PREVALENCE OF *Kudoa thyrsites* IN PACIFIC HAKE (*Merluccius productus*) AND THERMAL RESISTANCE OF *Kudoa thyrsites* AND *Kudoa paniformis* SPORES

by

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Abstract

Experiments were conducted to determine the potential of Pacific hake (*Merluccius productus*) fish meal to act as a vector of *Kudoa thyrsites* transmission to farmed Atlantic salmon (*Salmo salar*). This was done by determining the prevalence and intensity of *K. thyrsites* in Pacific hake destined for fish meal processing, developing a viability test for *K. thyrsites*, and determining the thermal resistance of *K. thyrsites*. Thermal resistance experiments were also performed for *K. paniformis*. Pacific hake samples were collected from the rendering plant in 2001 and 2002. The prevalence of *K. thyrsites* in the 2001 samples was 78.0%. Of these infections, 36.4% were light and 63.6% were moderate. The 2002 samples had a *K. thyrsites* prevalence of 80.1% infection, with 24.3% being light and 75.7% being moderate. Dyes used to test viability of other myxosporean parasites were examined for their suitability for *K. thyrsites* and *K. paniformis*. Methylene blue, fluorescein diacetate, and propidium iodide were suitable as a potential indicator of *K. thyrsites* viability. Only methylene blue was tested for *K.* paniformis, and it was found to be suitable. The decimal reduction times (D-value) of *K. paniformis* and *K. thyrsites* were determined using methylene blue as a viability test. The average D-value of *K. paniformis* in phosphate buffered saline (pH 7.0) was 18 hours at 53°C, while at 62°C and 69°C the average D-value was 4.5 hours and 0.80 hours, respectively. The z-value was 12C°. The average D-value for *K. thyrsites* at 43°C was 3.0 hours, and at 52°C and 60°C the average D-value was 1.4 hours and 0.030 hours, respectively. The z-value for *K. thyrsites* was 8.9C°. Given the available data, the likelihood of *K. thyrsites* surviving both fish meal and fish feed manufacturing are very low.

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1.0 Introduction

Kudoa thyrsites is a myxosporean parasite that has been problematic on salmon farms in British Columbia due to their association with softening effect on muscle tissue of Atlantic salmon (*Salmo salar*) (Whitaker and Kent, 1991; Whitaker et al., 1994). Wild fish species can also be infected with *K. thyrsites*. Some of these hosts include Pacific halibut (*Hippoglossus stenolepis*), lingcod (*Ophiodon elongates*) and Pacific hake (*Merluccius productus*) (Moran et al., 1999b).

Pacific hake is used to manufacture fish meal, which is subsequently used in the production of fish feed for farmed salmon. While it is known that Pacific hake can be infected with *K. thyrsites*, there are no current studies stating the level of infections. Therefore, Pacific hake that will be used for fish meal processing should be examined for the prevalence and intensity of *K. thyrsites*. In addition, it is unclear whether fish meal processing will inactivate *K. thyrsites* spores. Though the transmission of *K. thyrsites* is thought to be indirect, the life cycle has not yet been discovered. Therefore, there may be potential for fish meal to act as a vector for parasite transmission. In effect, studies need to be undertaken to determine the possibility of *K. thyrsites* surviving fish meal manufacturing.

Currently, there is no research available indicating whether temperatures and times used in the manufacturing of hake fish meal will eliminate *K. thyrsites* spores. At the same time, if the spores are not destroyed, fish feed manufacturers and salmon farmers fear that the fish meal may have the potential to act as a source of *K.*

thyrsites spores that may later infect farmed Atlantic salmon. If fish feed manufacturers could use Pacific hake fish meal for processing fish feed instead of importing fish meal from South America, it is estimated that fish feed manufacturers could save $1 million dollars per year (Mann, 2000).

Therefore, it was hypothesized that Pacific hake would be infected with *K. thyrsites* and that elevated temperatures would render *K. thyrsites* and *K. paniformis* nonviable. Thus, fish meal would not act as a vector for *K. thyrsites* transmission.

The main objective of the current study was to determine the possibility of fish meal to act as a vector for *K. thyrsites* transmission. To meet these objectives, several areas were explored. First, the prevalence and intensity of *K. thyrsites* infections in Pacific hake destined for fish meal processing was determined. Second, a viability test was developed for *K. thyrsites* spores, since as of yet, *K. thyrsites* remains unculturable. Third, the resistance of *K. thyrsites* spores to inactivation by high temperatures was determined. In addition, the heat resistance of *K. paniformis*, also found in Pacific hake, was determined. Fish oil was also examined for its potential to harbour *K. thyrsites*.

2.0 Literature Review

2.1 Myxosporea and *Kudoa*

There are over 1250 described species of parasitic organisms from the class Myxosporea (phylum Myxozoa). The majority of these organisms occur in freshwater, brackish and marine fishes, infecting the tissues and organ cavities of their hosts (Lom and Dykova, 1995). Myxosporea are divided into two orders: Bivalvulida, which have spores with two shell valves and one to four polar capsules, and Multivalvulida, which have spores with three to seven shell valves and one to seven polar capsules (Lom and Dykova, 1995). The family Kudoidae falls under the order Multivalvulida and contains the single genus *Kudoa* (Moran et al., 1999b).

Kudoa are marine myxosporean parasites that greatly impact product quality and decrease marketability of certain wild and farmed fishes. The genus is comprised of 44 described species distributed worldwide (Moran et al., 1999b). Most are histozoic parasites of the musculature causing soft flesh, possibly by secretion of an enzyme upon death of the host, and can also cause unsightly cysts within the flesh of fish making them aesthetically unacceptable (Tsuyuki et al., 1982; Moran et al., 1999b).

The species of concern on the west coast of British Columbia (BC) include *K. paniformis* and *K. thyrsites*. *K. paniformis* has only been found to infect Pacific hake (*Merluccius productus*). On the other hand, *K. thyrsites* has a broad host range that includes Pacific hake (*Merluccius productus*), Atlantic salmon (*Salmo salar*), Pacific salmon (*Onchorhynchus* spp.), lingcod (*Ophiodon elongates*) and Pacific halibut

(*Hippoglossus stenolepis*) (Moran et al., 1999a). Furthermore, *K. thyrsites* has a wide geographical distribution, found in areas such as Australia, South Africa, Chile, Ireland, and Japan (Whipps, 2002).

K. thyrsites spores are stellate in shape, consisting of 4 valves of unequal sizes. In lateral view, the spores appear subconical with the posterior corners of the valves forming sharp processes. Each valve contains a polar capsule that is pyriform and also unequal in size. One polar capsule is distinctly larger than the other three. In addition, each capsule contains a polar filament with 3.5 coils. The average width of *K. thyrsites* spores is 12.73 μm (Kabata and Whitaker, 1981; Kabata and Whitaker, 1985).

K. paniformis, on the other hand, is more rounded in shape "resembling loaves of bread depicted in ancient Roman paintings" (Kabata and Whitaker, 1981). Like *K. thyrsites*, four valves are present, each with a polar capsule that is pyriform in shape. The average width of *K. paniformis* spores is 5.90 μm (Kabata and Whitaker, 1981).

The full life cycle of *K. thyrsites* or *K. paniformis* has not been completely determined. Several myxosporean parasites have been shown to have or are suspected of having an intermediate host rather than being directly transmitted from fish to fish; however, it is possible that some marine myxosporeans have direct transmission without the need for an alternate host. In the case of indirect life cycles, oligochaetes have commonly been found to be the intermediate host (Kent et

al., 1994). The spore stage is referred to as the myxospore, while the stage that is found in the intermediate host is termed actinospore.

Needham (1994) suggested that Atlantic salmon smolts are probably infected with *K. thyrsites* by eating an intermediate host of *Kuoda*, but could be infected by other means, such as by krill added to smolt feeds and by "low temperature" herring meal used in manufacturing salmon feed. Recently, Moran et al. (1999a) investigated routes of *K. thyrsites* transmission to Atlantic salmon. This study represented one of the first experimental transmissions of a marine myxosporean parasite. Direct parasite transmissions from fish to fish by means of intubation were unsuccessful. The parasite was transmitted, however, by means of an injection of Coho salmon blood infected with *K. thyrsites* via the intraperitoneal route to Atlantic salmon. The most effective means of transmission was by natural exposure in seawater net-pens to infective stages of *K. thyrsites* within the environment. It was also observed that *K. thyrsites* could successfully sporulate in Atlantic salmon returned to freshwater after exposure to seawater.

Diamant (1997), on the other hand, was able to experimentally demonstrate fish to fish transmission of the marine myxosporean *Myxidium leei* in sea bream (*Sparus aurata*) using specific pathogen-free fish. He suggested that *M. leei* is transmitted between fish by ingestion of excreta from infected fish. Moran et al. (1999a) argued, however, that there is a possibility that *M. leei* was transmitted by vegetative

spores rather than myxospores since this particular parasite has many stages that may be released in fecal casts shed from infected fish.

2.2 *Kudoa* and Pacific Hake

Pacific hake (*Merluccius productus*) is commonly infected by the *Kudoa* parasite, namely *K. thyrsites* and *K. paniformis*. In a study by Tsuyuki et al. (1982), nearly 90% of Pacific hake (n=322) were infected with *Kudoa*; however, infections were not uniform, with many fish having only light infections. Furthermore, Tsuyuki et al. (1982) found that infections were generally heaviest in the anterior portion of the fillet and diminished towards the posterior end. Mixed infections were discovered in 18.3% of the Pacific hake, while 32.3% were infected with just *K. thyrsites* and 38.8% were infected with only *K. paniformis*. Interestingly, it was discovered that the Strait of Georgia hake were only infected with *K. thyrsites*; however, the offshore Pacific hake were infected with both *Kudoa* species. Whitaker et al. (1994) also noted that almost 100% of some catches of Pacific hake could be infected. These infections diminish the value of Pacific hake for further processing due to flesh softness and rapid postmortem deterioration, which is considered largely unacceptable to the industry (Kabata and Whitaker, 1986). Infections by both species form pseudocysts within the muscle fibres, appearing as white or black streaks running through the musculature, which adversely affect consumer acceptance and lower the market value (Whitaker and Kabata, 1987); however, *K. paniformis* is the species primarily responsible for soft flesh in Pacific hake (Tsuyuki et al., 1982).

2.3 Fish Meal in Farmed Salmon Diet

Pacific hake is used in fish meal production. Fish meal is the primary protein source and the largest cost experienced by fish feed manufacturers (IFFO, 1990). To manufacture a high quality fish meal, the temperature and time of heating must be controlled to ensure amino acids and vitamins are not adversely affected (March, 1998). Therefore, raw material should undergo the gentlest possible processing parameters. Studies have shown that meals prepared at less than 90°C have higher digestibility and support growth better for salmonids (IFFO, 1990).

The basic processes used in the manufacturing of fish meal include cooking, pressing, drying and grinding. The cooking process coagulates the protein and frees the oil and water within the fish. The subsequent pressing separates the solid matter from the liquids, which is a mixture of oil, water, vitamins, minerals, and the remaining solids composed of dissolved and suspended proteins. The solid matter is mainly proteins and bones. Following pressing, the liquid portion is centrifuged, which separates the oil from the stick water. The stick water is then added back to the solid matter where the complete mixture undergoes drying. Following drying, the meal is ground into the desired particle size. The separated oil is polished to extract any remaining impurities (FAO, Fishery Industries Division, 1986).

2.4 *Kudoa* and Atlantic Salmon

In BC, Atlantic salmon (*Salmo salar*) accounted for 81% of farmed salmon produced in 2000 (BC Salmon Farmers Association, 2002). Like Pacific hake, Atlantic salmon

are susceptible to *K. thyrsites* infections (Whitaker and Kent, 1991; Whitaker et al., 1994). Atlantic salmon, however, have not been found to be a host for *K. paniformis*. In November 1993, at a BC Salmon Farmers Association research and development (R&D) meeting, the *Kudoa* issue ranked in the top 10 of R&D priorities. Some members felt that it should have received higher priority, however, because of its potential negative economic impact on the whole industry (Conley, 1994). *Kudoa* continues to be problematic at some farms. At a workshop titled "Softflesh syndrome in farmed Atlantic salmon: a workshop to discuss ongoing and future research" held in Nanaimo, BC, on April 20, 2001, a conservative estimate of the total cost of *K. thyrsites* to the BC salmon aquaculture industry was $7.15 million (Hicks, 2001).

K. thyrsites was first reported in Atlantic salmon by Harrell and Scott (1985) in Washington State, and at that time was implicated as a cause of death of Atlantic salmon; however, *K. thyrsites* infections are not thought to be associated with mortality (Moran et al., 1999b). Whitaker and Kent (1991) also reported *K. thyrsites* infections in Atlantic salmon. In this case, smoked Atlantic salmon flesh had softened and discoloured areas. The smoking process consisted of holding the Atlantic salmon at 50°C for 10 hours. Lower smoking temperatures of 20-30°C reportedly did not prevent poor quality smoked fillets, which were apparently caused by the action of enzymes produced by the parasite. The enzymes are not destroyed by freezing and have been found to have optimal activity at 55-60°C (Tsuyuki et al., 1982).

When 32 fresh Atlantic salmon infected with *K. thyrsites* were compared to 32 uninfected fish, a correlation was found that suggested that *K. thyrsites* is a cause of muscle deterioration in Atlantic salmon. "A significant negative correlation was found between the firmness of flesh texture six days after harvest and the number of *K. thyrsites* spores in the muscle tissue." Furthermore, there appeared to be a threshold limit, 2.0×10^4 spores per gram of fish tissue, at which point infections began to show evidence of the soft flesh phenomenon (St-Hilaire et al., 1997a). Dawson-Coates et al. (2003), however, found that spore counts of 4.0×10^5 per gram of tissue resulted in inferior quality fillets and that counts of 7.5×10^5 per gram resulted in severe fillet deterioration. This difference between research groups is likely due to the differences in the trypsin treatment employed to release the *K. thyrsites* spores (Dawson-Coates et al., 2003). Dawson-Coates et al. (2003) used a method that was "a more efficient trypsin treatment of small amounts of tissue."

