THE EFFECT OF NATIVE AND LABORATORY SALINITY LEVELS ON SEED GERMINATION OF CAREX LYNGBYEI

by

Yu-Ting Cheng

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Name: Yu-Ting Cheng

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Examining Committee:

Chairman: Dr. G. Bojadziev

_____

Dr. D. Eaves
Senior Supervisor

Dr. R. Lockhart

Dr. C. Villegas

Dr. P. Kim
External Examiner

Date Approved: September 12, 1988

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The effect of native and laboratory salinity levels on seed germination of Carex Lyngbyei

Author:

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Yu-Ting Cheng

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ABSTRACT

Survival analysis is applied to study the germination of *Carex Lyngbyei* seeds for the first time. Of the four environmental covariates concerned, the difference among three deltas and the water salinity are found to have significant influence on the germination process. The results support the theory regarding seed germination in general.

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DEDICATION

To my family

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Carex Lyngbyei is a clonal, perennial sedge (marsh plant) that occupies estuarine marshes of the North American Pacific coast, from northern California to Alaska (Macdonald 1977). It is a plant which plays an essential role in the energetics and food-web dynamics of the Pacific estuaries. It provides food indirectly as detritus to fish and aquatic birds, its seeds are consumed directly by waterfowl, songbirds and other estuarine animals. Therefore *Carex Lyngbyei* is a critical plant species in marsh rehabilitation and creation projects designed to enhance wildlife and fish habitat.

Carex Lyngbyei is a species belonging to halophytic family. Studies on this family indicate that the capacity of a species to germinate and establish itself under saline conditions is one of the important factors governing its distribution (Ungar and Hogan 1970). There are many variables which affect the germination of *Carex Lyngbyei* seeds, such as salinity, nutrition and the temperature of the environment, etc. Among these variables, salinity seems to be the most important one. For example, when the salinity level is less than 5 ppt, *Carex Lyngbyei* seeds germinate quite well; but when the salinity level is greater than 10 ppt, the germination process is delayed greatly. A more notable differentiating characteristic of halophytes is their ability to remain dormant at high

salinities, and then germinate when conditions are better (i.e. fresh water is encountered).

The objective of this study is to determine the effect of salinity levels on the germination of *Carex Lyngbyei* seeds.

In the literature on the seed germination of halophytic species, statistical methods used to determine the effect of various environment variables are almost solely based on analyses of percentages, such as percentage of germination at different observational time points, or on mean time of germination. These methods are easy to use and usually give fairly good description of the pattern of seed germination, but it is not suitable for data that has been censored.

Although multivariable statistical methods have been employed in geographical studies (Bartlett and Noble, 1985), there seems to have been no application of multivariable methods to the study of seed germination. In this study, we try to study the effect of environmental variables on the germination of *Carex Lyngbyei* seeds by performing statistical survival analysis.

The idea is this: the history and the germination process of a *Carex Lyngbyei* seed are observed together with some environmental variables (we shall call these variables covariates later) such as salinity of the environment etc. If a seed germinates, we consider this as the "death" of the seed; if a seed does not germinate until the end of the observation

period, we consider the "life time" of the seed censored. With this in mind, the usual survival analysis framework fits our problem perfectly.

One advantage of using survival analysis methods in the above context is that the effect of several covariates on the germination of *Carex Lyngbyei* seeds can be studied simultaneously. Also, the whole germination process can be studied, while the usual method (such as percentage analysis) can study the germination process at a few observational time points.

The plan for this study is the following. A description of the data is given in chapter 2. In chapter 3, the models and the related statistical methods to be used are supplied. The data are analyzed in chapter 4, and the conclusions are presented in chapter 5, together with some discussion.

CHAPTER II

DATA

In February 1985, Susan R. Smythe, a Geography graduate student of Simon Fraser University, carried out a study on the germination of *Carex Lyngbyei* seeds.

Sampling Scheme

The sampling scheme for this study involved four sample sites from each of three river deltas.

The three river deltas, Squamish, Skagit and Nanaimo, were chosen on the basis of a study by Hutchinson (1988). The Squamish River delta is basically fresh to oligohaline (0.5 to 5 ppt salt) during the growing season, the Skagit River delta is oligohaline to mesohaline (5 to 18 ppt salt), and the Nanaimo River delta is essentially polyhaline (18 to 30 ppt salt). Within each delta, sampling was carried out along gradients of the physical variables deemed to be the abiotic factors with the greatest influence on plant growth - salinity and elevation. Two zones characterized by different salinity levels are chosen from each delta, then two zones with different elevation levels (high,low) are chosen from each salinity level. The resultant sampling scheme and the codes are illustrated in Figure 2.1. At each of the sample sites, the percentage of time submerged was recorded. Few seeds were found in NAFL and SKSL; seeds from

Squamish (Fresh)	Fresh	High (SQFH)
		Low (SQFL)
	Saline	High (SQSH)
		Low (SQSL)
Skagit (Intermediate)	Fresh	High (SKFH)
		Low (SKFL)
	Saline	High (SKSH)
		Low (SKSL)
Nanaimo (Saline)	Fresh	High (NAFH)
		Low (NAFL)
	Saline	High (NASH)
		Low (NASL)

Figure 2.1 Sampling scheme and the codes

these two sites are not available for use in the experiment. Because of low seed production in the NASH and NASL populations, the number of seeds for use in the germination experiment in these areas had to be reduced.

