities from 3 to 30 psu (or .3%-3%). These researchers also speculated that discrepancies from earlier studies could have been due to the unknowing use of different strains of *P. parvum*. The water associated with the fish kill in Morocco was characterized by an elevated salinity of 8.6-12.4% (Sabour et al. 2000).

Dickson and Kirst (1987) speculated that the success of *P. parvum* in variable saline environments may be due to its ability to synthesize compatible solutes. In this 1987 study of osmotic adjustment in marine algae, the researchers found that *P. parvum* showed an increase in DMSP (a tertiary sulphonium compound: B-

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dimethylsulphoniopropionate), as compared to other algae in this study, and an increase in the synthesis of an unknown polyol. The authors suggested that the increasing synthesis of these two molecules may aid in osmoregulation. They concluded that the control of compatible solute synthesis by *P. parvum* may give this microalga an advantage in environments with fluctuating salinities.

### ***Temperature***

Shilo and Aschner (1953) observed that temperatures greater than 30 C were inhibitory to the growth of *P. parvum*, and 35 C resulted in lysis. The authors also discovered that *P. parvum* cells survive 2 C for many days. In the 1958 study by McLaughlin, it was found that the three strains of *P. parvum* tested (1 Scottish strain and 2 Israeli strains) showed erratic growth above 32 C with death occurring at 34 C. A separate study by Larsen, Eikrem and Paasche (1993) found that the Denmark strain of *P. parvum* used had a growth temperature optimum of 26 C. The authors noted that this same strain was found to be severely limited at 10 C. The Danish, Norwegian and English strains of *P. parvum* tested by Larsen and Bryant (1998) exhibited a maximum growth rate at 15 C with two of the strains (Norwegian and Danish) tolerating a wide temperature range of 5 C to 30 C. The authors noted that this finding supports the notion that *P. parvum* is a eurythermal organism. The *P. parvum* outbreak in Morocco was characterized by water with moderate temperatures between 15C-23.5C (Sabour et al. 2000).



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### ***pH***

McLaughlin (1958) found that the success of *P. parvum* growth below pH 7 depended on the adjustment of concentrations of metal ions. The author discovered that the metal ions Fe, Zn, Mo, Cu or Co, with an increase in the concentration of any of these, resulted in increased growth with the adjustment of Fe concentrations determined to be the most important. The author also noted that, for the three strains tested, growth below pH 5.8 was erratic, and all cells remained viable to pH 5. The *P. parvum* outbreak in Morocco occurred in water with a pH of 7.67-9.04 (Sabour et al. 2000).

### ***Light***

Wynne and Rhee (1988) noticed that, in *P. parvum*, the activity of alkaline phosphatase is higher at saturation light intensities. The authors also noted that an increase in light intensities allows *P. parvum* to increase the speed at which it is able to take up phosphate from its environment, and it therefore seems that changes in light intensities have a profound affect on competition. However, it has been found that excessive illumination inhibits the growth of *P. parvum* (Padan et al. 1967).

### ***Growth in the Dark***

Rahat and Jahn (1965) discovered that heterotrophic growth of *P. parvum* is possible in the dark with high concentrations of glycerol available. They noted that the optimal concentration of glycerol was found to be lower in the light than in the dark. Chisholm and Brand (1981) found that *P. parvum* divided primarily in the dark period (L:D 14:10), and that this division is phased (synchronized) by the light/dark cycle.

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Jochem (1999) tested the dark survival strategies of *P. parvum*, and determined that *P. parvum* was a Type II cell when exposed to prolonged darkness (in Type II cells, metabolic activity continues 'as usual' in the dark resulting in a decrease in cell abundance). The author found that the surviving cells needed new energy upon illumination to refill exhausted cellular reserves before the cells could divide, and would therefore not be advantageous in long or short dark periods.

### ***Phosphorous***

It is known that phosphate is limiting to phytoplankton growth in the summer (Larsen et al. 1993). McLaughlin (1958) determined that *P. parvum* is able to satisfy its phosphate requirement from a wide range of compounds. The author noted that the three strains of *P. parvum* were indifferent to high or low levels of inorganic phosphate, and speculated that this may be due to the presence of many phosphatases. This obligate phototroph was also found to graze bacteria, especially when phosphate is limiting, and it therefore seems that bacteria may be a source of phosphate for this microalga when phosphate is scarce (Nygaard and Tobiesen 1993).