In addition, some factors were found to increase the chance of fish becoming infected with the *K. thyrsites* parasite. Sexually mature fish were more likely to be infected with *K. thyrsites* than sexually immature fish (St-Hilaire et al., 1998). During experiments conducted in Departure Bay near Nanaimo BC, it was found that infections were "readily contracted in the summer and fall but not through the winter and early spring months" (Moran and Kent, 1999). Furthermore, it was discovered that "smolts become infected with *K. thyrsites* shortly after their transfer to the seawater net-pens." It is believed that "the compromised immunity of the host, both

at smoltification and sexual maturation, permits the parasite to proliferate or reinfect the fish" (Moran et al., 1999b).

Post mortem deterioration may not occur until several days after harvest. Therefore, one would ideally like to detect *K. thyrsites* infections before harvest because of the parasites' aesthetic effects. Many times, this may mean that the soft flesh may appear once the fish have reached the customer. Sakanari (1994) mentions several methods to possibly detect and quantitate *K. thyrsites* infections. Both microscopy and polymerase chain reaction (PCR) can be used to detect *K. thyrsites*; however, macroscopic observation is not feasible. Moran et al. (1999a) and St-Hilaire et al. (1997b) describe similar procedures to prepare wet mounts for the observation of the *K. thyrsites* myxospore stage. Muscle tissue is mixed with saline, minced with scalpels and pressed between plexiglass plates. The resulting extract is then observed with a phase contrast microscope. This procedure requires that the parasite has sporulated and contains the recognizable myxospore stage specific to *K. thyrsites.* St-Hilaire (1997b) also developed a method that involves preparing wet mounts using the *Hyohyoideus ventralis* muscle of the operculum, resulting in no observable signs of sampling on commercial products. This procedure was reported to have a sensitivity of 79.0% and a specificity of 94.6%. When used with heavily infected fish, the sensitivity jumped to 93%. Hervio et al. (1997) developed a sensitive and specific PCR assay for the detection of *K. thyrsites.* This procedure may be useful for early or light infections and has the advantage of having the ability to detect all stages of the parasite. Myxospores, however, are the only consistent

stage detected by wet mounts (Moran et al., 1999b). Currently, there are no immunological techniques available for an inexpensive, rapid and quantitative assay for *K. thyrsites*. Chase et al. (2001), however, developed monoclonal antibodies specific for *K. thyrsites* spore antigens.

2.5 Heat Resistance and Measuring Viability

Thermal resistance of microorganisms is often measured by determining their decimal reduction time (D-value). Organisms are exposed to a specific temperature in an appropriate medium and sampled at specific time intervals. At each time, the microorganisms are assessed for viability. The D-value is then determined by plotting the log number of survivors against the time the organisms were sampled. This plot is often referred to as a survivor curve. The D-value from each survivor curve can then be calculated by determining how long it takes to reduce the population by one log value or 90%. The D-value may also be calculated as $D=(t/(\log_{10}x-\log_{10}y))$ where D represents the decimal reduction time; t is the time in minutes; and x and y represents the number of microorganisms before and after exposing them to these conditions, respectively. Alternatively, the D-value is also equivalent to the negative inverse of the slope of the survivor curve. D-values will decrease with increasing temperatures (Heldman and Hartel, 1997; Singh and Heldman, 1993).

There are several different techniques used for D-value determination. Those described by Stumbo (1973) include the thermal death time tube method, the

thermal death time can method, the "tank" method, the flask method, the thermoresistometer method, the unsealed thermal death time tube method, and the capillary method. Each method has its own inherent advantages and disadvantages. Some such disadvantages listed by Mikolajcik and Rajkowski (1980) for these various methods include that they "(*a*) lacked accuracy, (*b*) required extensive manipulation of samples and test systems, (*c*) were time consuming, (*d*) were limited to temperatures <100 C and/or (*e*) required excessive amounts of test organisms."

Kooiman and Geers (1975) described a screw-capped tube technique for heat resistance of bacterial spores that eliminated many of the drawbacks of previous methods. For example, this method allowed for temperatures greater than 100°C and allowed accurate monitoring of heating and cooling time and temperature; however, a separate tube for each holding time was required, and it can be difficult to achieve a uniform inoculum in all tubes. Modifications of this procedure have been used by other researchers (Mikolajcik and Rajkowski, 1980; Makki and Durance, 1996) using a serum bottle in which only a single bottle was required with samples being withdrawn at selected time intervals. A disadvantage of this method is that more than one minute is required to re-establish temperature equilibrium after addition of the inoculum.

Another parameter, z-value, is the "temperature increase required to cause a one log cycle reduction in the decimal reduction time" (Heldman and Hartel, 1997). The

z-value is also referred to as the thermal resistance constant. It can be graphically determined by plotting the log of the D-value against temperature, the thermal death time curve. The z-value is then calculated as the negative inverse of the slope. A large z-value indicates that at a higher temperature of exposure there would be a small change in D-value, thus providing information on the relative resistance of a microorganism to different temperatures (Heldman and Hartel, 1997).

Currently, there is no documentation indicating the temperature sensitivity of *K. paniformis* or *K. thyrsites* spores, although the optimum activity of the enzyme has been found to be in the range of 55°C to 60°C (Tsuyuki et al., 1982). The temperature sensitivity of *Myxobolus cerebralis* (formerly *Myxosoma cerebralis*), an organism of the same class (Myxosporea), has been investigated. Hoffman and Putz (1969) demonstrated that *M. cerebralis* spores survived freezing at –20°C for at least 18 days. In a follow-up study, Hoffman and Putz (1971) discovered that spores of *M. cerebralis* could survive at least 2 months at –20°C, and could probably survive much longer. El-Matoubli and Hoffmann (1991) confirmed that the same parasite could survive at -20°C for at least 3 months. Furthermore, they suggested that resistance to freezing may be a common attribute of *Myxobolus* spores since spores of *M. cotti* and *M. pavlovskii* exhibited the same properties.

Hoffman and Putz (1969) also exposed *M. cerebralis* spores to heat ranging from room temperature to 100°C in 0.85% saline in 50 ml beakers. Spores were observed microscopically. Those that appeared distorted were presumed dead.

They discovered that *M. cerebralis* spores held at 60°C, 80°C, and 100°C for 10 min were killed. Spores that were held at 40°C and at room temperature showed no change.

Hoffman and Markiw (1977) used 0.08% aqueous methylene blue to determine the efficiency of heat treatment in killing *M. cerebralis* spores. Spores allowing methylene blue to enter were presumed dead. One hundred percent of *M. cerebralis* spores held at 90°C for 10 min were presumed dead after staining with methylene blue. Spores held at 80°C for 10 min were 100% stained except one out of the five trials that was 88% stained. At 70°C, 60°C, and 50°C for 10 min, an average of 60%, 34%, and 24% of the spores were stained, respectively. The control, those maintained at room temperature, had an average of 22% spores presumed killed. Methylene blue has been used for the assessment of viability of microorganisms and is based on membrane integrity. Spores that take up the dye are considered to be non-viable as indicated by a loss of membrane selectivity. Therefore, viable cells with an intact cytoplasmic membrane will not be stained (Breeuwer and Abee, 2000).

The combination of fluorescein diacetate (FDA) and propidium iodide (PI) for the determination of viability of the myxosporean stage of *Myxobolus artus* spores and the actinosporean stage spores of *M. cultus* was found to be a suitable method by Yokoyama et al. (1997). Using this double staining technique, viable spores stained green with FDA and non-viable spores stained red with PI. In principle, FDA, a

nonpolar ester, is deacylated (or hydrolyzed) by intracellular cytoplasmic esterases to yield fluorescein, a polar compound which accumulates only inside viable intact cells. Fluorescein exhibits green fluorescence when excited with blue light. PI, on the other hand, permeates only through the membrane of dead cells, intercalates with DNA and RNA, and the resulting complex emits a red fluorescence. Like methylene blue, PI is also based on membrane integrity (Yokoyama et al., 1997; Jones and Senft, 1985).

Like methylene blue and propidium iodide, trypan blue is a viability stain based on membrane integrity. Viable cells exclude the dye resulting in a distinction between unstained viable cells and blue stained nonviable cells (Mishell et al., 1980). The usefulness of using trypan blue is limited since cells must be quantified within 3-5 minutes and reportedly trypan blue overestimates cell viability (Altman et al., 1993). Hoffman and Markiw (1977) attempted to use trypan blue to assay the viability of *M. cerebralis* spores but found that the spores did not stain. They presumed that the resistance of the spores was due to the spore wall and polar capsule plugs of normal spores resisting the entrance of the chemical. Hirazawa et al. (2001) also used trypan blue to determine the viability of *Kudoa shiomitsui* spores after treatment with caprylic acid solution. Spores that had burst dyed blue; however, there was no indication whether all of the visually intact spores were unstained or if some had also become blue.

2.6 Summary

Given the previous information, Pacific hake used for fish meal processing will likely be infected with both *K. thyrsites* and *K. paniformis*. While *K. paniformis* is associated with the soft flesh found in Pacific hake, *K. thyrsites* is thought to be responsible for the softening of Atlantic salmon fillets. No information, however, is available on the thermal resistance of either parasite, nor is there a method to determine the viability of either organism. Previous studies, though, have used trypan blue, methylene blue, fluorescein diacetate, and propidium iodide to assess the viability of other myxosporean parasites. These dyes should be tested for their suitability as an indicator of viability for *K. thyrsites* and *K. paniformis*. An appropriate viability test should then be used for thermal resistance studies of *K. thyrsites* and *K. paniformis*.

3.0 Materials and Methods

3.1 Prevalence and Intensity of *K. thyrsites* Infections

Pacific hake fish samples were provided by the fish meal rendering plant during the 2001 and 2002 hake season, and were sampled from the receiving pit. Three trips were made during the 2001 season, and two trips were made in the 2002 season. All fish were offshore hake. These were transported to the lab on ice in a cooler. Samples were frozen until they were analyzed. There were several types of samples including whole fish, mid-tail sections (with no heads), head-mid sections (with no tails), frames (whole fish with the fillets removed), head sections, mid sections, and tail sections. For whole fish, samples were excised from the left fillet from three regions: the cranio-dorsal, medial lateral, and caudal regions. Mid-tail and head-mid sections were also sampled from the left fillet, but from the medial lateral and caudal sections, and cranio-dorsal and medial lateral sections, respectively. Frames were sampled from the cranio-dorsal region and from anywhere along the skeleton where flesh remained. For all samples the skin and bones were removed. Samples were frozen at -18°C until analysis.

Atlantic salmon fillets were provided by fish farms in British Columbia. All fillets were sampled from the cranio-dorsal, medial lateral, and caudal regions. The skin and bones were removed. All samples were kept at 4°C for immediate analysis.

The prevalence and intensity of *Kudoa* infections were determined using a technique demonstrated by Dawson-Coates (2000), which is similar to the procedure described

by Moran et al. (1999a) and St-Hilaire et al. (1997b). At room temperature, 2.5-5.0 ml of phosphate-buffered saline (pH 7.4, 0.137M NaCl, 0.007M K_2HPO_4, 0.002M KH_2PO_4) was added to 3-4 g of fish tissue. The muscle tissue was then minced with scalpels to release spores from the flesh. The minced material was then pressed between two Plexiglas plates (5" x 7") and the exuding liquid was collected into a 15 ml conical centrifuge tube (Corning Incorporated, Corning, NY, 430290). The liquid was left undisturbed for at least 5 min to allow the spores to settle. A wet mount was prepared by placing a drop collected near the bottom of the tube onto a microscope slide and laying a glass cover slip over the drop. The slide was examined with a phase contrast microscope (Zeiss, West Germany, D-7082) at a magnification of 200 times using an S-shaped search pattern. The term "light infection" was used to describe slides containing 5 spores or less. A moderate infection described slides containing more than 5 per slide but less than 20 per field, while a heavy infection was used to describe wet mounts with 20 or more spores per field.

3.2 Trypsin Digestion to Release *Kudoa* Spores from Fish Meal

The trypsin digestion was based on a procedure by St-Hilaire et al. (1997a). Six fishmeal samples were examined in duplicate. Eight grams of fish meal was mixed with 27 ml of physiological saline in a sterile plastic bag. The bag was placed in a Seward Stomacher lab blender (Model: 400 Circulator, Norfolk, UK) for 3 minutes at 260 rpm. Five grams of the slurry was transferred to a 150ml Erlenmeyer flask to which 100ml of 0.5% trypsin solution (pH=7.2; Sigma Chemical Co., St. Louis MO, T-7409) was added. The trypsin activity was 1,800 BAEE (N-benzoyl-L-arginine

ethyl ester) units per milligram of solid. One BAEE unit will produce a ΔA_{253} of 0.001 per minute at pH 7.6 at 25°C using BAEE as a substrate (reaction volume = 3.2 ml; 1 cm light path). The mixture was shaken vigorously. The flask was then placed in a shaker bath (Lab-Line Instruments, Inc., Melrose Park, IL, Model 3543) at 37°C and gently agitated at 110 rpm for 30 minutes. The flask was shaken manually and replaced in the agitated water bath for an additional 30 minutes. To concentrate the samples, the trypsin mixture was divided into two 50ml centrifuge tubes and centrifuged at 3000 x g for 15min at 10°C. The supernatant was decanted and 3 ml of water was added to each pellet. This was mixed with a vortex. Wet mounts were prepared by pipetting 20 µl of the suspension onto glass slides and screened for *Kudoa thyrsites* spores using phase contrast illumination at 200 times magnification.