Germination Experiments

Most halophytes will greatly reduce germination at salinities ≥ 10 ppt and remain dormant until the conditions are better for them to germinate again. It is because of this nature of the *Carex Lyngbyei*, the experiment was, therefore, conducted into two phases; phase I and phase II. Phase I is where seeds were placed in the different salinity levels namely 0, 5, 10, 15 and 30 ppt. This is to imitate the natural condition of *Carex Lyngbyei* seeds which have encountered different salinity levels. The salinity levels are classified as:

0 and 5 ppt refer to fresh and low salinity level. 10 ppt refers to a fairly high salinity level. 15 ppt refers to high salinity level and 30 ppt is considered as sea water level. Phase II is to imitate the situation where seeds that have remained dormant after encountering high salinity levels, resume the germination process when the conditions are better.

In phase I, the germination tests, which determine whether or not a seed germinates and the time it takes to germinate, were carried out on two sheets of Whatman No. 1 filter paper in 100 mm petri dishes. Fifty seeds were placed in each dish.

Initially, five salinity treatment levels were prepared, (0, 5, 10, 15, 30 ppt), produced by adding distilled water to seawater. Within each treatment level, four replicate dishes (five ml of solution were added to each dish) were used per site except for NASH and NASL for which only two replicated dishes were used. During this first phase, the dishes were examined 4, 7, 10, 13, 20, 27, 34 and 41 days after the experiment was initiated. At these times, seeds that had germinated (defined as emergence of the radicle or plumule) were counted and removed, and filter paper was replaced to prevent salinity build-up through evaporation. Water loss was corrected twice a week. Of the 9000 seeds, 885 seeds germinated in 0 ppt treatment and 189 seeds germinated in 5, 10, 15, 30 ppt treatments.

In phase II, seeds that did not germinate in treatment 5, 10, 15, 30 ppt after 47 days (there had been no germination since day 41), were rinsed in distilled water and transfered to new filter paper in clean petri dishes. Each dish was then treated with 5 ml of distilled water. The dishes were inspected 4, 8, 12, 17, 20, 26, 33, 40, 47, 54, and 61 days afterwards. In this phase, 2828 seeds out of the total 7011 seeds germinated.

The number of Squamish, Skagit and Nanaimo seeds which germinated in each salinity treatment within each counting period in phase I and phase II are presented in Appendix I.

Defining Covariates

Based on the above design and experiment, we define the response variable and covariates to be analyzed as below.

1. TOBS : Survival time
   (the time that took a seed to germinate)
   (in days after initiation of Phase I or II)
2. DONM : Donor marsh
   1 = Nanaimo river delta (DON1)
   2 = Skagit river delta (DON2)
   3 = Squamish river delta (DON3)
3. TSAL : Treatment salinity magnitude (0, 5, 10, 15, 30)
4. SITE : 1 = Fresh and High
   2 = Saline and High
   3 = Fresh and Low
   4 = Saline and Low
5. PSUB : Percent annual submergence

Among the above variables, survival time (TOBS) is the response variable, DONM, TSAL, SITE, and PSUB are covariates. In particular, PSUB is considered as the history of *Carex Lyngbyei* seeds.

CHAPTER III

STATISTICAL METHODOLOGY

For ease of reference, the statistical models and methods to be used in this study are gathered in this chapter. To do this, the basic concepts of survival distributions are reviewed first, then the survival models, the methods of estimation and the techniques for fitting survival models to data are described in detail.

3.1 Survival Distribution and its Estimation

3.1.1 Survival Distribution

Let T be a nonnegative random variable representing the survival time (also called lifetime or failure time) of an individual. The probability distribution of T can be specified in many ways, three of which are particularly useful in survival analysis, namely, the survival function, the probability density function, and the hazard function. Definitions and relationships among these three representations are given below.

1. *Survival Function S(t)* is the probability that an individual survives until at least time t (t>0). That is, S(t) is the probability that T is greater then t (t>0), or in symbols,

$$S(t) = Pr(T > t) \qquad 0 \leq t < +\infty.$$

Related to S(t) is the cumulative distribution funtion F(t),

$$F(t) = Pr(T \leq t)$$

thus

$$S(t) = 1 - F(t).$$

2. *Probability Density Function f(t)* is the rate that an individual dies at time t. Assuming that F(t) is differentiable, the probability density function of T is

$$f(t) = \frac{dF(t)}{dt}. \qquad 0 \leq t < +\infty.$$

3. *Hazard Function h(t)* specifies the instantaneous rate of death at time t conditional upon survival to time t, that is,

$$h(t) = \frac{f(t)}{S(t)} .$$

The cumulative hazard function of T is defined as

$$H(t) = \int_0^t h(u)du = -\ln S(t).$$

The relationships among S(t), f(t), and h(t) are as the following:

$$\text{(i)} \quad f(t) = -\frac{dS(t)}{dt},$$

$$\text{(ii)} \quad h(t) = -\frac{d\ln S(t)}{dt},$$

$$\text{(iii)} \quad S(t) = \exp\left(-\int_0^t h(u)du\right).$$

When the survival process of each individual is observed together a number of covariates denoted by a vector $\underset{\sim}{z} = (z_1, \cdots, z_s)^T$, the above functions are denoted by $f(t;\underset{\sim}{z})$, $S(t;\underset{\sim}{z})$ and $h(t;\underset{\sim}{z})$, respectively.

3.1.2 Product Limit Estimate of the Survival Function

There are several methods of estimating survival functions. One of them, which is suitable for survival analysis, is called the product limit method.