### ***Nitrogen***

McLaughlin (1958) found that ammonia is a good source of nitrogen for *P. parvum* in the acid pH range. The author discovered that in acidic media, ammonium salts, the amino acids aspartic and glutamic acid, alanine, methionine, histidine, proline, glycine, tyrosine, serine, leucine, and isoleucine all can be utilized as a nitrogen source by this organism. In alkaline media, nitrate, creatine, asparagines, arginine, alanine,

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histidine, methionine and acetyl-urea were found by the author to be good sources of nitrogen. Syrett (1962) reported that *P. parvum* is not able to utilize urea as a nitrogen source. Methionine and ethionine can be utilized as sole nitrogen sources by *P. parvum*, and they are not inhibitory at high concentrations (Rahat and Reich 1963).

### ***Nutrients and Eutrophication***

Increases in the concentrations of nitrogen and phosphorous (and other nutrients) in water ultimately leads to eutrophication. The introduction of phosphorous to waterways may be from agricultural runoff (including fish ponds and aquaculture) and domestic sources. Nitrogen also comes from agriculture and is also introduced through airborne nitrogen precipitation from traffic emissions (Finnish Environmental Administration 2001). Holdway, Watson and Moss (1978) noted that there could be a relationship between the degree of eutrophication and population sizes of *P. parvum*. This is likely since eutrophication is known to cause an increase in phytoplankton and algae along with other aquatic plant life (Finnish Environmental Administration 2001).

Holdway, Watson and Moss (1978) noted that, in the Thurne system of Norfolk Broads, England, *P. parvum* competes poorly with *Chlorococcalean*, small cyanophyten and diatoms. They suggested that an increase of phosphorous and nitrogen may ease this competition and allow *P. parvum* to capture available nutrients more quickly. The authors noted that increases in fertility in the Hicking Broad-Horsey Mare-Heigham Sound area of River Thurne system caused eutrophication followed by heavy phytoplankton growth and a decrease in submerged macrophytes in these areas where *P.*

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*parvum* blooms occurred (Martham Broad, also in the Thurne system, was noted as having submerged macrophytes and also lower levels of *P. parvum* cells). They believed that the increase in nitrogen in the Thurne system was most probably from agriculture, and phosphorous-loading was likely from a large population of black-headed gulls. The authors remarked that a connection may be seen in the 1938 bloom of *P. parvum* in Ketting Nor, Denmark where it was noted that gulls were polluting the water causing it to turn turbid followed by the disappearance of macrophytes.

Kaartvedt, Johnsen, Aksnes and Lie (1991) noted that currents in the Hylsfjorden branch of the Sandsfjorden system, Norway, were weak which led to a long residence time of the brackish water in this fjord branch. The authors suggested that this resulted in low advective loss of *P. parvum*, relatively high temperatures and depletion in nitrogen and silica derived from freshwater with the low silicate levels favoring the proliferation of flagellates over diatoms. They speculated that low exchange rates and benthic settlement (*P. parvum* was found associated with the benthic green macroalgae *Cladophora* spp., and on nets of fish farms) of *P. parvum* could have facilitated an increased efficiency in the use of nutrients supplied by fish farms. They noted that the discharge of a hydroelectric power plant just after the first observed fish mortalities caused advection of *P. parvum* and its associated toxin throughout the Sandsfjord system. The authors suggested that subsequent large amounts of additional freshwater runoff from other sources aided in the dispersal of the algae, and may have also played a part in phosphorous limitation since the freshwater input contained low concentrations of

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phosphates. Overall, the authors concluded that fertilization associated with fish farming seems to have created a favorable environment for a *P. parvum* bloom in the Sandsfjord system. The association of *P. parvum* with *Cladophora* sp. and other macroalgae was tested by Johnsen and Lein (1989) with the conclusion that *P. parvum* is attracted to macroalgae (*P. parvum* grown in nutrient-poor solutions swam toward *Cladophora* sp.). The authors offered the possible explanation that *P. parvum* is chemotactic and may attach to macroalgae (via the haptonema) that give off dissolved organic matter when the concentrations of nutrients in the water are low. They also suggested that microalgae with the ability to utilize organic matter given off by macroalgae would have a definite advantage over other autotrophic algae.

In the Morocco *P. parvum* bloom, the water was high in organic matter, and characterized by elevated levels of total nitrogen, limited concentrations of nitrates and undetectable amounts of orthophosphates. This eutrophic, phosphorous-limiting environment is believed to have lead to the extensive fish mortalities (Sabour et al. 2000).

Wynne and Rhee (1988) found extracellular alkaline phosphatase activity to be highest in *P. parvum* when compared to the other species of algae tested. These authors discovered that phosphate uptake and enzyme activity increased with an increase in the N:P ratio, and concluded that this would give *P. parvum* a competitive advantage in phosphate-limited environments. They also determined that a decrease in phosphate

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concentrations were found to cause a disruption in the membrane synthesis of *P. parvum* that may lead to leakage of intercellular molecules including toxins.