Spiked samples were prepared in the same manner as described above except that approximately 10^6 *K. thyrsites* spores were added prior to incubation at 37°C.

3.3 Isolation of *Kudoa* Spores from Fish Flesh

The spore isolation procedure is based on the protocol described by Chase et al. (2001) with minor modifications. As recommended, the prevalence and intensity of infection was first determined before isolating spores using the procedure previously described. Samples containing at least 50 spores per field using 200 times magnification are optimal for isolation; however, the Atlantic salmon samples used in the present study were not this highly infected with *K. thyrsites*. Instead, samples

containing a minimum of 15 spores per field were used for isolation. Pacific hake, samples with at least 50 *K. paniformis* spores per field were used for spore isolation.

The isolation procedure was performed in a cold room at 4°C. Fish tissue weighing 7 – 10 g with the skin and bones removed was placed in a cell dissociation sieve (Sigma Chemical Co., St. Louis, MO, S-1145) containing a layer of 100 um nylon mesh (Nitex®, Sefar America Inc., Depew, NY) followed by a 250 µm metal screen (Sigma Chemical Co., St. Louis, MO, S-1020). A petri plate was placed underneath the sieve to collect the exuding fluids. The spores were washed through the mesh using 100 ml of Hank's balanced salt solution (HBSS) containing approximately 0.02% w/v sodium bicarbonate (Life Technologies, HBSS Cat. No. 14060-057, Sodium bicarbonate solution 7.5% (w/v) Cat. No. 25080-094). The plunger of a 3cc syringe (Becton Dickinson and Co., Franklin Lakes, NJ, No. 309585) was used to break apart the muscle and to facilitate the flow of buffer through the sieve. The fluid in the Petri plate was collected into two 50ml conical tubes (Corning Incorporated, Corning, NY, 430053) using a plastic transfer pipette and centrifuged at 1500 x g (Beckman, GS-6 Centrifuge) for 15 min at 5°C. The liquid was decanted and the remaining pellets were collected into a 50 ml conical centrifuge with a transfer pipette and resuspended with HBSS to a total volume of 20 ml. Discontinuous layers of 15% and 30% Percoll (made with PBS pH 7.0; Sigma Chemical Co., St. Louis, MO, P-1644) gradients of 2.5 ml each were prepared for spore separation in 15 ml conical centrifuge tubes. This was done by first pipetting 2.5 ml of 15% Percoll into a 15 ml tube. Using a 3cc syringe equipped with a pipetting needle (16

gauge, 15cm long, Aldrich Chemical Co., Milwaukee, WI, Cat. No. Z26130-6), 2.5 ml of the 30% Percoll layer was pipetted into the 15 ml tube below the 15% layer. This was done carefully so the layers did not mix. One millilitre of the resuspended spore/HBSS suspension was layered on top of the Percoll gradients (a total of 20 tubes) and centrifuged at 1500 x g for 30 min at 5°C. Following centrifugation, the layers were removed with a syringe equipped with a long pipetting needle until only a little bit of liquid (approximately 1ml) and the pellet remained. After rinsing the pipetting needle with PBS, a new syringe was attached and was used to transfer the remaining contents of the tubes into new 15 ml conical centrifuge tubes to a maximum volume of 4 ml. The tubes were then topped up to 15ml with PBS, and the percoll was then washed out by centrifuging the tubes at 1500 x g for 15 min at 5°C. The liquid was decanted and replaced with 15 ml of PBS.

3.4 Enumerating *Kudoa* Spores

Kudoa spores were counted using a Neubauer hemocytometer (Hausser Scientific, Horsham, PA) covered with a thick glass cover slip. The chamber was carefully filled by means of capillary action through the use of a p20 pipetman (Gilson, Inc., Middleton, WI) filled to 20 µl. The tip on the pipetman was brought to the very edge of the cover slip (without touching it) and the liquid was released slowly, being careful not to overfill or underfill the chamber. The spores were then counted using 100x magnification. A minimum of three 1 mm squares were counted. To calculate the number of spores per millilitre the average count per 1 mm square was then

multiplied by 10^4. Total spores were therefore calculated by multiplying the number of spores per millilitre by the original volume from which the spores were removed.

3.5 Development of a Viability Test for *Kudoa* Spores

In all cases, spores held in 1% formalin (Fisher Scientific, Fair Lawn, NJ, F79-1) overnight acted as a negative control. Before testing, the formalin was washed out and replaced with PBS.

3.5.1 Trypan Blue

The trypan blue viability test was based on the procedure described by Altman et al. (1993). A 0.4% (w/v) trypan blue solution (Sigma Chemical Co., St. Louis, MO., T-0776) was prepared in 0.81% NaCl (Fisher Scientific, Fair Lawn, NJ, S271-3) and 0.06% (w/v) dibasic potassium phosphate (K_2HPO_4). One hundred microlitres of spore suspension was transferred to a microcentrifuge tube (Fisher Scientific, Nepean, ON, Cat. No. 05-407-25A) and was incubated for 5 minutes at room temperature with 100 µl of 0.4% (w/v) trypan blue solution. Spores were examined by phase contrast microscopy at 200x magnification. Spores that did not turn blue were considered viable, while spores that stained blue were considered nonviable.

3.5.2 Methylene Blue

The methylene blue test was based on the technique used by Hoffman and Markiw (1977). A 0.25% (w/v) aqueous methylene blue solution (Sigma Chemical Co., St. Louis, MO, M-9140) was prepared. Thirty-six microlitres of 0.25% aqueous

methylene blue solution was added to 74 µl of spore suspension, resulting in a final concentration of methylene blue of 0.08%. The mixture was left to stand for 3 minutes at room temperature. Spores were then examined by phase contrast microscopy at 200x magnification. Spores that did not turn blue were considered viable, while spores that stained blue were considered nonviable.

3.5.3 Fluorescein Diacetate (FDA) and Propidium Iodide (PI)

The FDA and PI viability tests were performed according to the procedure described by Yokoyama et al. (1997) with minor modifications. Stock solutions of FDA (Sigma Chemical Co., St. Louis, MO, F-7378) and PI (Sigma Chemical Co., St. Louis, MO, P-4170) were prepared in advance. The FDA stock solution consisted of 5 mg/ml of FDA dissolved in acetone and was stored in 1ml aliquots at –18°C in microcentrifuge tubes. The PI stock solution, which was also the working solution, was composed of 0.02 mg/ml of PI dissolved in distilled water and was also stored in 1ml aliquots at –18°C. The working solution of FDA was prepared by diluting 20 µl of FDA stock solution with 4.2 ml of distilled water. For the viability assay, 25 µl of freshly diluted FDA solution was combined with 25 µl of PI solution and 50 µl of spore suspension in a microcentrifuge tube. The tube was mixed with a vortex and left undisturbed in the dark at 5°C for 15 minutes. The spores were observed with a fluorescence microscope. Both green and red fluorescing cells were observed simultaneously by using an exciter filter band pass of 450-490nm (blue) with a 510 chromatic beam splitter and a long pass barrier filter of 520nm (Zeiss, West Germany, Filter Set 48

79 09). Spores fluorescing green with FDA were considered viable, while spores fluorescing red with PI were considered non-viable.

3.6 Heat Resistance Determinations

3.6.1 Decimal Reduction Time (D-value) of *Kudoa* Spores

Heat treatments were performed according to the procedures of Mikolajcik and Rajkowski (1980) and Makki and Durance (1996) with some modifications. For each heat treatment, 4 ml of buffer (PBS, pH 7.0) was placed into a 7 ml Supelco clear glass vial sealed with a PTFE/silicone septum and polypropylene hole screw cap (Supelco, Bellefonte, PA, 27210). These glass vials were pretreated to prevent adherence of the spores to the glass. This required coating the glass surface with 5% DMDCS (dimethyldichlorosilane) in toluene (Supelco, Bellefonte, PA, 33065-U) for 15 seconds. The surface was then rinsed two times with toluene (OmniSolv, BDH Chemicals, Toronto, ON, Code B90339). Following this, the surface was then rinsed with methanol (Fisher Scientific, Fair Lawn, NJ, A412-20) three times, and dried with nitrogen (Compressed, Praxair Canada, Inc., Mississauga, ON, medical grade).

The filled vials were placed in an agitated water bath set at the desired temperature. The temperature of the buffer was monitored using a copper-constantan thermocouple probe (T type). When the buffer reached the appropriate temperature, 200 µl of the spore suspension was injected through the seal of the vial using a gas

tight syringe (Hamilton Co., Reno, NV). *K. thyrsites* spores were isolated from Atlantic salmon, and *K. paniformis* spores were isolated from Pacific hake.

When the entire contents again reached the desired temperature (zero time), which took between one and two minutes, 400 µl of the buffer/spore suspension was withdrawn from the vial and placed into a microcentrifuge tube in an ice/water bath. The viability was determined using methylene blue. One slide was examined at each sampling time, with three sets of 50 spores being counted and averaged to determine the percent viability. The spores were also enumerated as previously described. Samples were also withdrawn at appropriate time intervals thereafter. The survivors were calculated as the total number of spores multiplied by the proportion of spores that were still viable. A minimum of three replicates was performed at each temperature.

3.6.2 Calculation of Kinetic Parameters

The log of the number of survivors was plotted against the corresponding time of sampling for each trial at a particular temperature. Linear regression analysis was used to determine the best fit line and the coefficient of determination for each trial was determined using Excel (Microsoft Corporation, 2002). The D-value was calculated as the negative inverse of the slope of the regression line.

The z-value was calculated by plotting the log of the D-values against the temperatures at which they were determined. Linear regression analysis was

performed to determine the best fit line and the coefficient of determination for each trial using Excel (Microsoft Corporation, 2002). The z-value was calculated as the negative inverse of the slope of the regression line.

3.7 Examination of Fish Oil as a Source of *Kudoa* Spores

Wet mounts were prepared from fish oil obtained from the fish meal rendering plant to see if spores could be detected. In addition, 200 µl of *K. thyrsites* spore suspension was added to 1 ml of fish oil and to 1 ml of phosphate buffered saline. Both preparations were heated at 96°C for 15 min. All samples were examined with a phase contrast microscope at 200x magnification.

4.0 Results and Discussion

4.1 Prevalence and Intensity of *K. thyrsites* Infections

A total of 254 Pacific hake samples were collected from the fish meal rendering plant from the receiving pit between July 2001 and October 2001. These were transported to the lab on ice in a cooler. Of these 254 samples, 56 samples were whole fish, 185 were heads, 8 were midsections, and 1 was a tail section. In addition, there were 3 samples with head and midsections (i.e. no tail), and 1 sample with mid and tail sections (i.e. no head). The prevalence of *K. thyrsites* infection in Pacific hake collected from the fish meal rendering plant was 78.0% (Table 1). Of these infections, 36.4% were light and 63.6% were moderate. There were no heavy infections.

During the 2002 hake season, a total of 211 samples were obtained. Of these samples 1 was a whole fish, 113 were heads, 47 were mid-sections, 10 were tails, 6 were mid and tail sections, and 34 were frames (i.e. fillets removed with only the head and rack remaining). The prevalence of *K. thyrsites* infection in these samples was 80.1% infection, with 24.3% being light and 75.7% being moderate (Table 2).

The prevalence of *K. thyrsites* was higher than reported in earlier studies. Tsuyuki et al. (1982) found the parasite in 163 of 322 (50.6%) Pacific hake. Kudo et al. (1987) found that in a sample of 562 Pacific hake, 315 (56.0%) were infected. Morado and Sparks (1986) found a prevalence of 24% in a total of 178 samples. These differences in prevalences may be a result of the variety of samples collected

Table 1. Prevalence and intensity of *K. thyrsites* infections in Pacific hake collected in 2001.

Portion	Total Number	Prevalence and Intensity			
		None	Light	Moderate	Heavy
Whole	56	7 (12.5%)*	3 (5.4%)	46 (82.1%)	0
Head	185	44 (23.8%)	65 (35.1%)	76 (41.1%)	0
Mid	8	4 (50.0%)	2 (25.0%)	2 (25.0%)	0
Tail	1	0	1 (100.0%)	0	0
Head-Mid	3	1 (33.3%)	0	2 (66.7%)	0
Mid-Tail	1	0	1 (100.0%)	0	0
Total	254	56 (22.0%)	72 (28.3%)	126 (49.6%)	0

* Percentage of total number of portion type (i.e. 12.5% of whole samples have no infection).
Percentages are read across each row.

Table 2. Prevalence and intensity of *K. thyrsites* infections in Pacific hake collected in 2002.

Portion	Total Number	Prevalence and Intensity			
		None	Light	Moderate	Heavy
Whole	1	0	0	1 (100%)	0
Head	113	12 (10.6%)*	15 (13.3%)	86 (76.1%)	0
Mid	47	18 (38.3%)	10 (21.3%)	19 (40.4%)	0
Tail	10	3 (30.0%)	2 (20.0%)	5 (50.0%)	0
Mid-Tail	6	1 (16.7%)	2 (33.3%)	3 (50.0%)	0
Frame	34	8 (23.5%)	12 (35.3%)	14 (41.2%)	0
Total	211	42 (19.9%)	41 (19.4%)	128 (60.7%)	0

* Percentage of total number of portion type (i.e. 10.6% of head samples have no infection).
Percentages are read across each row.

from the fish meal rendering plant. Previous studies only looked at whole fish, while the current study examined whole fish, head, mid, and tail portions, head-mid portions, mid-tail portions, and frames. Head portions dominate in numbers. Previous studies indicate that the most heavily infected part of Pacific hake is directly behind the head, with intensity decreasing toward the tail (Kabata and Whitaker, 1986). Therefore, the current results may not apply to the entire population, but to only the fish sampled. Looking at whole fish alone, 50 out of 57 fish were infected with *K. thyrsites*. The distribution of the parasite in the 50 infected fish can be seen in Table 3. In addition, although measured differently, the current results agree with Tsuyuki et al. (1982) in that *K. thyrsites* infections in Pacific hake do not appear to result in heavy infections.