Let $t_1, t_2, \cdots, t_k$ represent the observed failure times in a sample of size n from a population with survival function S(t). Suppose that d_i individuals died at t_i ($i = 1, \cdots, k$) and m_i individuals are censored in the interval $[t_i, t_{i+1})$. Then $n_i = (m_i+d_i)+ \cdots + (m_k+d_k)$ is the number of individuals at risk at a time just prior to t_i. If a censored time equals a failure time t_i, the convention is that the censored time is included in the set of n_i individuals at risk at t_i. The survival function is thus estimated by

$$\hat{S}(t) = \prod_{i:t_i<t} [\ (n_i - d_i)/n_i\ ] .$$

The above estimate is called the product limit estimate (also known as the Kaplan-Meier estimate). It is obtained by making the conditional probability of death at each t_i agree exactly with the observed conditional relative frequency of death at t_i given by d_i/n_i. This will be used to estimate the survival curve in section 4.1.2.

3.2 K-sample Mantel's Test

Suppose one wishes to test whether the survival curves obtained for the groups are equal, one approach would involve the use of K-sample Mantel's test.

Let k be the number of groups (or categories of a covariate) for individuals whose survival distributions are to be compared. Let $t_1 < t_2 < \cdots < t_h$ be the times at which deaths occurred among the k groups and let n be the total number of individuals. The null hypothesis is that the k groups have the same survival distribution.

At time t_i, let n_{ij} be the number of individuals in group j in the study (that is, whose observation time t is greater than or equal to t_i). Let x_{ij} be the number of individuals who died at exactly time t_i in group j. (If there are no tied times, x_{ij} is zero for all but one group; $x_{ij} = 1$ for the group where death occurs.)

Conditioned on the n_{ij} and the sum $x_{i+} = \sum_{j=1}^{k} x_{ij}$, the vector $\underline{x}_i = (x_{i1},\cdots,x_{i(k-1)})^T$ has a k-1 dimensional hypergeometric distribution with mean vector

$$E(\underline{x}_i) = (E(x_{i1}),\cdots,E(x_{i(k-1)}))^T,$$

where

$$E(x_{ij}) = (x_{i+}n_{ij})/n_{i+}, \quad i = 1, 2, \cdots, h,$$
$$j = 1, 2, \cdots, k-1.$$

The covariance matrix V_i of $\underline{x}_i$ has elements

$$\text{cov}(x_{ij},x_{il}) = \frac{n_{ij}(\delta_{jl} - n_{il}/n_{i+})x_{i+}(n_{i+} - x_{i+})}{n_{i+}(n_{i+} - 1)},$$

where $\delta_{jl} = 1$ if $j = l$

$= 0$ if $j \neq l$

$n_{i+} = \sum_{j=1}^{k} n_{ij}$, $j, l = 1,2,\cdots,k-1$.

Let

$$E = \sum_{i=1}^{h} E(\underset{\sim}{x}_i)$$

$$O = \sum_{i=1}^{h} \underset{\sim}{x}_i,$$

$$V = \sum_{i=1}^{h} V_i.$$

Then the j-th element of O - E is like $\sum_{i=1}^{h}(x_{ij} - x_{i+}n_{ij}/n_{i+})$.

Mantel's test statistic is

$$X_M^2 = (\underset{\sim}{O} - \underset{\sim}{E})^T \underset{\sim}{V}^{-1}(\underset{\sim}{O} - \underset{\sim}{E}),$$

which is asymptotically distributed as chi-square with k-1 degrees of freedom and large values of X_M^2 indicate that the null hypothesis is false (Lawless 1982). We perform Mantel's test in section 4.1.2.

3.3 Cox Proportional Hazards Model

Let $h(t;\underset{\sim}{z})$ represent the hazard function at time t for an individual with covariates $\underset{\sim}{z} = (z_1,\cdots,z_s)^T$. The *Cox proportional hazards model* (Cox, 1972) specifies that

$$h(t;\underset{\sim}{z}) = h_0(t)\exp(\underset{\sim}{\beta}^T\underset{\sim}{z}) \quad . \qquad (1)$$

where β is a vector of s regression parameters and $h_0(t)$ is an arbitrary unspecified base-line hazard function for an individual with covariate vector $z = 0$. Since $h_0(t)$ is an arbitrary funtion (distribution-free), the Cox model is only semiparametric. Although the function $\exp(\beta^T z)$ is usually used in practice because it naturally guarantees that $h(t) \geq 0$ for all values β, other non-negative functions of the covariates could be used in principal. The fundamental assumption implied by model (1) is that the covariates taken together have the same multiplicative effect on the hazard at all points in time. That is, the ratio of the hazard functions for two individuals with different covariate vectors does not depend on time. The survival function and the density function, take the following forms under model (1).

$$f(t;z) = h_0(t) \exp(\beta^T z) \exp(- e^{\beta^T z} \int_0^t h(u)du), \qquad (2)$$

$$S(t;z) = (S_0(t))^{\exp(\beta^T z)}, \qquad (3)$$

$$S_0(t) = \exp(- \int_0^t h_0(u)du). \qquad (4)$$

When a covariate does not affect the hazard multiplicatively, Kalbfleisch and Prentice (1980) suggest stratifying the data so that cases within each stratum conform to the proportional hazards model. Suppose there is a covariate that occurs on q levels and for which (1) may be violated, we define the hazard function for an individual in the jth stratum of this covariate as

$$h_j(t;\underline{z}) = h_{0j}(t)\exp(\underline{\beta}^T\underline{z}) \qquad j=1,\cdots,q. \qquad (5)$$

That is, individuals in the same stratum have proportional hazard functions, but this is not necessarily the case for individuals in different strata. In (5) it is also assumed that the relative effect of the regressor variables is the same in all strata.