### ***Vitamins***

Past studies indicate that vitamin B12 and thiamine are absolutely required for the growth of *Prymnesium parvum* (McLaughlin 1958, Shilo and Sarig 1989). Biotin was found not to be necessary for growth (McLaughlin 1958). Droop (1962) noted that thiamine is a component of the enzyme thiamine pyrophosphate (cocarboxylase), but no algae are known to be able to utilize the complete enzyme in place of thiamine as some bacteria do (the enzyme is probably less permeable). He found that *P. parvum* requires the pyrimidine component of thiamine, but does not require the thiazole component of thiamine. The author also noted that pyrimidine-requiring organisms require the molecule some 200 times more than vitamin B12. Another study indicates that with a given concentration of vitamin B12, growth in the light equals growth in the dark, and that this outcome may mean that B12 is not required for the immediate metabolism of the photosynthetic product (Rahat and Jahn 1965). Rahat and Reich (1963) discerned that a small portion of the B12 molecule is utilized in methyl metabolism for methionine or methyl group synthesis, and the rest of vitamin B12 (majority) used in other metabolic processes. They suggested that this may be why there is no sparing of B12 in the presence of methionine. The authors also found that some B12 analogs were found to inhibit the growth of *P. parvum*.

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## Toxin Characteristics

### *Structure*

The toxin of *Prymnesium parvum* has been found to be composed of a collection of substances and not a single component (Shilo and Sarig 1989). It was noted in one study that the *P. parvum* toxin was proteinaceous, acid-labile, thermostable, and non-dialyzable (Prescott 1968). Padilla (1970) noted the finding by Paster in 1968 that hemolysin, the hemolytic component of the *P. parvum* toxin, is a lipopolysaccharide. Padilla observed glycerol enhancement of hemolysin production, and suggested this shows that synthesis of hemolysin is dependent on carbohydrate and lipid metabolism. It was also implied by the author that hemolysin may be a structural component of *P. parvum* membranes; a notion supported by previous research that gave evidence that toxins of *P. parvum* are a heterogeneous mixture of phosphate-containing proteolipids. Dafni, Ulitzer and Shilo (1972) found a correlation between toxin formation (hemolysin) and presence of membrane vesicles. The authors noted that the observations of this study and past studies suggest that the *P. parvum* toxin appears in conditions where growth factors are limited and growth is disturbed. Because of this, they hypothesized that the toxin may be a product of imbalanced cell membrane metabolism. In one experiment, hemolysin was separated into six components with the major component, hemolysin I, determined to be a mixture of 1'-*O*-octadecatetraenoyl-3'-*O*-(6-*O*-*B*-*D*-galactopyranosyl-

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*B-D-galactopyranosyl)-glycerol and 1'-O-octadecapentaenoyl-3'-O-(6-O-B-D-galactopyranosyl-B-D-galactopyranosyl)-glycerol* (Kozakai et al. 1982).

Yariv and Hestrin (1961) noted that the *P. parvum* toxin, prymnesin, was soluble in methanol and n-propanol water solvent systems thereby distinguishing the toxin from simple protein and polysaccharides. They ascertained that prymnesin was a lipid with both non-polar and polar moieties. The authors recognized that the observed properties of prymnesin (ichthyotoxicity, hemolytic activity, non-dialysability against water, general solubility features, formation of insoluble inactive complexes with certain alcohols, and capacity to precipitate by Mg hydroxide and ammonium sulphate) were similar to the properties of saponins. However, they also noted that the action of prymnesin requires a cofactor whereas saponins do not require a cofactor. Paster (1973) described prymnesin as a high molecular weight glycolipid with a detergent-like structure. It has also been hypothesized that *P. parvum* toxins are plastid components or that toxin synthesis is partially plastid mediated (Guillard and Keller 1984). The hypothesis that the toxin, a proteophospholipid, is a membrane precursor is supported by the fact that there is a 10-to 20-fold increase in the toxins (ichthyotoxin, hemolysin and cytotoxin) when phosphate is limiting (Shilo and Sarig 1989).

Igarashi, Satake and Yasumoto (1999) have recently reported the structural elucidation (Figure 1) of the *P. parvum* toxin. They found that *P. parvum* produces two glycosidic toxins they named prymnesin-1 (C<sub>107</sub>H<sub>154</sub>Cl<sub>3</sub>NO<sub>44</sub>) and prymnesin-2 (C<sub>96</sub>H<sub>136</sub>Cl<sub>3</sub>NO<sub>35</sub>). The authors also concluded that prymnesin-1 and prymnesin-2 have biological activities that are almost the same. They noted that both prymnesin-1 and



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prymnesin-2 express potent hemolytic activity greater than that of a Merck plant saponin, and that both exhibit ichthyotoxicity.