A total of twenty Atlantic salmon fillets were received from various fish farms in British Columbia. These fillets were deemed infected with *K. thyrsites* by personnel at the processing plants. Using the procedure previously described, sixteen fillets had moderate *K. thyrsites* infections, while two fillets had light *K. thyrsites* infections. The remaining two were not infected with the parasite.

4.2 Trypsin Digestion to Release *Kudoa* Spores

A wet mount prepared directly from fish meal diluted with PBS failed to show any signs of *K. thyrsites* spores. This is expected since there would be a dilution effect due to the large mass of Pacific hake used to process fish meal, especially since not all fish are infected and those that were examined carried only light or moderate

Table 3. Intensity of *K. thyrsites* infections in whole Pacific hake collected in 2001 and 2002 (n=50).

Portion	Prevalence and Intensity			
	None	Light	Moderate	Heavy
Head	2	10	38	0
Mid	5	6	39	0
Tail	7	10	33	0

infections. The eight gram sample examined would also be only a portion of a fish, considering that 100% of fish converts into approximately 16% fish meal (Jones, 2001). Therefore, an 8 g sample of fish meal results in only 50 g of fish. An adult hake normally weighs 1.4 kg (Rodger, 1991). In addition, it is possible that during fish meal production the spores may tightly adhere to the fish flesh. Therefore, the spores would not be immediately recognizable in the wet mount preparations. It is also possible that due to the heat process, the spores may be unrecognizable in appearance.

Therefore, the trypsin digestion was attempted on fishmeal samples from the 2001 and 2002 processing year (3 from each year) to see if spores could be found in the fish meal. Upon performing the digestion procedure, however, no *K. thyrsites* spores were detected by preparation of wet mounts from the fluid portion of the digest. Spores were, however, detectable in fish meal samples spiked with approximately 10^6 *K. thyrsites* spores. An average of 20 spores per slide were identified. St. Hilaire et al. (1997) used a similar trypsin digestion to release spores from fresh, uncooked Atlantic salmon. The nature of heating fish to manufacture fish meal, however, could render spores unrecognizable due to morphological changes attributed to high temperatures and mechanical forces involved in hake meal manufacturing.

4.3 Development of a Viability Test for *Kudoa* spores

As of yet, there is no accurate means of determining the viability of *K. paniformis* or *K. thyrsites*. Until the life cycle of both organisms is discovered, this will likely remain true. Dyes, however, have been applied to assess viability based on factors such as membrane integrity and enzyme activity (Breeuwer and Abee, 2000). In the current study, the proposed dyes for development of a viability test for *Kudoa* spores included trypan blue, methylene blue, fluorescein diacetate, and propidium iodide.

Formalin treated *K. thyrsites* spores excluded the trypan blue dye and remained unstained; however, one would expect that formalin would cause inactivation of the spores and thus result in uptake of the trypan blue dye. Formalin is a solution of formaldehyde, and formaldehyde is used as a disinfectant to kill viruses, bacteria, fungi, and parasite (World Health Organization, 1989). It is suspected that formaldehyde exerts its effects by alkylation of nucleic acids (Russell, 1991).

In preliminary studies, *K. thyrsites* spores heated at 78°C for 10 minutes were not able to exclude trypan blue and stained blue. This suggests that the heating altered the ability of the spores to exclude the dye, possibly by damaging the spores. These results indicate that trypan blue would not be a reliable indicator of the viability of *K. thyrsites* spores since formalin treated spores remained unstained. Hofffman and Markiw (1977) also attempted to use trypan blue to assay the viability of *M. cerebralis* spores but found that the spores did not stain. They presumed that the

resistance of the spores was due to the spore wall and polar capsule plugs of normal spores resisting the entrance of the chemical.

With methylene blue and propidium iodide, *K. thyrsites* spores were able to exclude the dye when unheated and pick up the dye after exposure to formalin. For example, all formalin treated spores took up methylene blue (Figure 1) or propidium iodide. In addition, there was no fluorescence of fluorescein diacetate when spores were treated with formalin.

Both fluorescein diacetate and propidium iodide were used simultaneously to determine viability (Figures 2 and 3). There were incidents, however, when spores fluoresced both green and red (Figure 4). Fluorescein diacetate is deacylated by intracellular cytoplasmic esterases to yield fluorescein, a polar compound which accumulates only inside viable intact cells. The fluorescein exhibits green fluorescence when excited with blue light (Yokoyama et al., 1997; Jones and Senft, 1985). In these cases of double staining, the polar capsules stained green, while the remainder of the spore stained red. This suggests that the polar capsule cells were intact and esterase activity was still present within the polar capsule cells, but the membrane of the spore valves had been compromised. Therefore, the spores had the ability to fluoresce red as well. Under these circumstances, would spores be considered viable or nonviable? Because the spore valves stained red, it suggests that the membrane integrity has been compromised and thus, should be considered

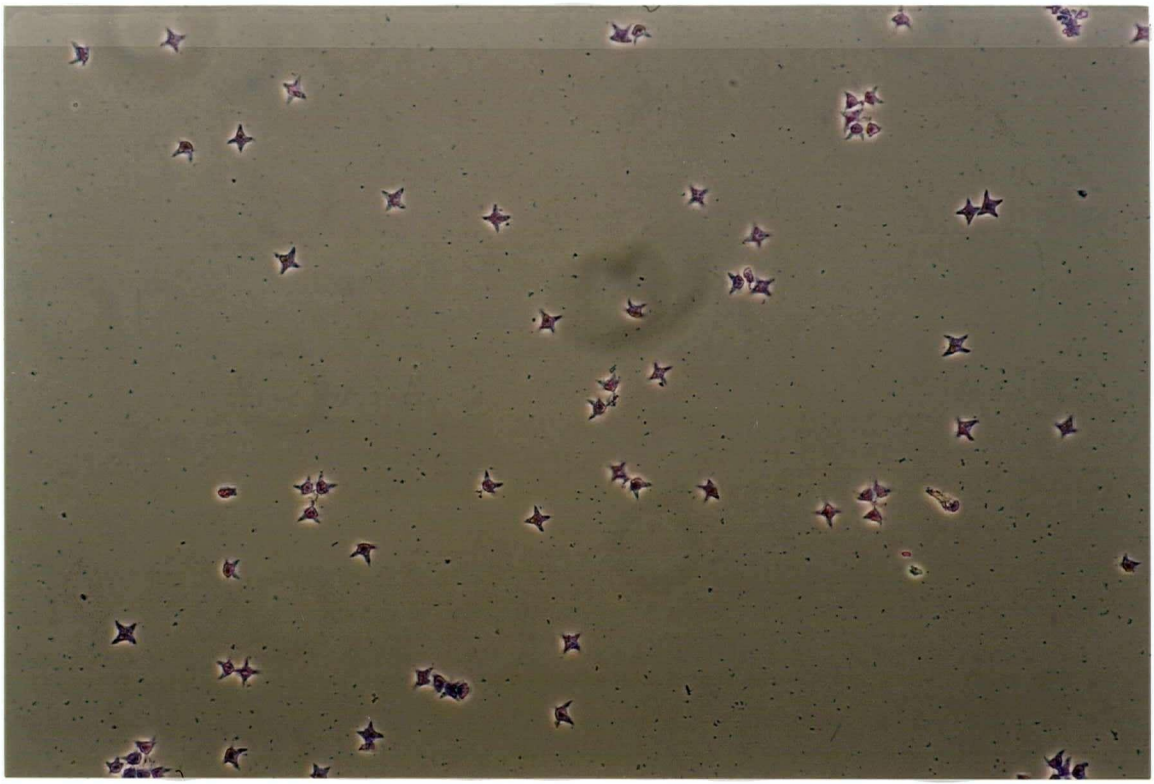


Figure 1. *K. thyrsites* (formalin-treated) spores stained with methylene blue. (200 x magnification)

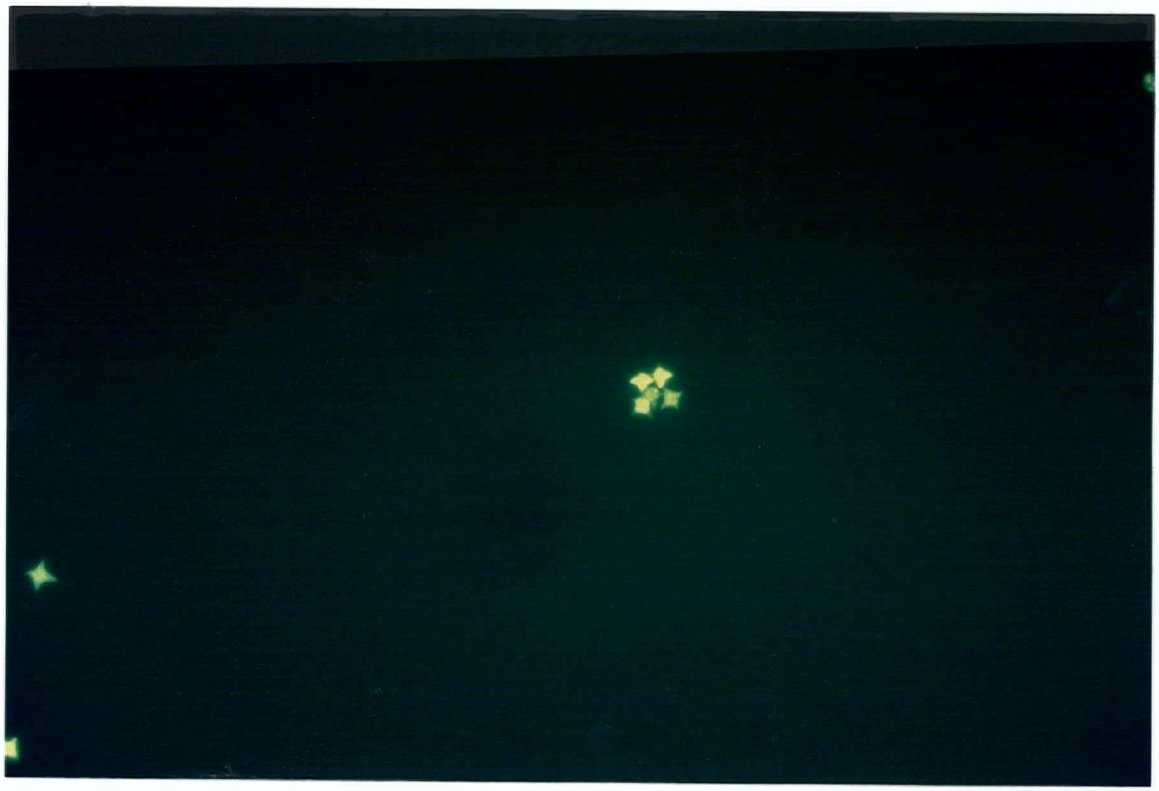


Figure 2. *K. thyrsites* spores stained with fluorescein diacetate. (200 x magnification)

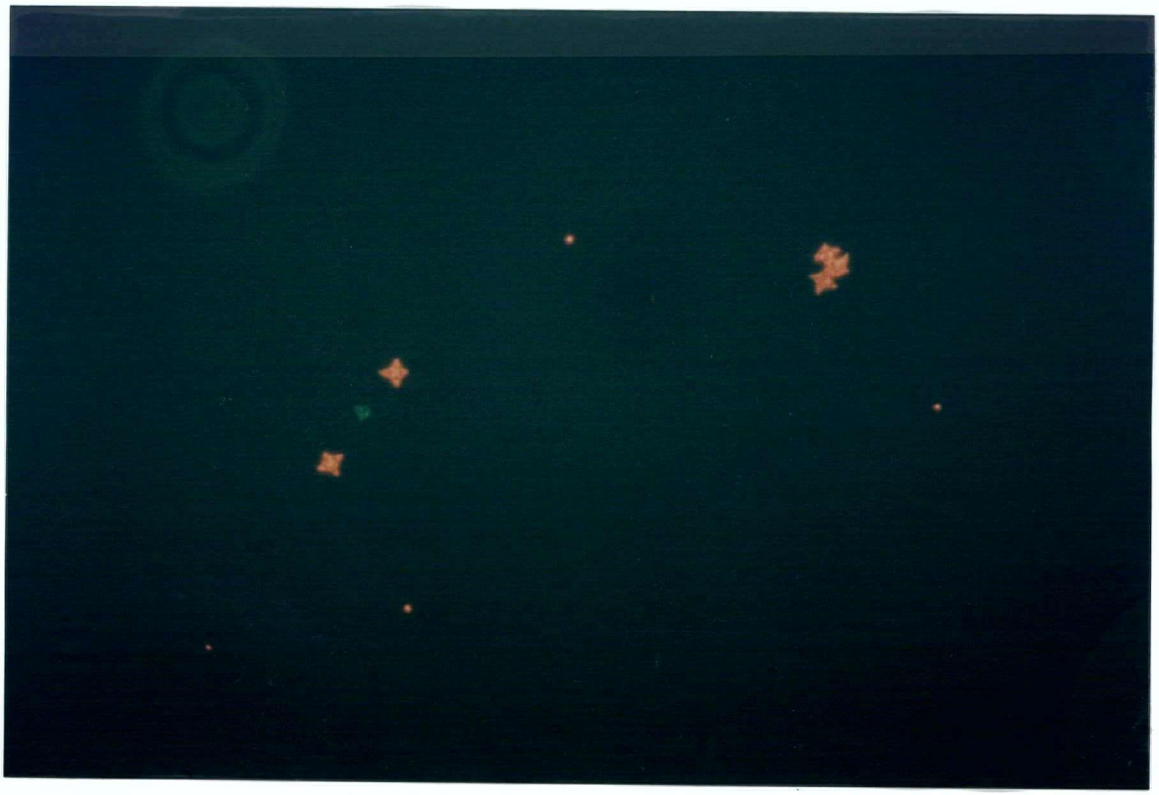


Figure 3. *K. thyrsites* spores stained with propidium iodide (red). One spore is stained with fluorescein diacetate (green). (200 x magnification)