3.4 Maximum Likelihood Estimation

As mentioned before, $\underline{z}$ is a vector of s measured covariates, and $\underline{\beta}$ is a vector of s regression parameters in model (1). In this section, the method of partial likelihood (Cox,1975) is applied to estimate the parameters $\underline{\beta}$.

As before, let $t_1 < t_2 < \cdots < t_k$ represent k distinct time to death among n observed individuals. Let R_i be the group of individuals at risk (not previously dead or censored) at observed death time t_i. If all individuals were alike, then the probability of death for the particular individual would simply be $1/R_i$. However, the individuals are not all alike, so one must weight the probability for each individual according to its hazard as given by equation (1). If there are no ties in death time, then the conditional probability under the proportional hazard model that an individual with covariate vector $\underline{z}_i$ dies at time t_i given that the set R_i is at risk is:

$$h(t_i;\underline{z}_i) \;/\; \sum_{j\in R_i} h(t_i;\underline{z}_j) \qquad i = 1,\cdots,k\ .$$

Because the base-line hazard, $h_0(t)$, is shared by all individuals, it cancels out from this probability and the contribution to the probability at each distinct death time is given by

$$\exp(\underline{\beta}^T\underline{z}_i) \;/\; \sum_{j\in R_i} \exp(\underline{\beta}^T\underline{z}_j) \qquad i = 1,\cdots,k\ .$$

Multiplying these probabilities together for each of the k death times gives the *partial likelihood function*:

$$L(\underline{\beta}) = \prod_{i=1}^{k} [\ \exp(\underline{\beta}^T\underline{z}_i) \;/\; \sum_{j\in R_i} \exp(\underline{\beta}^T\underline{z}_j)\ ].$$

When there are ties among death times, a modified likelihood function (Breslow, 1974) is:

$$L(\underline{\beta}) = \prod_{i=1}^{k} \{\ \exp(\underline{\beta}^T\underline{s}_i) \;/\; [\ \sum_{j\in R_i} \exp(\underline{\beta}^T\underline{z}_j)]^{m_i}\ \}, \qquad (6)$$

where m_i is the number of deaths at t_i and $\underline{s}_i$ is the vector sum of the covariates of the m_i individuals.

The estimation of the parameters $\underline{\beta}$ is based on the partial likelihood (6) in this study. By using the Newton-Raphson method, the partial likelihood (6) is maximized to get a maximum partial likelihood estimate $\hat{\underline{\beta}}$ of $\underline{\beta}$. It has been shown by Tsiatis (1981) that the maximum partial likelihood estimates thus obtained are consistent and asymptotically normal, therefore asymptotically, the behavior of $\hat{\underline{\beta}}$ from maximizing the partial

likelihood function (6) is just like that of ordinary maximum likelihood estimates.

With the stratified model (5), a partial likelihood function $L_j(\beta)$ of the form (6) is obtained for each stratum, and then the overall partial likelihood function for β is

$$L(\beta) = \prod_{i=1}^{q} L_i(\beta). \qquad (7)$$

By maximizing (7), we can get a m.p.l.e. $\hat{\beta}$ of β.

3.5 A Graphical Method for Checking the Cox Model Assumptions

When fitting the Cox model to the data, it is necessary to check whether the proportionality assumption holds or not. The proportionality assumption requires that the ratio of hazard functions for two individuals with different vectors of covariates does not depend on time. Suppose there is a covariate that has q levels (or strata), we define the hazard function for an individual in jth level (or stratum) of this covariate as

$$h_j(t;z) = h_{0j}(t)\exp(\beta^T z) \qquad j = 1, 2, \cdots, q .$$

Let $S_j(t;z)$ be the survival function for the jth level of this covariate :

$$S_j(t;z) = \exp[\, -\exp(\beta^T z) \int_0^t h_{0j}(u)du \,].$$

If we take natural logarithms twice of both sides, we obtain

$$\ln[-\ln S_j(t;\underline{z})] = \underline{\beta}^T\underline{z} + \ln[\int_0^t h_{0j}(u)du]. \qquad (8)$$

The proportionality assumption says that the ratio of hazard functions does not depend on time, that is, the ratio $h_{0j}(t)$ / $h_{0i}(t)$ must be constant. If the ratio $h_{0j}(t)$ / $h_{0i}(t)$ is constant, then plotting log cumulative hazard functions, $\ln[\int_0^t h_{0j}(u)du]$ and $\ln[\int_0^t h_{0i}(u)du]$, should yield constant difference between the curves. Thus the equation (8) can be used to check whether this covariate can be studied by proportional hazards model by plotting

$$\ln[-\ln \hat{S}_j(t;\bar{\underline{z}})] \quad \text{versus} \quad t, \quad j = 1, \cdots, q.$$

Such plots for any two values of j should exhibit approximately constant differences over time (i.e. they should appear parallel), where $\hat{S}_j(t;\bar{\underline{z}})$ is the estimator of $S_j(t;\bar{\underline{z}})$ and $\bar{\underline{z}}$ is the mean vector of the covariates from stratum j.

In this project, we use the BMDP package to draw the graphs of $\ln[-\ln \hat{S}_j(t;\bar{\underline{z}})]$ versus t where $\hat{S}_j$, $j = 1,\cdots,q$, is estimated by using the estimated cumulative hazard function $\hat{H}_j(t;\bar{\underline{z}})$.