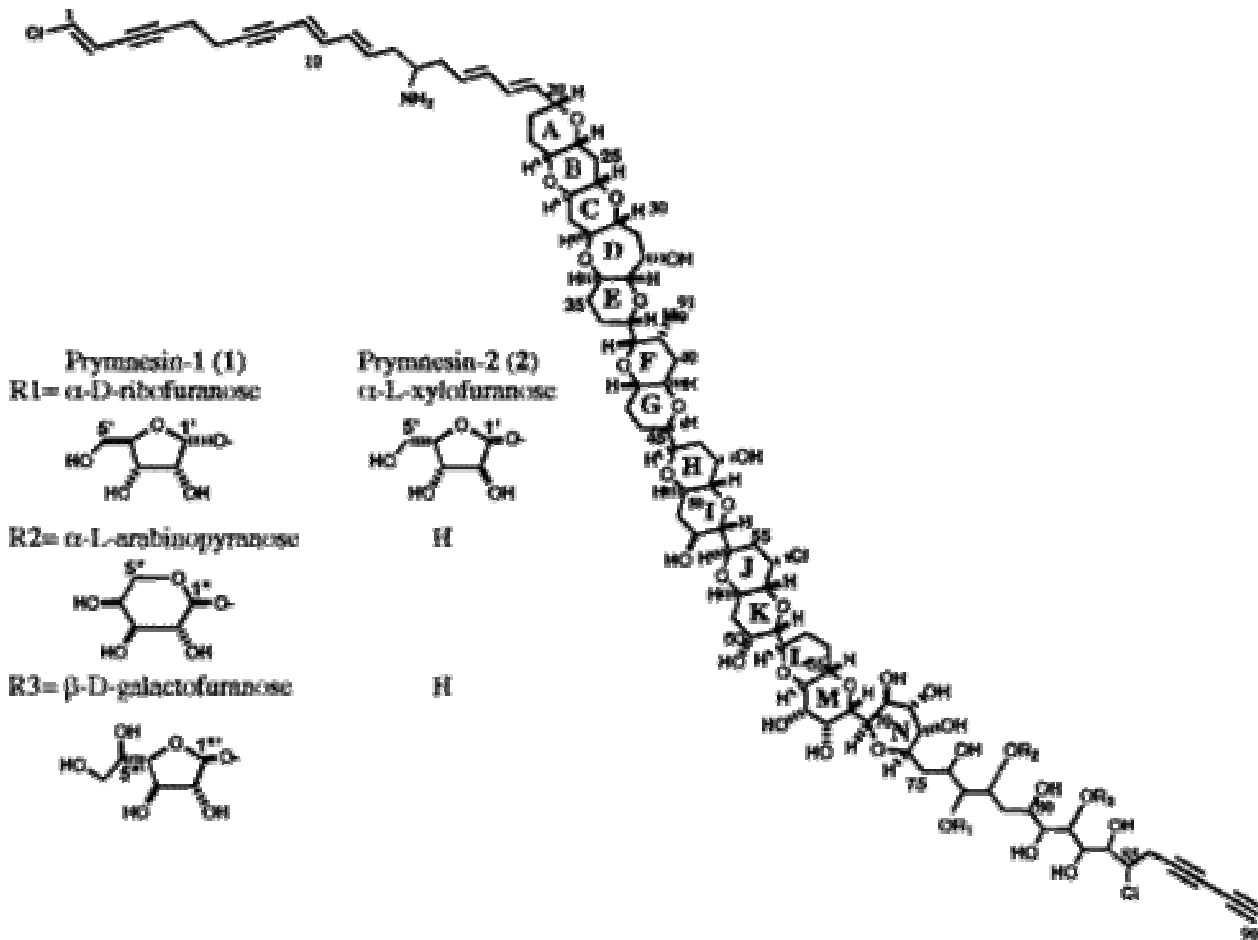
### ***Target and Action of Toxin***

*P. parvum* produces soluble toxic principles: an ichthyotoxin, hemolysin and cytotoxin (Ulitzer and Shilo 1964). The *P. parvum* ichthyotoxin is toxic to gill-breathing species such as fish, mollusks, arthropods, and to the gill-breathing stage of amphibians (Paster 1973). The ichthyotoxin targets the permeability mechanism of the gill (Yariv and Hestrin 1961). Ulitzer and Shilo (1966) noted that toxicity occurs in two stages. The first stage is reversible damage to the gill tissues (i.e. permeability) that occurs only with a cation synergist and suitable pH. They described the second stage as mortality due to a response to toxicants already present in the water including the *P. parvum* toxin itself.