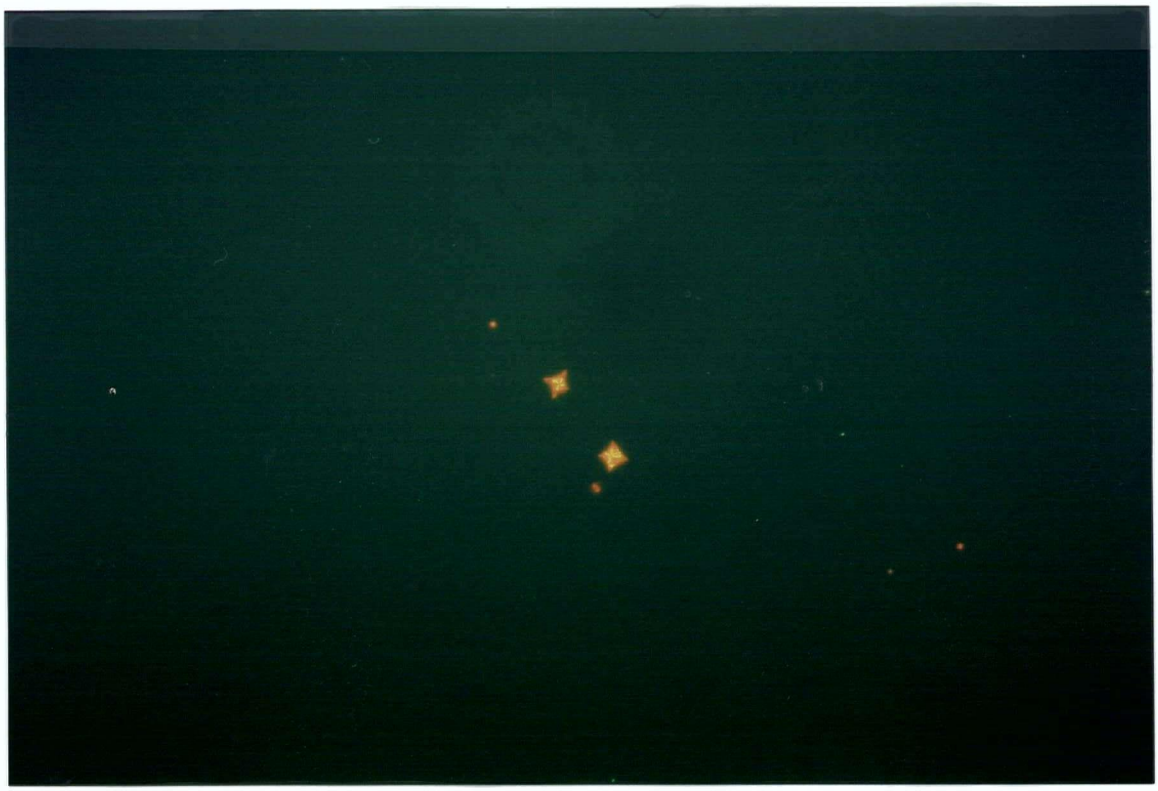


Figure 4. *K. thyrsites* polar capsules fluorescing green, and the remainder of the spore fluorescing red. (200 x magnification)

nonviable. Yokoyama et al. (1997) found that the polar capsules of *Myxobolus artus* myxosporean stages stained green and sporoplasm cells stained red. The same group also found that "*Myxobolus cultus* actinosporean stage spores stained bright green in the sporoplasm cells, and red in the spore processes" (Yokoyama et al., 1997). They did not address, however, whether these double-stained spores would be considered viable or nonviable.

The only dye tested for *K. paniformis* was methylene blue. The negative controls, those treated with formalin, all stained blue. In addition, spores heated briefly at 90°C also picked up the dye indicating that they were nonviable. Freshly isolated spores remained largely unstained.

As previously mentioned, there is no way of culturing or growing *Kudoa* spores outside of the fish host, and there is no reliable way to test viability. Methylene blue, however, was chosen as an indicator of potential loss of viability.

For practicality only methylene blue was used for heat resistance determinations. On many occasions, spores prepared with fluorescein diacetate and propidium iodide were not visible to the human eye using fluorescence microscopy but were visible in photographs taken of the same slides (exposure time of 1 minute). Therefore, there was a possibility of miscounting spores.

4.4 Heat Resistance Determinations

The D-value of both *K. thyrsites* and *K. paniformis* was determined at three different temperatures (Tables 4 and 5). Only phosphate buffered saline was used for determining D-value since particulate matter and protein would also stain using methylene blue. The log survivor curves for *K. paniformis* in phosphate buffered saline (PBS, pH 7) are displayed in Figures 5-7. The three temperatures used were 53°C, 62°C, and 69°C. The average D-value at 53°C was 18 hours, while at 62°C and 69°C the average D-value was 4.5 hours and 0.80 hours, respectively. D-value determinations were also attempted at 90°C; however, 100% of the *K. paniformis* spores were stained blue at the 0-time sampling mark (the time at which PBS and spores reached 90°C following inoculation of the preheated PBS), which took about 1 minute. The thermal death time curve for *K. paniformis* can be viewed in Figure 8. The resulting z-value was 12C°.

The log survivor curves for *K. thyrsites* are shown in Figures 9-11. Experimental temperatures included 43°C, 52°C, and 60°C. The average D-value at 43°C was 3.0 hours, and at 52°C and 60°C the average D-value was 1.4 hours and 0.030 hours, respectively. The z-value for *K. thyrsites* was 8.9C° (Figure 12). The present study provides the first reports about thermal resistance of *K. thyrsites* and *K. paniformis* spores.

Table 4. Decimal reduction times (D-value) of *K. paniformis*.

Temperature (°C)	Equation	r^2	D-value (h)
53	y = -0.0550x + 6.3545	0.8077	18
53	y = -0.0602x + 6.3330	0.9187	17
53	y = -0.0522x + 6.3103	0.8797	19
62	y = -0.2313x + 6.5071	0.9654	4.3
62	y = -0.1464x + 6.5416	0.9135	6.8
62	y = -0.2480x + 6.3072	0.9931	4.0
62	y = -0.2690x + 6.2914	0.9011	3.7
62	y = -0.2632x + 6.2034	0.9528	3.8
69	y = -1.3532x + 5.7934	0.9762	0.74
69	y = -1.2225x + 5.8718	0.9798	0.82
69	y = -1.1713x + 5.9241	0.9735	0.85

Table 5. Decimal reduction times (D-value) of *K. thyrsites*.

Temperature (°C)	Equation	r^2	D-value (h)
43	y = -0.4044x + 5.5341	0.9091	2.5
43	y = -0.3244x + 5.5101	0.9338	3.1
43	y = -0.3004x + 5.6989	0.8859	3.3
43	y = -0.3194x + 5.7836	0.7628	3.1
52	y = -0.6870x + 5.3333	0.9836	1.5
52	y = -0.7832x + 5.5273	0.9873	1.3
52	y = -0.6589x + 5.2249	0.9990	1.5
60	y = -31.596x + 5.7795	0.9984	0.032
60	y = -38.487x + 5.3558	0.9724	0.026
60	y = -32.082x + 5.3512	0.9872	0.031

Figure 5. Survivor curve at 53°C for *K. paniformis* in phosphate buffered saline pH 7.0

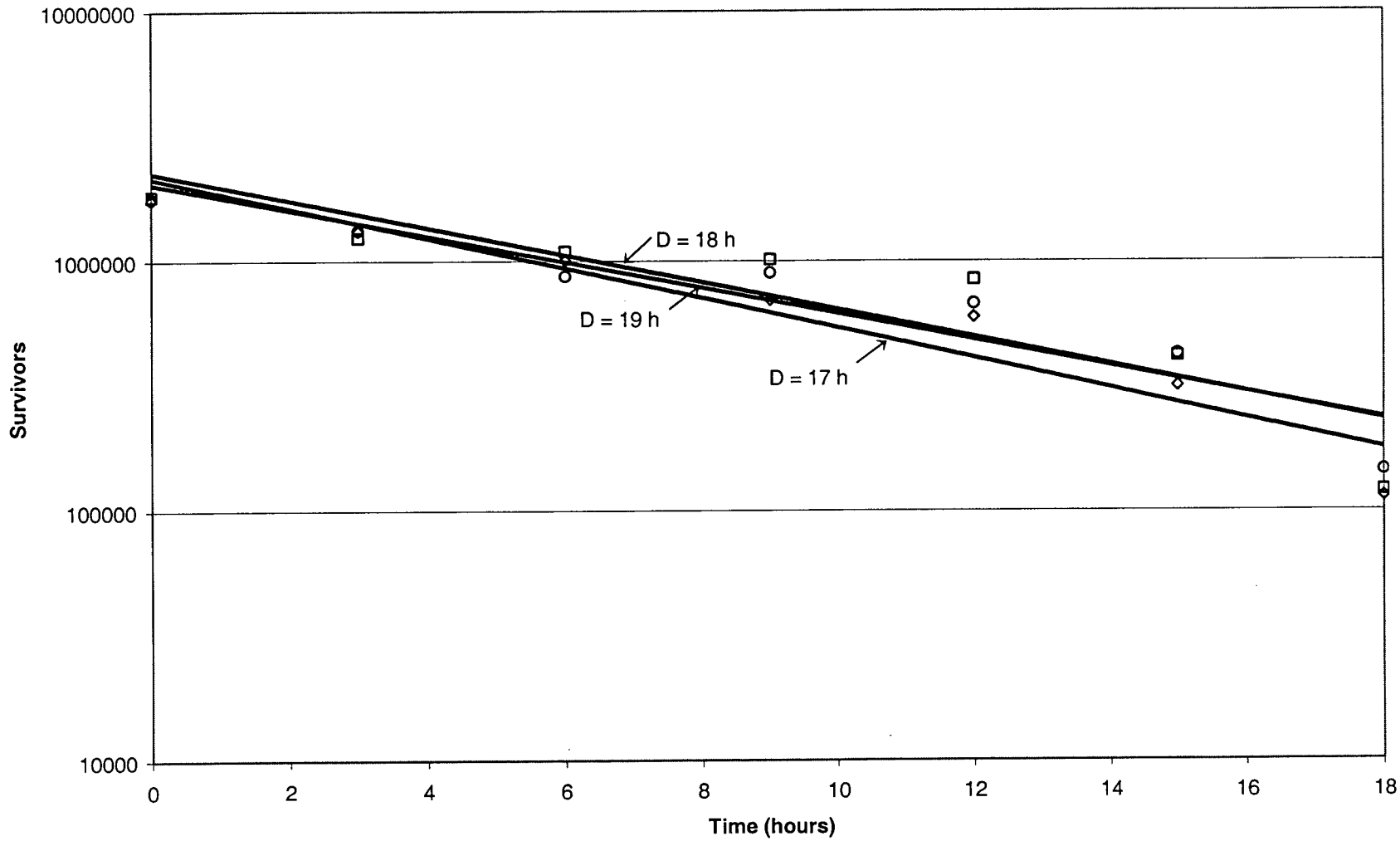


Figure 6. Survivor curve at 62°C for *K. paniformis* in phosphate buffered saline pH 7.0

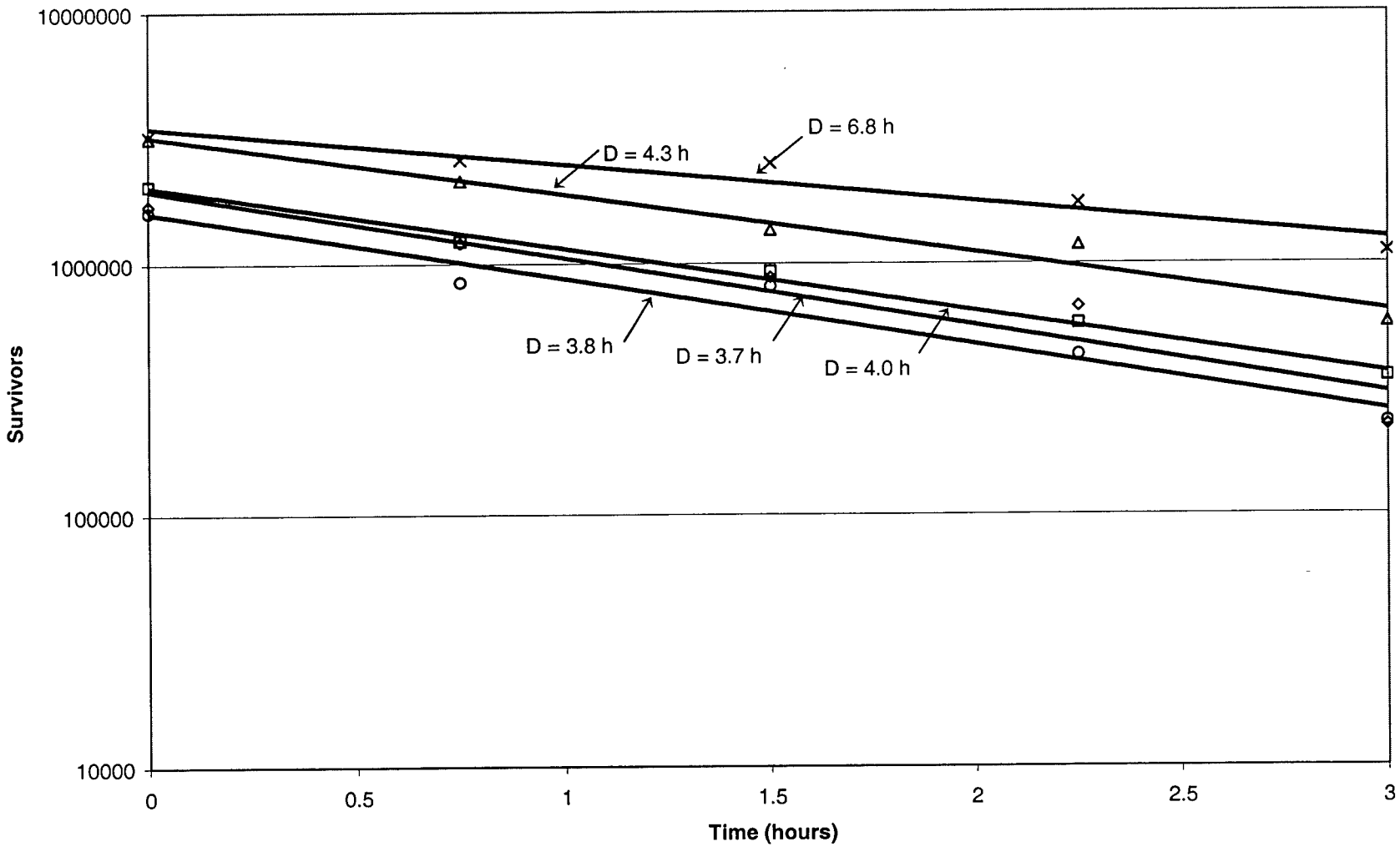


Figure 7. Survivor curve at 69°C for *K. paniformis* in phosphate buffered saline pH 7.0