For the entire unstratifed sample, this function $\hat{H}(t;\underline{z})$ is calculated using the method of Link (1979):

$$\hat{H}(t;\underline{z}) = \exp(\hat{\underline{\beta}}^T\underline{z}) \int_0^t \hat{h}_0(u)du$$

$$= \exp(\hat{\underline{\beta}}^T\underline{z}) \left[\sum_{i=1}^{k}(t_i - t_{i-1})\hat{h}_{0i} + (t-t_k)\hat{h}_{0k+1}\right] \qquad (9)$$

where

$\hat{\underline{\beta}}$ is a m.p.l.e. (by maximizing equation 7)

$$\hat{h}_{0i} = [\ 1\ /\ (t_i - t_{i-1})\ ] \cdot [\ m_i\ /\ \sum_{r \in R_i} \exp(\hat{\beta}^T z_r)\ ]$$

and $t_k < t$, $t_{k+1} \geq t$, $t_0 = 0$.

The overall $\ln[-\ln \hat{S}(t;\bar{z})]$ versus t plot for the entire data set is obtained by plotting the function $\ln[\hat{H}(t;\bar{z})]$ versus t.

For an informal graphical test of the proportional hazards assumption, this calculation can be carried out separately for each stratum j, getting H_j and then $\ln[-\ln \hat{S}_j(t;\bar{z})] = \ln[\hat{H}_j(t;\bar{z})]$.

CHAPTER IV

STATISTICAL ANALYSIS

We are now ready to analyze the data described in Chapter 2. As mentioned before, the seed germination experiment was carried out in two Phases, therefore, the analyses to be done also fall into two parts, one for Phase I, one for Phase II.

The approach to the statistical analyses to be done consists of model identification and model fitting. The interpretation of and conclusions from the fitted models will be discussed in Chapter 5.

4.1 Analysis of Phase I Data

In Phase I, the germination of each seed was observed, together with four covariates, namely, DONM, TSAL, SITE, and PSUB. The observation lasted for 47 days and the time of seed germination is recorded. Because of its non-parametric nature (therefore, wider applicability), the Cox proportional hazards model is fitted to Phase I data.

4.1.1 Checking the Proportionality Assumption

As the first step of fitting a Cox proportional hazards model, the graphical method described in section 3.4 is used to check the proportionality assumption. There are four covariates: DONM, TSAL, SITE, and PSUB. Among them, there are two covariates

(DONM and SITE) which are categorical. The other two covariates are continuous. For covariates DONM and SITE, we can take their own categories to form strata. For the other two continuous covariates, (TSAL and PSUB) appropriate cut points must be found to form strata.

PSUB can be divided into three categories, namely, 0 - 30 %, 51 - 70 %, and 71 - 100 %. The absence of 31 - 50 % is due to the experiment. For TSAL, strata are formed according to the fact that seeds of most halophytic plants germinate best under freshwater conditions; the germination is fairly good at salinities below 5 ppt and is delayed at salinities above 10 ppt (Ungar 1982). Following the above discussion, the coding information for checking the proportionality assumption is summarized in Table 4.1. The results shown in Figure 4.1-4.4 used the method which we have mentioned in section 3.5.

In Figure 4.1, the two curves representing DON1 and DON3 cross each other at the beginning. The time of seed germination in DON3 occured at a later period than DON1 and DON2. This suggests that DONM is probably best not included as a covariate in our model in phase I. In Figure 4.2, only a few seeds germinate in the begining period for TSA3 (above 10 ppt). So the plot of TSA3 appears to be only a short curve. The plots of TSA1 and TSA2 are seen to have approximately constant differences over time, as well as the plots in Figure 4.3 - 4.4. On the whole, this suggests that the proportionality assumption is appropriate for TSAL, SITE, and PSUB but not DONM.

TABLE 4.1

Coding information for checking proportionality assumption in Phase I

Covariates	Levels	Codes	Names	Base - level
DONM	Nanaimo	1	DON1	DON1
	Skagit	2	DON2	
	Squamish	3	DON3	
TSAL	0 ppt	1	TSA1	TSA3
	1 - 5 ppt	2	TSA2	
	10 above ppt	3	TSA3	
SITE	High and Fresh	1	SIT1	SIT1
	High and Saline	2	SIT2	
	Low and Fresh	3	SIT3	
	Low and Saline	4	SIT4	
PSUB	0 - 30 %	1	SUB1	SUB3
	51 - 70 %	2	SUB2	
	71 - 100 %	3	SUB3	

To accomodate covariate DONM, we allow the strata of DONM to have different base-line hazard functions. Again, the proportionality assumption for TSAL, SITE and PSUB will be checked within each level of DONM. Since only a few seeds germinate in DON1, it is very difficult for us to check the proportionality assumption in this level. Besides this, we are also unable to draw the graph of PSUB in DON2 level because the PSUB in DON2 level falls into one category only. Therefore only