Dissolved potassium and calcium are necessary for developing extracellular micelles important for toxicity (Glass et al. 1991). The ichthyotoxin (now in micelles) requires activation by cofactors such as calcium, magnesium, streptomycin and sodium. (Shilo and Sarig 1989, Yariv and Hestrin 1961). Ulitzer and Shilo (1964) observed that neomycin, spermine and other polyamines can activate ichthyotoxicity with spermine being the most active. They concluded that, in the presence of more than one cofactor, the resulting toxicity was not always additive. Instead, the authors found that the toxicity depends on the specific activity of each cofactor present and their relative concentrations.

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They also observed that calcium has the ability to mask other cofactors. The authors found that the presence of calcium (low activity) in the presence of a cofactor that



**Figure 1.** Pymnesin-1 (1) and Pymnesin-2 (2) structure. Relative stereochemistry is shown for the rings A-N of pymnesin-1 and pymnesin-2 (From Igarashi et al. 1999).

normally expresses high toxin activity will cause the activity of the toxin to decrease.

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Ulitzer and Shilo (1966) discovered that the *P. parvum* ichthyotoxin was also augmented by the cation DADPA (3,3-diaminodipropylamine) in lab with the ichthyotoxin increasing the sensitivity of *Gambusia* to toxicants already present in the media. Padilla and Martin (1973) noted that calcium and streptomycin have proven to be slightly synergistic, neomycin slightly more synergistic, spermine induces a four-fold increase in ichthyotoxicity, and DADPA induces a two-fold increase. They also speculated that the toxin/cation complex could interact with charged groups on toxin molecules reducing the degree of ionization and making them more reactive with the membrane.

Paster (1973) noted that the attachment of prymnesin to gill cell membranes most likely occurs where molecules such as lecithin and cholesterol are found, and attachment imposes a rearrangement on the membrane making it more permeable. The fact that prymnesin interacts with cholesterol in attack of erythrocyte membranes may support this idea (Padilla and Martin 1973). It has also been speculated that the cofactors may alter the permeability of the gills, thereby increasing the rate of absorption of the toxin into the circulation (Spiegelstein et al. 1969). Increased permeability of the gill membrane imposed by prymnesin causes fish to become more susceptible to compounds in water like CaCl and streptomycin sulphate (Yariv and Hestrin 1961). The increased permeability of the gills may even cause an increased susceptibility to the toxin's cytotoxic and hemolytic activity (Ulitzer and Shilo 1966). Spiegelstein, Reich and

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Bergmann (1969) used two methods to observe the effects of the ichthyotoxin on *Gambusia*. They found that in the immersion method (fish in an ichthyotoxin solution), the toxicity occurs as follows: the toxin enters the gills (via capillaries), enters the dorsal aortas, and then travels to the brain. The authors noted that in the intraperitoneal injection method, the toxin first enters the circulation where it travels to the liver, then enters the hepatic vein, the heart, the aorta and finally the brain. They recalled that the toxin is acid-labile and suggested that it may be altered (inactivated) in the GI tract and liver. The authors suggested that this could be why the toxin is non-toxic to non-gill breathers, but toxic to gill breathers.

### ***Accumulation***

It has been reported that the ichthyotoxin accumulates during the stationary phase of growth, and the hemolytic toxin accumulates during log phase (Padilla 1970). Simonsen and Moestrup (1997) determined that the hemolytic compounds within the *P. parvum* cells are the highest in late exponential growth phase and decreased during stationary phase. The authors then discovered hemolytic activity in the medium during stationary phase.

### ***Population Density***

Shilo and Aschner (1953) discovered that fish peptone and egg yolk increases the density of *P. parvum* cultures. The authors ruled out the idea of toxigenic variants due to an observed decrease in toxicity accompanied by an increase in cell proliferation. They often observed an inverse relationship between cell count and toxicity. Shilo (1967) also

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found a lack of correlation between toxicity and cell density. The author hypothesized that the ability to form toxins may be determined by genetic factors. The author based this hypothesis on the knowledge that different strains of *Microcystis aeruginosa* and *Anabena flos-aquae* were shown to differ markedly in their toxin productivity. The author also found non-toxigenic strains of this alga.

### ***Salinity***

Reich and Rotberg claimed that the activity of the ichthyotoxin of *P. parvum* is inversely proportional to salt concentrations (Reich and Parnas 1962). Ulitzer and Shilo (1964) also found that a decrease in salinity equals an increase in ichthyotoxicity, and that ichthyotoxicity decreases as salinity increases. In a later study, increased salinity decreased the uptake of trypan blue (i.e. toxicity) in the gills of fish (Ulitzer and Shilo 1966). Paster (1973) observed that a NaCl range of 0.3%-5% was optimal for toxin production in *P. parvum*. However, Larsen and Bryant (1998) noted that variable salinity did not have significant effects on toxicity.