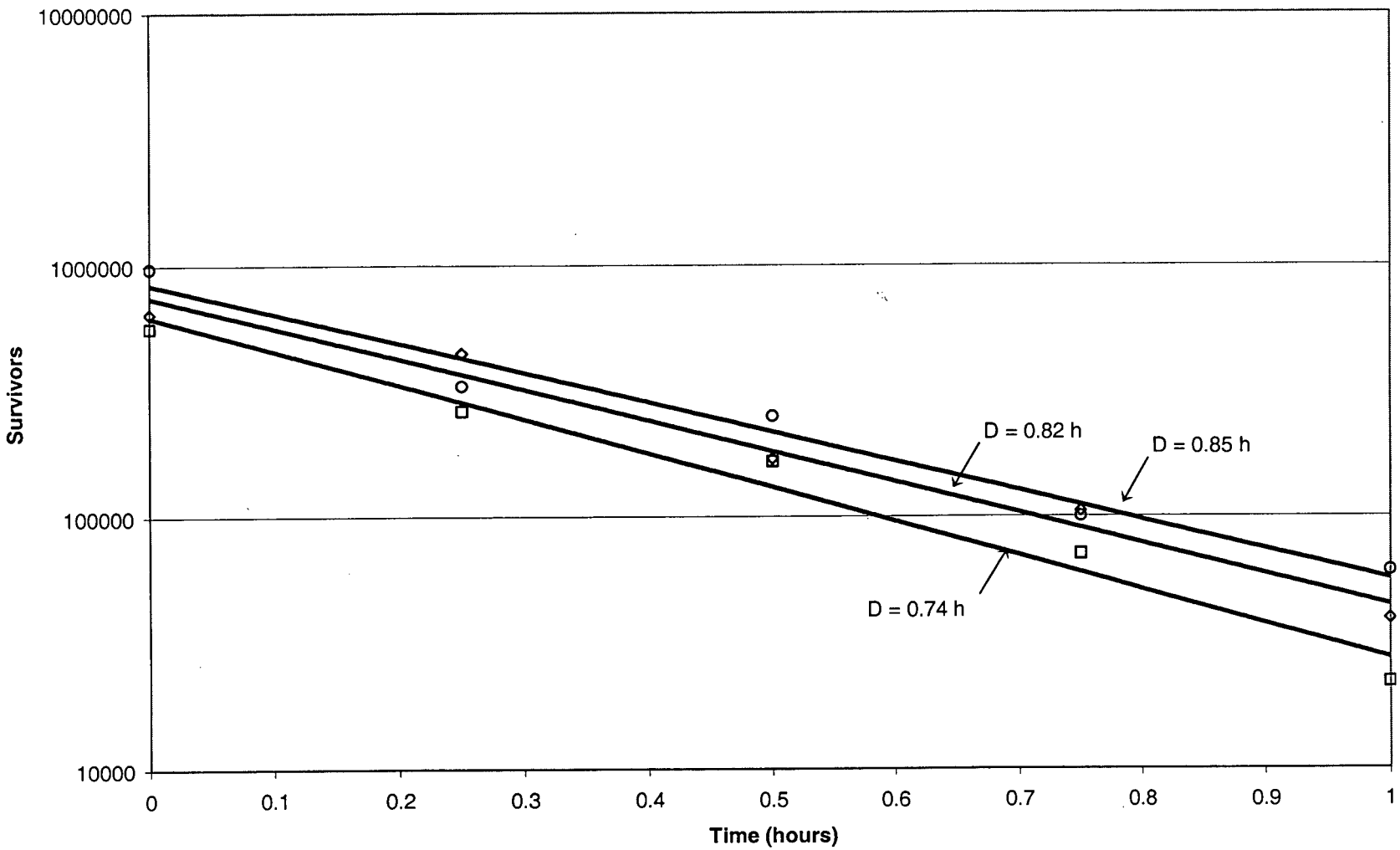


Figure 8. Thermal death temperature sensitivity curve for *K. paniformis* in phosphate buffered saline pH 7.0.

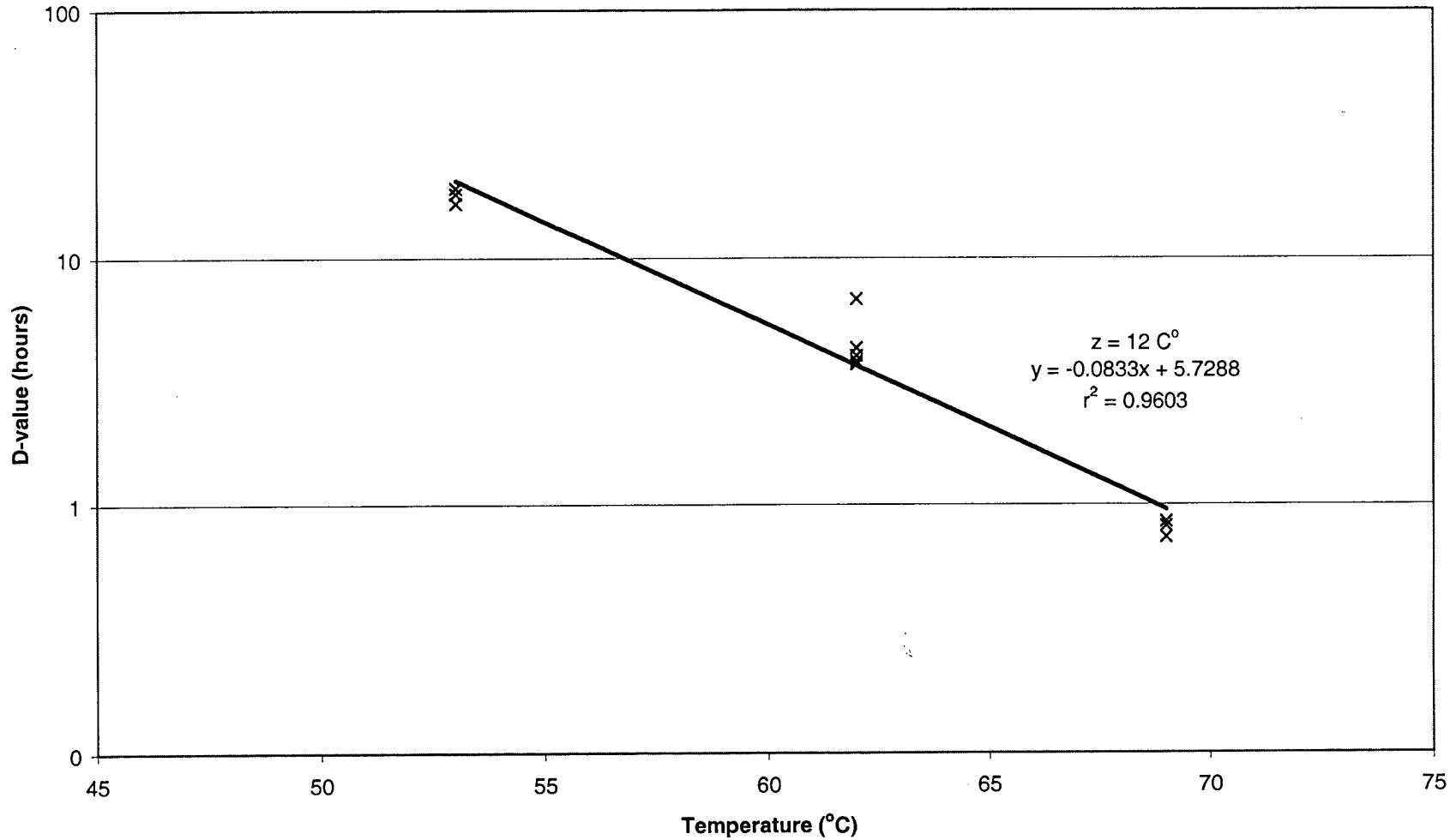


Figure 9. Survivor curve at 43°C for *K.thyrsites* in phosphate buffered saline pH 7.0

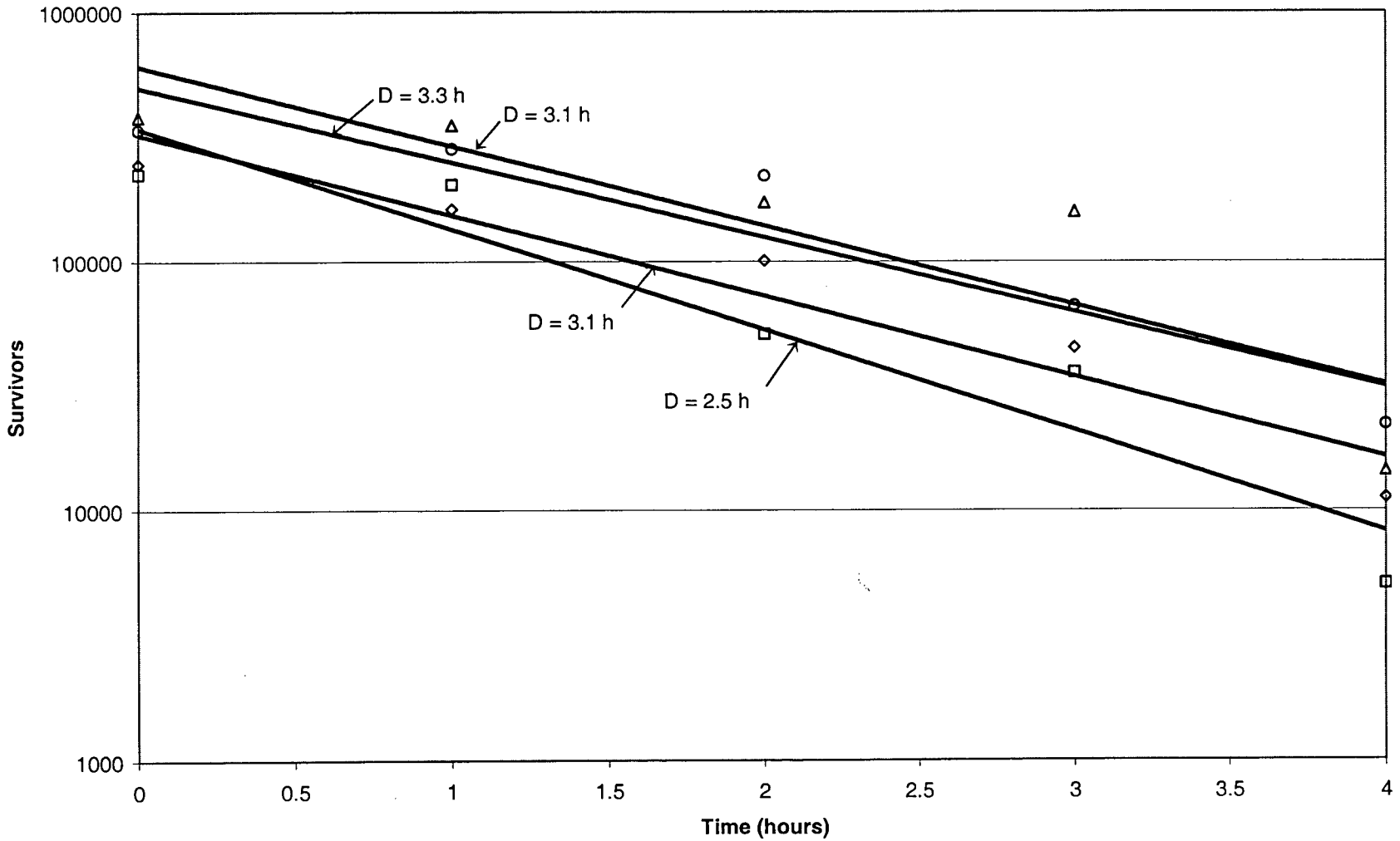


Figure 10. Survivor curve at 53°C for *K.thyrsites* in phosphate buffered saline pH 7.0