FIGURE 4.1

Checking Proportionality for DONM in Phase I

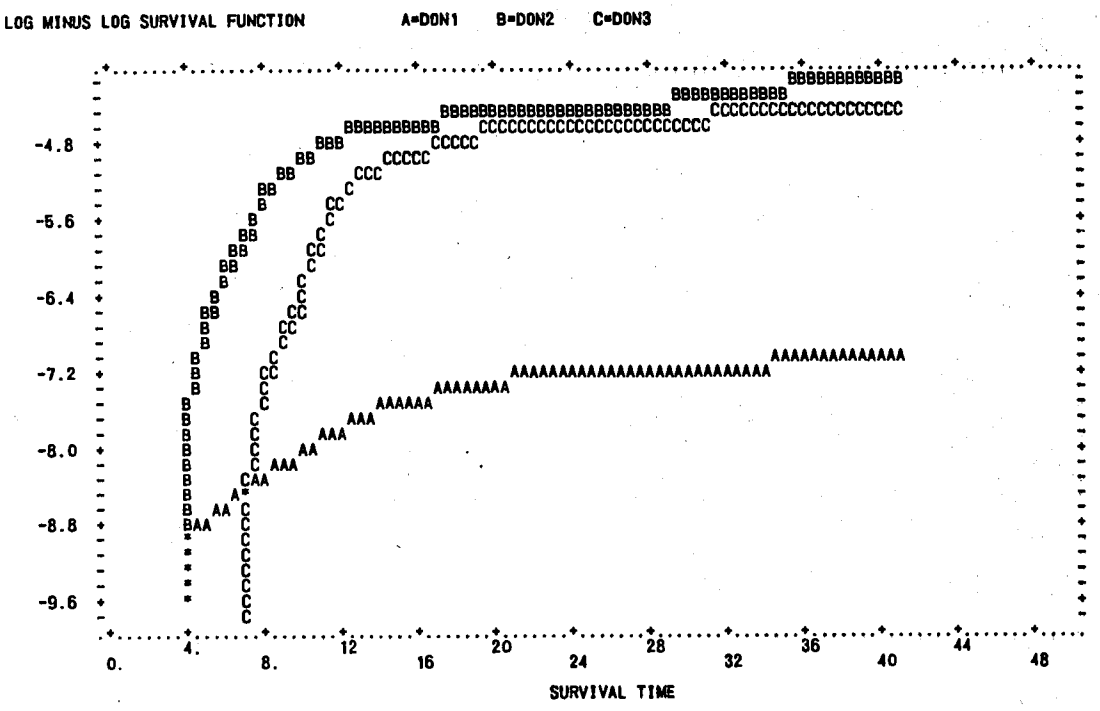


FIGURE 4.2

Checking Proportionality for TSAL in Phase I

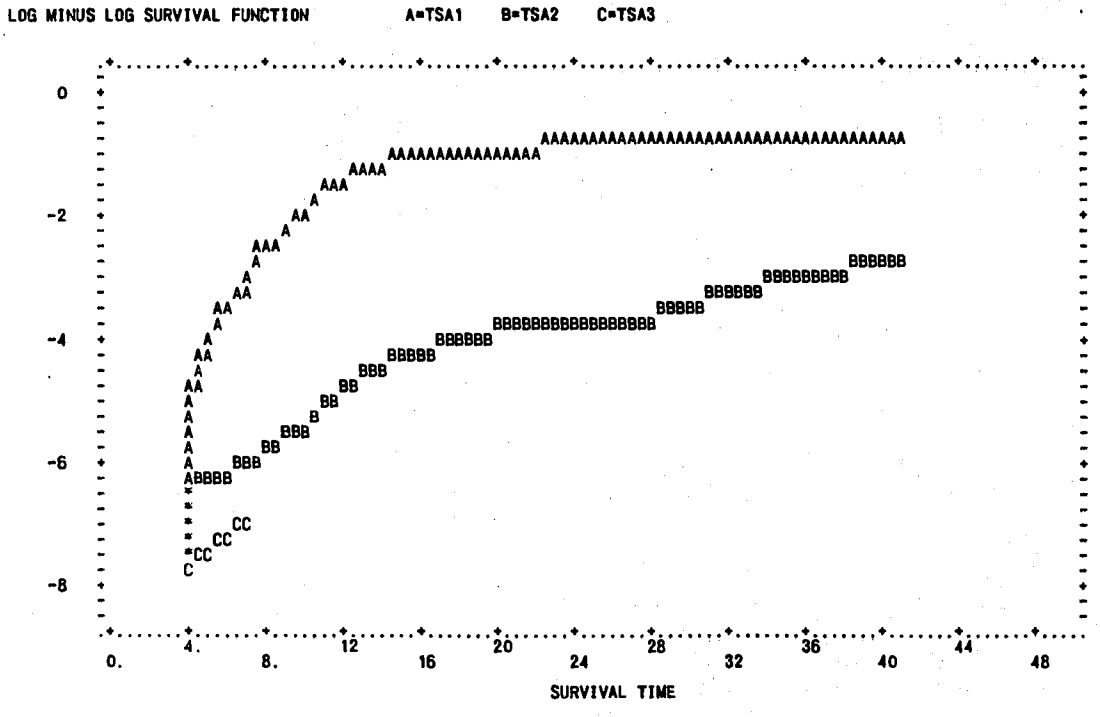


FIGURE 4.3

Checking Proportionality for SITE in Phase I

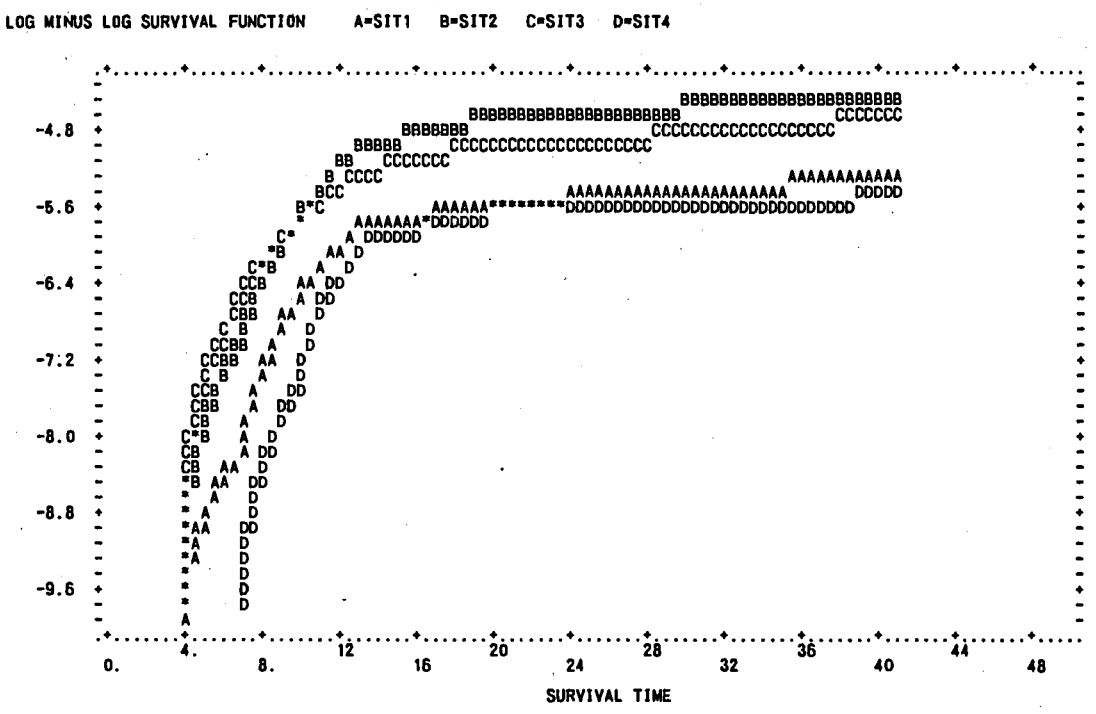
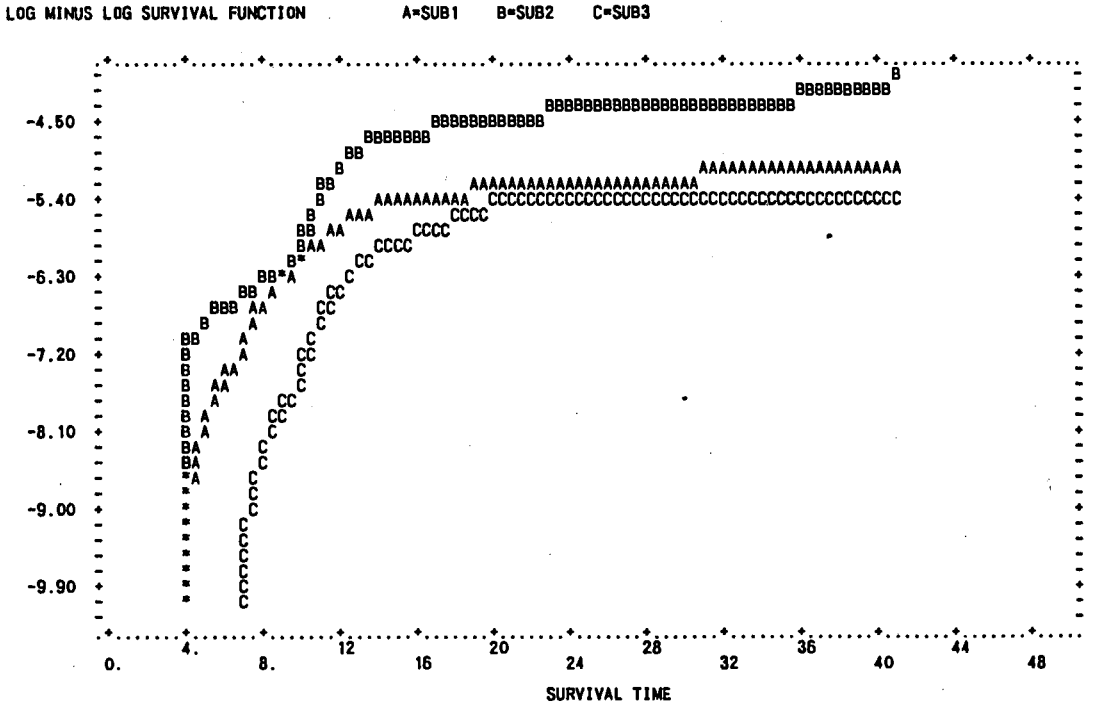


FIGURE 4.4

Checking Proportionality for PSUB in Phase I



the plots of TSAL, SITE, and PSUB in the level of DON3 and plots of TSAL, SITE in the level of DON2 are presented in Figure 4.5 - 4.9. However, only three curves of SITE appear in Figure 4.6 because seeds from one of the sites are not available for this experiment as mentioned in chapter 2. The plots from these figures show that the proportionality assumption for TSAL, SITE, and PSUB is reasonable with the possible exception of TSAL in DON3 (Fig. 4.7). In Figure 4.7, seeds germinate rapidly at TSA1 (0 ppt) in the begining period of the observation and then decline afterward. At TSA2 (5 ppt), seeds germinate rather slowly for the first five weeks and then germinate rapidly in week six. However, since the others covariates do seem to have a proportional effect on survival, we proceed to fit a stratified proportional hazards model to the data.

4.1.2 Fitting Cox Proportional Hazards Model

Specifically, model (5) of section 3.2 is to be fitted. In the present context, each seed is associated with a covariate vector $\underset{\sim}{z} = (z_1, \cdots, z_7)^T$ of seven components. We define the hazard function for an individual in the jth stratum of DONM as

$$h_j(t;\underset{\sim}{z}) = h_{0j}(t)\exp(\sum_{i=1}^{7}\beta_i z_i), \qquad (9)$$

for $j = 1, 2, 3$, where h_{0j} is the base-line hazard function for jth stratum. The p.m.l.e.'s (partial maximum likelihood estimates) along with their estimated standard errors are given in Table 4.2. The calculations were done using a Newton-Raphson iteration as described in section 3.4.