### ***Temperature***

Shilo and Ashner (1953) found that at 80 C and 97 C toxicity declined rapidly while 62 C caused a slow decline in toxicity. They also observed that at room temperature and 4 C there was no decrease in toxicity. Yariv and Hestrin (1961) noted that prymnesin solutions in water showed a decrease in titer when kept at 35 C for 60 minutes, but returning the solution to a pH of 4 restored toxin activity. Paster also discerned the thermo-sensitivity of the toxin in 1968 (Stabell et al. 1993). Ulitzer and

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Shilo (1964) noted that an increase in temperature in the range of 10 C to 30 C caused an increase in the rate of mortality of minnows with the titer of the toxin unaffected.

However, Larsen and Bryant (1998) concluded that variable temperatures in the range of 5 C to 30 C do not have significant effects on toxicity thereby contradicting the research by Ulitzer and Shilo in 1964.

### ***Light***

Shilo and Aschner (1953) concluded that light augments toxin production. These authors found water containing *P. parvum* to be more toxic in light than in dark. Parnas, Reich and Bergmann (1962) found that the *P. parvum* toxin is sensitive to light. The experiments conducted by these authors revealed that UV causes 100% inactivation of the toxin with the upper limit of inactivation by visible light at 520 nm (50% inactivation). Reich and Parnas (1962) noted that, in their first experiment, ichthyotoxicity decreased gradually with exposure to light. The authors also found that, in the dark, toxicity rises reaching a maximum in 7.5 hours. In the second experiment, the observed similar results: toxicity increased in the dark accompanied by a pH drop in dark to between 7.0 and 7.1. The pH rose in the light to 8.0-8.1 due to, the researchers speculated, photosynthetic activity. They hypothesized that the desistance of ichthyotoxicity was either due to inactivation by light or to the delay of toxin formation due to increased photosynthesis in light.

Rahat and Jahn (1965) observed that dark cultures in their study were more toxic, even with less cells, than light cultures (this agrees with idea that the extracellular toxin is

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inactivated by light). The authors also concluded that light is not needed to make the *P. parvum* toxin, and that previous assays of the toxin are only the net result of toxin production and inactivation. A study by Padan, Ginzburg and Shilo (1967) showed that the ichthyotoxin and hemolysin are both sensitive to inactivation by light. The authors also concluded that light is needed for the appearance of extracellular hemolysin. They noted that the equilibrium between the appearance of hemolytic activity and inactivation appears to be in favor of toxin accumulation in low light (60 foot candles). Spiegelstein, Reich and Bergmann (1969) determined that the ichthyotoxin is produced in the dark equally well as in the light (light not necessary). Paster (1973) observed inactivation of prymnesin by visible light (400nm-510nm) and UV light (225nm). The toxin of the closely related, flagellated algae, *Phaeocystis pouchetii*, is also believed to be photosensitive (Stabell et al. 1999). However, Larsen and Bryant (1998) have concluded that variable salinity, light and temperature do not have significant effects on toxicity. These authors believe that growth phase and nutrient status probably have a greater impact.

### ***pH***

Shilo and Aschner (1953) deduced that *P. parvum* toxicity was independent of pH in the range of 7.5-9.0; toxicity decreased rapidly at pH less than 7.5 and was completely inactive at 6.0. The authors hypothesized that the inactivating effect was due to the hydrogen cation. McLaughlin (1958) concluded that toxicity decreased at pH 6.0-6.5 (or become non-toxic), and toxicity returns when the solution is brought back to a neutral pH. The author also found that acid grown cultures are less toxic than alkaline

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grown cultures. Ulitzer and Shilo(1964) noted that there is a correlation between elevated pH and toxicity. Ulitzer and Shilo (1966) observed that the gills of *Gambusia* became darkly stained at pH 9, but that no trypan blue staining (i.e. toxicity) was observed at a pH of 7. Padilla (1970) noted an increase in pH caused a decrease in hemolysis (pH range 5.5-8.0). Padilla and Martin (1973) noticed that maximum hemolytic activity occurred at pH 5.5. The authors also found that cytotoxicity is arrested by pH 6.4, and that maximum binding of the toxin was observed in the pH range of 4.6-5.5.

Shilo and Sarig (1989) found that a pH higher than 8 was necessary for cation activation, and a pH of 7 and lower equals little ichthyotoxic activity with the toxicity increasing to a pH of 9. They also determined that, when a cation is complexed with the toxin at high pH, the ichthyotoxicity is expressed even at low pH (6-7).

### ***Phosphate and Nitrogen***

Dafni, Ulitzer and Shilo (1972) found that a decrease in phosphate caused an increase in toxicity. The authors speculated that a phosphate-limiting environment could cause a disturbance in the formation of membrane phospholipids that may lead to leakiness (and the toxin escaping). They noted that the cell volume of *P. parvum* increased as the concentration of phosphate decreased, and it was hypothesized that swelling was due to osmotic imbalance (leakiness) or disturbance in regular cell division. Paster (1973) also found *P. parvum* to be more toxic in phosphate-poor media.