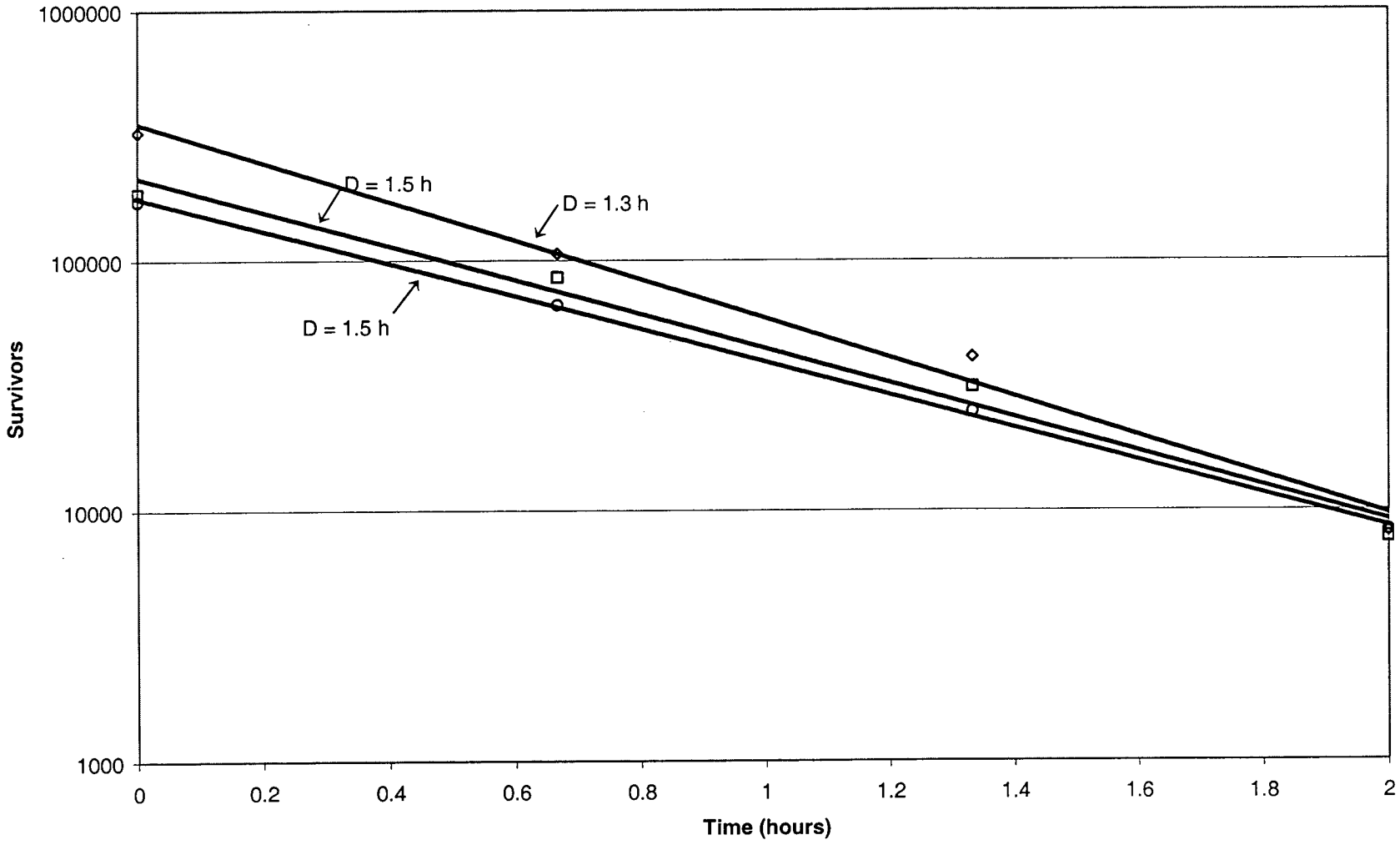


Figure 11. Survivor curve at 60°C for *K. thyrsites* in phosphate buffered saline pH 7.0

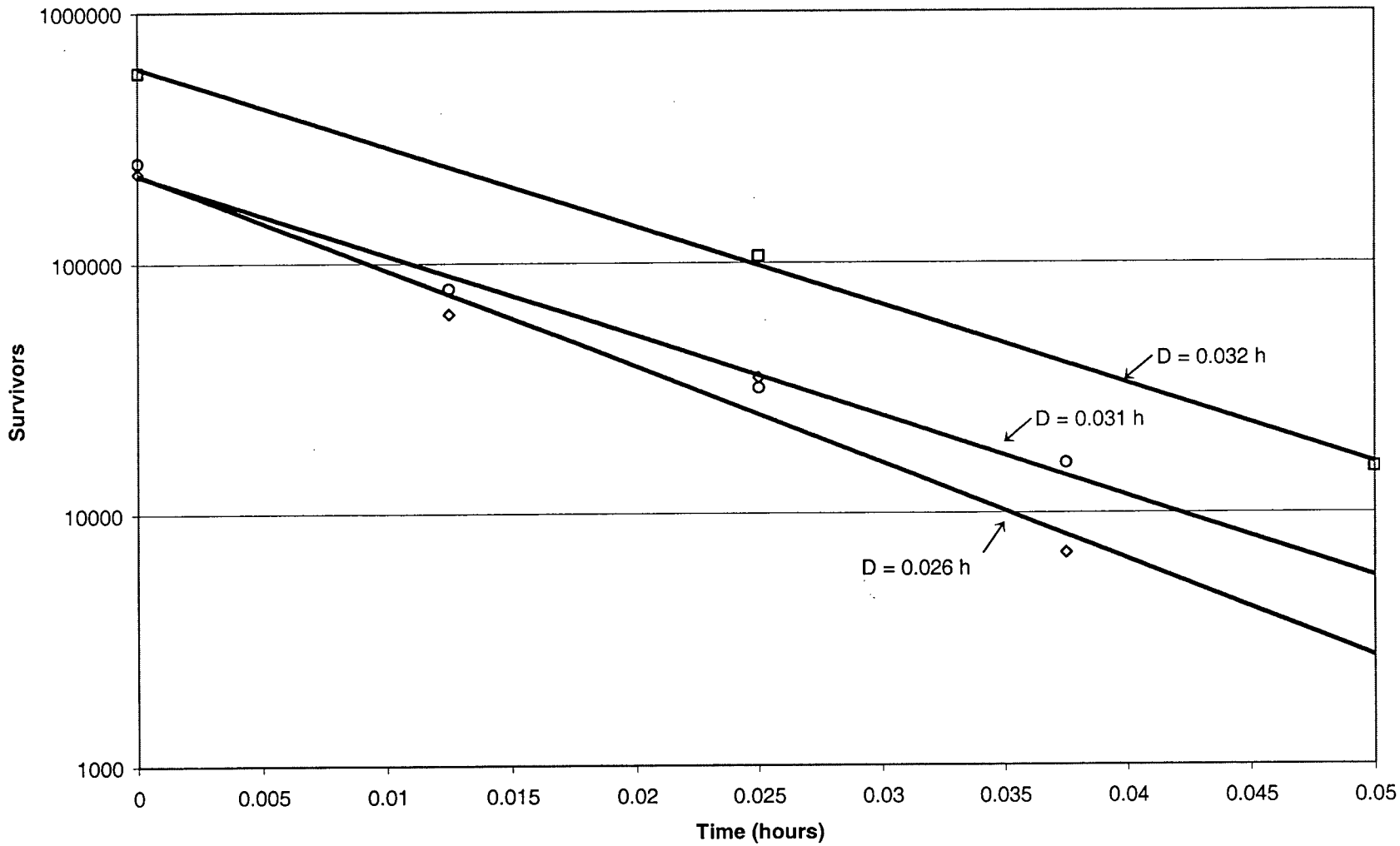
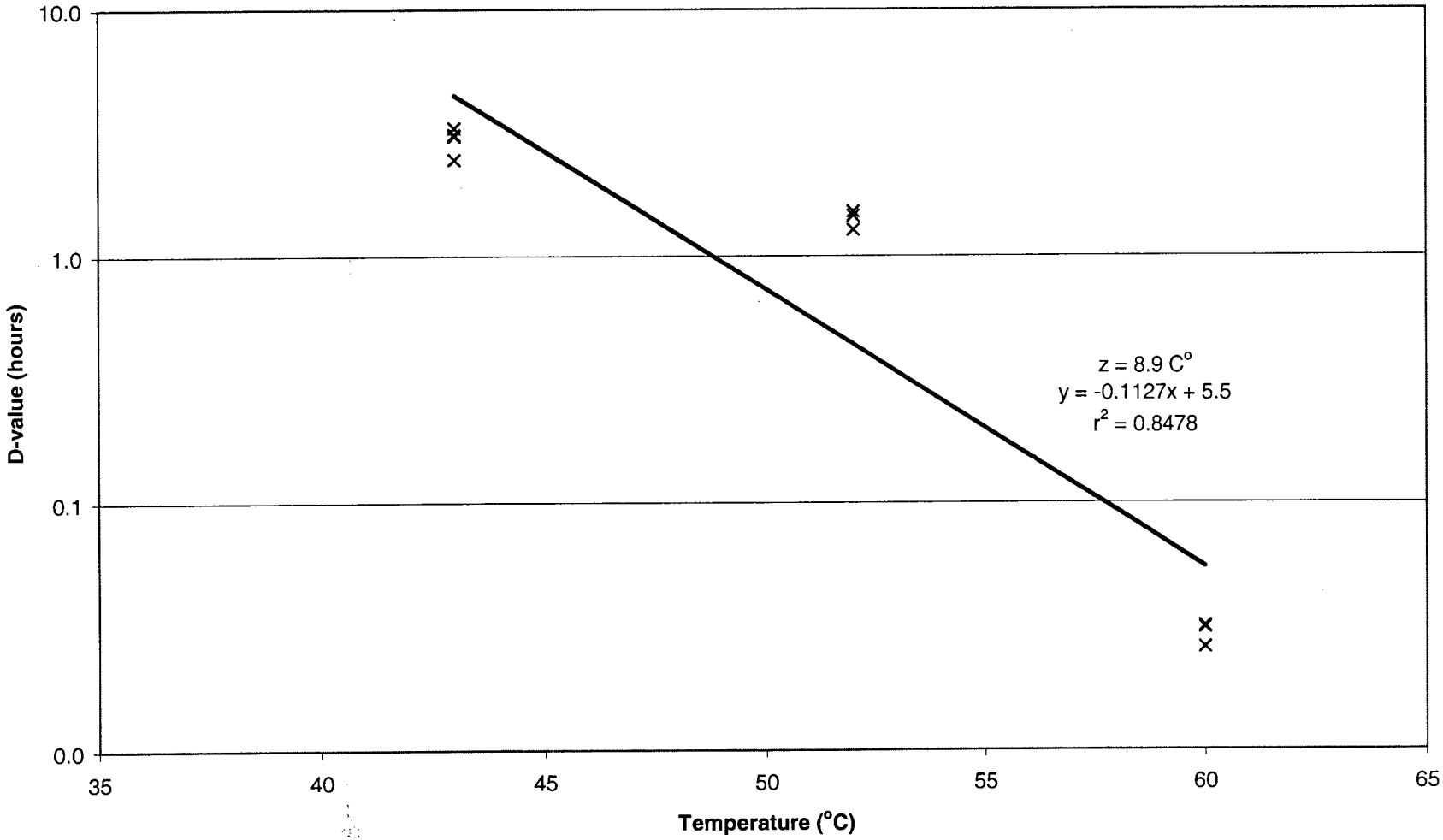


Figure 12. Thermal death temperature sensitivity curve for *K.thyrsites* in phosphate buffered saline pH 7.0



The cooking unit operation of fish meal manufacturing at the rendering plant in question occurs at a minimum of 85°C and a maximum of 95°C for approximately 15 minutes (Figure 13). Extrapolating from the thermal death time curve (Figure 4), the D-value of *K. paniformis* at 85°C would be 0.044 hours or 2.7 minutes. Atlantic salmon, though, have not been found to be a host for *K. paniformis* (Moran et al., 1999b).

The concern about using Pacific hake fish meal for manufacturing fish feed lies in the presence of *K. thyrsites*, which has been associated with post-mortem myoliquefaction of Atlantic salmon fillets. In the present study, *K. thyrsites* was less heat resistant than *K. paniformis*. Extrapolating from Figure 12, *K. thyrsites* would have a D-value of 8.3×10^{-5} hours or 0.30 seconds at 85°C. This means that every 0.30 seconds, 90% of *K. thyrsites* spores present would become nonviable in a PBS system (pH 7) at 85°C. Therefore, if one started with 1×10^{6} spores, 1×10^{5} would remain after heating at 85°C for 0.30 seconds. If the same system was heated for a total of 2.1 seconds, one would expect only 1 spore to survive. In a fish system, we would expect that the spores may survive for a longer period of time. One of the main reasons is that proteins may have a "protective effect on microorganisms" (Jay, 2000). This has been demonstrated with bacterial populations. For example, *Streptococcus faecalis* had a D-value at 140°F of 1-5 minutes in saline; however, in various foods the D-value at the same temperature increased to 10.5-15.3 minutes (Spencer, 1967). Reported D-values of *Salmonella senftenberg* 775W at 65°C in 0.1M phosphate buffer (pH 6.5) was 0.29 minutes. In liquid whole egg (pH ~6.6), the