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Holdway, Watson and Moss (1978) noted that, with substantial concentrations of nitrogen and phosphorous, *P. parvum* will not produce or release toxins. The fish kills in the Sandsfjord system in Norway were determined to be mostly due to phosphorous-limited growth of *P. parvum*; the phosphate-limited environment was considered to be the major factor influencing increased toxicity (Kaartvedt et al. 1991). Larsen, Eikrem and Paasche (1993) found that phosphate-limitation caused an increase in toxicity of a Denmark strain of *P. parvum* in lab. Simonsen and Moestrup (1997) observed an increase in the size of *P. parvum*, *Chrysochromulina polylepsis*, *Chrysochromulina hirta*, and *Isochrysis* spp. cells accompanied by increased toxicity when phosphate was limited. They also noted that the dinoflagellate *Alexandrium tamarense* has been noted to show increased toxicity with a decrease in phosphate concentrations. Johansson and Graneli (1999) discerned that nitrogen limitation causes an increase in toxicity, and also found that phosphate limitation causes increased toxicity as well. The authors hypothesized that the N:P ratio could be the governing factor of toxicity in *P. parvum*, and that a change in the N:P ratio by nutrient inputs could lead to toxicity (an unbalanced N:P ratio could result from eutrophication). The authors admitted that the reason for toxin production by *P. parvum* is uncertain, but speculated that the toxin could be produced because of the need to wipeout competition during nutrient limitation. Wynne and Rhee (1986) concluded that changes in the light regime can alter the optimum cellular N:P ratio in *P. parvum* thereby greatly influencing nutrient requirements and species interrelationships.

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### ***Glycerol***

Glycerol was found to increase the growth rate and toxin synthesis in *P. parvum* (Padilla 1970). Cheng and Antia (1970) found that *P. parvum* is able to metabolize glycerol in high and low concentrations. The authors implied that glycerol pollution may stimulate *P. parvum* thereby causing blooms in light as well as in the absence of light. They found that *P. parvum* responded rapidly to high glycerol concentrations, and appeared to become 'spent out' with rapid cell lysis following an early growth peak.

### ***Toxin Inhibitors***

Padilla (1970) noticed that hemolysis is inhibited by high pHs with a maximum toxicity at pH 5.5, 50% at pH 7, and 10% at pH 8. Paster (1973) found that lecithin, cholesterol and cephalin inhibit the hemolytic affect in small quantities, and concluded that these lipid compounds must compete with the toxin for the target site. The author also noted that the bacteria *Proteus vulgaris* and *Bacillus subtilis* decrease the potency of *P. parvum* cultures. Padilla and Martin (1973) inferred that cholesterol, cephalin and the *Gymnodinium breve* toxin exert a protective influence. It has also been observed that NaCl inhibits *P. parvum* toxin activity (Shilo and Sarig 1989).

### **Successful and Possible Control Methods**

Moshe Shilo and Miriam Shilo (1953) noted that ammonium sulphate has a lytic effect on *P. parvum*. They found lytic activity to be a function of temperature in the range of 2 C-30 C with lytic activity increasing as temperature increases. The authors also found that lytic activity decreases dramatically when temperatures below 10 C are reached. The authors also discovered that the lytic activity of ammonium sulphate was a

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function of pH in the pH range of 6.5-9.5 with activity increasing as pH increases. They suggested that this shows that free ammonia, not the ammonium ion, is responsible for lysis. The addition of ammonia to water for control of *P. parvum* is also effective (Glass et al. 1991). The addition of ammonium sulphate or ammonia to contaminated water controls *P. parvum* in the following manner: trapping and concentration of the protonated ammonia ion in the *P. parvum* cell due to a pH difference between the inside and outside of the cell is followed by the entry of water, swelling and lysis (Shilo and Sarig 1989).

Unslaked lime (CaO) was found to reduce the amount of ammonium sulphate or ammonia needed for complete lysis by a factor of three; unslaked lime markedly enhances the effectiveness of ammonium sulphate and ammonia because it increases the pH of water when added (Shilo and Shilo 1953). However, ammonia and ammonium sulphate are counteracted by an increase in NaCl concentrations (McLaughlin 1958). Removal of fish from contaminated water and then placing the fish in non-contaminated water was found to reverse the gill permeability effect of the toxin (Glass et al. 1991).

Shilo and Aschner (1953) found that oxygen and air decrease toxicity when bubbled through a solution of the toxin. The authors also discerned that potassium permanganate and sodium hypochloride destroy toxicity. They also noted that adsorbents such as kaolin, Norit A (acid washed), activated charcoal, calcium sulphate and pond-bottom soils have also been shown to detoxify cultures of *P. parvum*. In addition, the authors observed that the bacteria *Proteus vulgaris* and *Bacillus subtilis* decreased the

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toxicity in cultures by 50% in one hour. Paster (1973) also revealed that *Proteus vulgaris* and *Bacillus subtilis* decreased toxicity of *P. parvum* cultures. Simonsen and Moestrup (1997) speculated that the *C. polylepsis* toxin decomposition in dark may be explained by bacterial activity, and the same may be true for the *P. parvum* toxin.

In Palestine, a 1:100,000 of copper sulphate was used to successfully control *P. parvum* (Reichenbach-Klinke 1973). Introduction of acetic acid and other weak electrolytes is reported to cause *P. parvum* cells to lyse (Glass et al. 1991). McLaughlin (1958) noted that organic algicides or lowering the pH decreases toxicity. Glass, Linam and Ralph (1991) noted that a pH less than 6 and greater than 9 reportedly inactivates the toxin. The authors added that increasing NaCl concentrations decreases toxicity probably by replacing a cofactor (Ca<sup>++</sup> and/or Mg<sup>++</sup>) needed to activate the toxin. They also noted that UV and strong visible light have also been found to destroy prymnesin in lab. Nygaard and Tobiesen (1993) noted that *P. parvum* grazes bacteria when phosphate is limited. These authors believe that *P. parvum* utilizes certain species of bacteria when nutrients are limited. They suggested that the presence of these bacteria could decrease toxicity. Wynne and Rhee (1988) noted that detecting the activity of phosphatase in the water could be used to determine if the environment is limited in phosphate concentrations, and that this could be used to predict toxic blooms of *P. parvum*.

### **Conclusion and Areas of Further Research**

The biological and ecological significance of the synthesis and release of the *Prymnesium parvum* toxin is not clear. However, it is now known that this microalga

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produces two glycosidic toxins, prymnesin-1 and prymnesin-2, collectively called prymnesin, and that the two similar toxins have both hemolytic and ichthyotoxic activity (Igarashi et al. 1999). Does prymnesin have negative effects on the competitors of *P. parvum* that would lend an advantage to the growth and success of this flagellate? It has been proposed that a critical concentration of a “growth-initiating factor” is required to start division in this species and yield blooms, but this factor (if it exists) has not been described and is an area of additional research (Glass et al. 1991). If a “growth-initiating factor” is discovered, it could lead to an effective means of controlling *P. parvum*. The targeting of alkaline phosphates for the control of this microalga is another area that will require additional research if it is indeed possible. The synthesis of DMSP and the unknown polyol believed to aid in the osmoregulation of *P. parvum* needs to be studied further. This, too, may lead to an effective control of this microalga.

Additional research is needed to determine which types of bacteria cause a decrease in *P. parvum* toxicity (Nygaard and Tobiesen 1993). These bacteria could be potential biological control agents, and prove to be more practical in the control of *P. parvum* blooms that cover large areas in sensitive aquatic environments.

It seems that the most important factor governing the toxicity of *P. parvum* blooms is the relative amounts of nitrogen and phosphorous found in the water, and that limitation of both of these nutrients seems to cause an increase in toxicity (Johansson and Graneli 1999). Additional research must be conducted in this area to determine if an unbalanced N:P ratio indeed leads to increased toxicity. If the N:P ratio proves to be the

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most important factor, research must be conducted to determine how the optimum N:P ratio for *P. parvum* can be restored. The sources for the imbalance must also be studied since it seems that nitrogen and phosphorous inputs from agriculture, aquaculture and other sources may be involved. *P. parvum* has been found to graze bacteria as a source of phosphate (Nygaard and Tobiesen 1993). More research in this area may lead to ways of treating *P. parvum* blooms in phosphate-limited environments by supplying this microalga with bacteria it is known to utilize as a source of phosphate.

Glycerol has been found to enhance the growth of *P. parvum* (Cheng and Antia 1970). Are there any sources of glycerol pollution in the areas where fish kills occurred in Texas? The investigation of future fish kills should include the detection of glycerol in the aquatic environment since this may cause *P. parvum* blooms.

The importance of the microscopic algae *Prymnesium parvum* can be seen in the millions of fish killed across the globe, and the ensuing economic losses it creates. The recent appearance of *P. parvum* in Texas, and the recurring fish kills caused by the toxins released, is a cause for concern. This problem is one that must be addressed soon for history has shown us that *P. parvum* is ever present once the organism first appears. Steps must be taken to further understand the ecology of this organism, its toxin and causes of toxic blooms in an attempt to decrease the number of occurrences we see in the future.

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