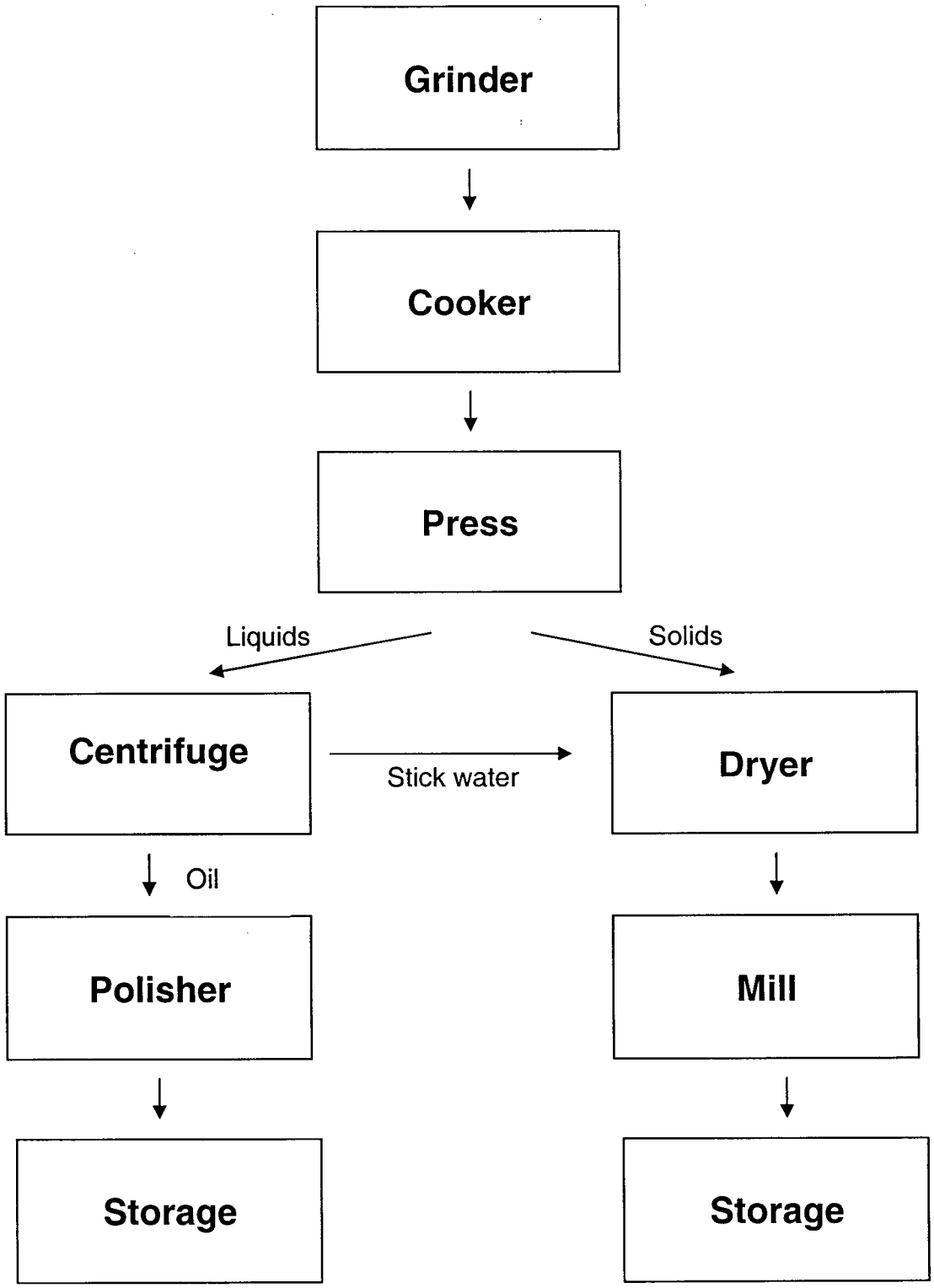


Figure 13. Flow chart of fish meal processing.

D-value was 9.0 minutes at 60°C (Jay, 2000). The mechanism by which proteins protect microorganisms against heat, however, are still unknown (Manas et al., 2001). Manas et al. (2001) determined that the protein fraction and divalent cations were responsible for the protective effect in milk.

Drying (Figure 13) is performed following cooking in the fish meal manufacturing process. The cooked meal is dried for one hour, with the dryer having a midpoint temperature of 120°C. At the start of drying, the fish meal has a higher water activity than at the end of drying. Previous studies indicate that microbial cells are more resistant to heat at lower water activities (Jay, 2000; Joslyn, 1991). Therefore, it is important to avoid any cross-contamination after drying since microorganisms will be protected by the lower water activity of the fish meal. In order to manufacture the fish meal into fish feed additional heating processes occur, such as extrusion that would further reduce the chance of *K. thyrsites* spores surviving.

Another possible source of *K. thyrsites* spores in the fish meal rendering process is the fish oil. All wet mounts prepared directly from the fish oil failed to show any signs of *K. thyrsites* when observed with a phase contrast microscope at 200x magnification. When an aqueous suspension of spores (200µl) was mixed with the oil (1ml), the *K. thyrsites* spores tended to clump together in the aqueous phase. After heating the oil and spore mixture at 96°C some spores became mixed in the oil phase and some remained in the aqueous phase. Spores in both phases appeared darker in colour. The part between the sharp processes of the spores appeared

shrunken inwards compared to their original morphology. The spores also tended to clump together. *K. thyrsites* spores heated in PBS alone appeared darker in colour than those heated in the oil preparation. In addition, some spores also appeared shrunken inward between the sharp processes. These spores also tended to clump together. It is likely that *K. thyrsites* spores would fractionate to the aqueous phase during fish meal manufacturing, especially during the centrifuging operation where oil is separated from the aqueous portion of the cooked fish meal mash.

The fish meal rendering plant is not only concerned about end product quality, but is equally concerned about biological, physical, and chemical hazards. Therefore, the plant has a hazard analysis and critical control points (HACCP) system present. The plant is designed so that the likelihood of cross contamination between raw, unprocessed fish and the manufactured fish meal is extremely low. Both the plant schematics and the actual plant were examined. These observations confirmed that cross-contamination was unlikely to occur. Given the current processing conditions, the likelihood of *K. thyrsites* spores surviving the manufacturing process is low. In addition, the *K. thyrsites* parasite is accustomed to seawater temperatures, which therefore increases the likelihood that *K. thyrsites* could not survive the fish meal manufacturing process and subsequent fish feed manufacturing.

If, however, *K. thyrsites* survived fish meal processing and subsequent fish feed manufacturing there is the possibility that *K. thyrsites* may be introduced into naïve

areas if an intermediate host exists. Feed that is not consumed by farmed salmon will break down over time. If *K. thyrsites* remains viable, the intermediate host may become infected and subsequently infect farmed salmon. Although the likelihood of *K. thyrsites* surviving fish meal and fish feed manufacturing is low, this possibility needs to be considered.

5.0 Conclusion

The prevalence of *K. thyrsites* spores in Pacific hake was about 79%, while the prevalence of *K. thyrsites* spores in the Atlantic salmon fillets was 18 out of 20. The present study represents the first heat resistance studies with *K. thyrsites* and *K. paniformis*. The accuracy of the methylene blue test to assess viability is unknown; therefore, it can only be used as an indicator of potential loss of viability. When a method for accurately determining the viability of these parasites exists, the results should be re-examined. In this study, *K. paniformis* was more resistant to heat than *K. thyrsites*. Given the data obtained from this study, however, it is unlikely that *K. thyrsites* will survive fish meal manufacturing.

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Appendix

Temperature Dependence of pH on Buffers

The buffers tested included PBS (0.137M NaCl, 0.007M K_2HPO_4, 0.002M KH2PO_4) and HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid], 0.137M NaCl, 0.01M HEPES) buffer (Sigma Chemical Co., St. Louis, MO, H-3375). In order to determine the most appropriate buffer to use for temperature resistance of *Kudoa*, the change in pH was measured as a result of change in temperature. The pH of the buffer was first measured at ambient temperature. The buffer was then placed into a water bath (Blue M Electric Company, Blue Island, IL, MSB-1122A-1) set at the experimental temperature. When the buffer had reached the desired temperature, the sample was removed and the pH was measured immediately.

It was originally intended that the resistance of *K. thyrsites* to high and low temperatures would be determined. Therefore, preliminary work to determine appropriate buffers for the experiments was performed. Because HEPES buffer is commonly used in low temperature experiments with microorganisms, the buffer was tested for its ability to resist changes in pH at higher temperature to determine its appropriateness as a buffer for D-value studies. The other buffer tested was PBS, since phosphate buffers are commonly used for D-value determinations. The pH change as a result of a change in temperature was measured following heating the buffer at the appropriate temperature until the desired temperature was achieved (Tables 5 to 10). Changes in pH as a result of a change in temperature were greater for HEPES buffer than PBS. In addition, as the temperature increased the

differences in pH also increased. However, pH change was greater in HEPES buffer than in PBS. Therefore, PBS appears to be a more appropriate buffer to use for the temperature sensitivity experiments since the pH changes less with the tested temperature than HEPES buffer.

Table 1. Change in pH of PBS and HEPES buffer after change from room temperature to 40°C.

PBS				HEPES			
Temp (°C)	**pH**	**Temp (°C)**	**PH**	**Temp (°C)**	**pH**	**Temp (°C)**	**pH**
24.04	7.14	41.06	7.12	30.03	7.10	40.87	7.03
24.45	7.15	43.37	7.12	32.40	7.10	41.25	7.00
24.51	7.14	41.58	7.11	30.68	7.10	42.82	7.01
24.53	7.14	42.75	7.10	31.24	7.10	42.51	7.00
24.90	7.15	41.62	7.11	31.04	7.12	42.01	7.01
*24.49**	*7.14*	*42.08*	*7.11*	*31.08*	*7.10*	*41.89*	*7.01*
*0.31***	*0.01*	*0.95*	*0.01*	*0.87*	*0.01*	*0.82*	*0.01*

* The mean of the column values.

** The standard deviation of the column values.

Table 2. Change in pH of PBS and HEPES buffer after change from room temperature to 50°C.

PBS				HEPES			
Temp (°C)	**pH**	**Temp (°C)**	**PH**	**Temp (°C)**	**pH**	**Temp (°C)**	**pH**
31.01	7.13	51.66	7.08	31.35	7.09	51.86	6.94
30.88	7.12	50.08	7.08	31.36	7.10	52.45	6.93
30.99	7.13	53.28	7.09	31.37	7.10	51.97	6.93
31.10	7.14	50.47	7.09	31.37	7.09	51.47	6.93
31.13	7.14	51.26	7.09	31.47	7.09	52.33	6.93
31.02	*7.13*	*51.35*	*7.09*	*31.38*	*7.09*	*52.02*	*6.93*
0.10	*0.01*	*1.25*	*0.01*	*0.05*	*0.01*	*0.39*	*0.00*

* The mean of the column values.

** The standard deviation of the column values.

Table 3. Change in pH of PBS and HEPES buffer after change from room temperature to 60°C.

PBS				HEPES			
Temp (°C)	**pH**	**Temp (°C)**	**PH**	**Temp (°C)**	**pH**	**Temp (°C)**	**pH**
24.98	7.13	59.55	7.07	24.64	7.15	60.13	6.90
25.03	7.14	59.11	7.08	25.14	7.17	61.71	6.89
25.22	7.13	61.01	7.09	25.30	7.14	60.58	6.89
25.42	7.12	59.04	7.07	25.54	7.14	62.52	6.89
25.45	7.11	60.57	7.09	25.54	7.14	60.31	6.88
25.22	*7.13*	*59.86*	*7.08*	*25.23*	*7.15*	*61.05*	*6.89*
0.22	*0.01*	*0.89*	*0.01*	*0.37*	*0.01*	*1.03*	*0.01*

* The mean of the column values.

** The standard deviation of the column values.

Table 4. Change in pH of PBS and HEPES buffer after change from room temperature to 70°C.

PBS				HEPES			
Temp (°C)	**pH**	**Temp (°C)**	**PH**	**Temp (°C)**	**pH**	**Temp (°C)**	**pH**
25.77	7.12	70.80	7.06	25.86	7.15	70.41	6.81
26.55	7.11	70.52	7.05	26.49	7.12	70.23	6.77
26.12	7.11	70.98	7.05	26.10	7.14	70.73	6.75
26.33	7.13	70.42	7.06	26.32	7.14	70.35	6.76
26.57	7.12	70.74	7.06	26.69	7.13	70.90	6.74
26.27	*7.12*	*70.69*	*7.06*	*26.29*	*7.14*	*70.52*	*6.77*
0.33	*0.01*	*0.22*	*0.01*	*0.32*	*0.01*	*0.28*	*0.03*

* The mean of the column values.

** The standard deviation of the column values.

Table 5. Change in pH of PBS and HEPES buffer after change from room temperature to 80°C.

PBS				HEPES			
Temp (°C)	**pH**	**Temp (°C)**	**PH**	**Temp (°C)**	**pH**	**Temp (°C)**	**pH**
26.43	7.11	80.09	7.07	26.64	7.15	80.14	6.69
26.36	7.12	80.15	7.06	26.82	7.12	80.42	6.70
26.37	7.12	80.28	7.08	26.66	7.14	80.76	6.64
26.40	7.13	80.22	7.06	26.90	7.14	80.23	6.68
26.52	7.12	80.31	7.07	26.25	7.13	80.08	6.67
26.42	*7.12*	*80.21*	*7.07*	*26.65*	*7.14*	*80.33*	*6.68*
0.06	*0.01*	*0.09*	*0.01*	*0.25*	*0.01*	*0.27*	*0.02*

* The mean of the column values.

** The standard deviation of the column values.

Table 6. Change in pH of PBS and HEPES buffer after change from room temperature to 90°C.

PBS				HEPES			
Temp (°C)	**pH**	**Temp (°C)**	**PH**	**Temp (°C)**	**pH**	**Temp (°C)**	**pH**
26.52	7.11	88.57	7.03	26.82	7.12	88.36	6.65
27.08	7.11	87.92	7.04	27.18	7.12	87.90	6.65
26.98	7.12	87.96	7.05	27.44	7.12	88.16	6.68
27.14	7.14	88.56	7.06	27.25	7.11	88.04	6.64
27.07	7.12	89.10	7.06				
26.96	*7.12*	*88.42*	*7.05*	*27.17*	*7.12*	*88.12*	*6.66*
0.25	*0.01*	*0.49*	*0.01*	*0.26*	*0.01*	*0.19*	*0.02*

* The mean of the column values.

** The standard deviation of the column values.