FIGURE 4.5

Checking Proportionality for TSAL within DON2 in Phase I

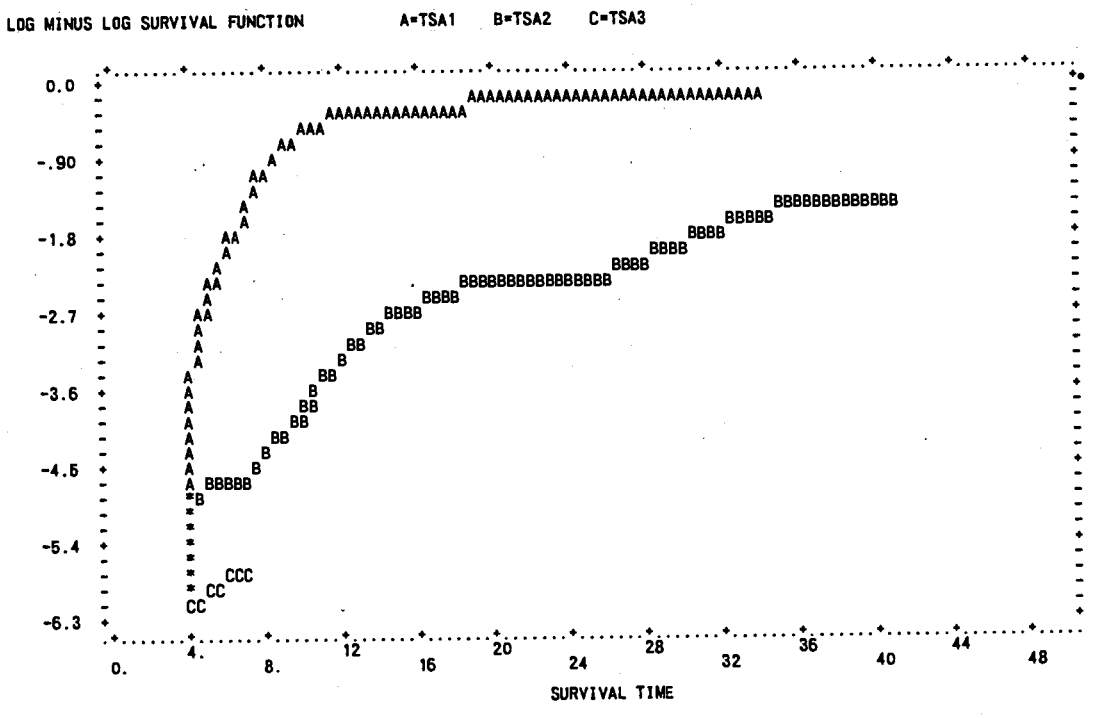


FIGURE 4.6

Checking Proportionality for SITE within DON2 in Phase I

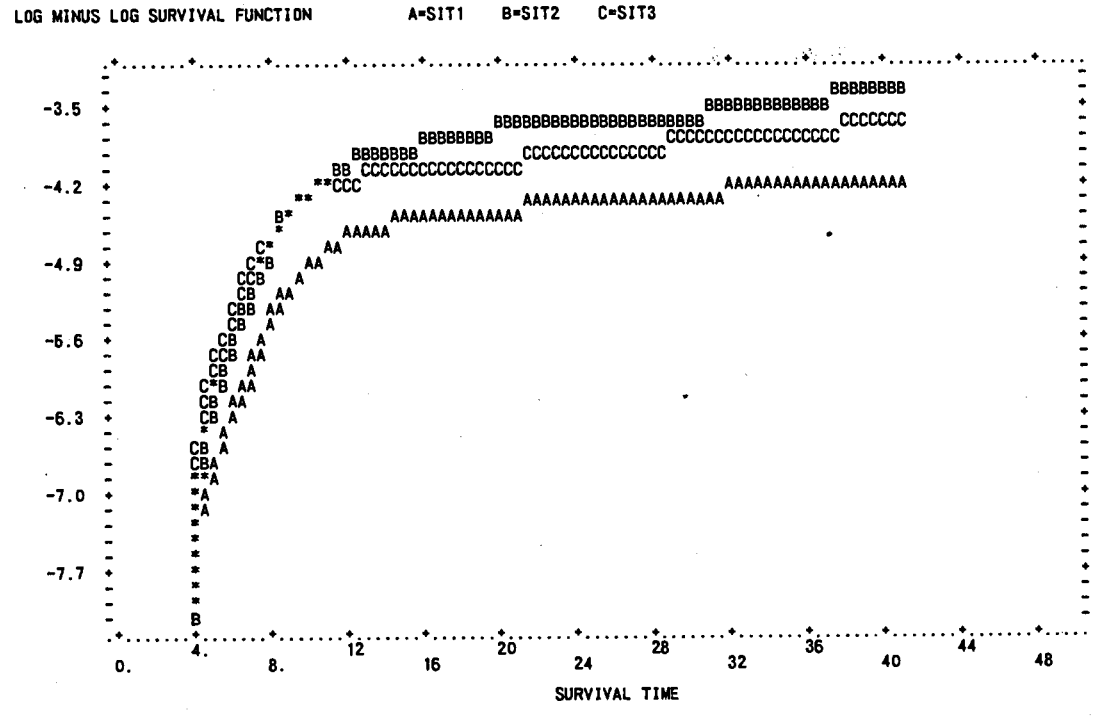


FIGURE 4.7

Checking Proportionality for TSAL within DON3 in Phase I

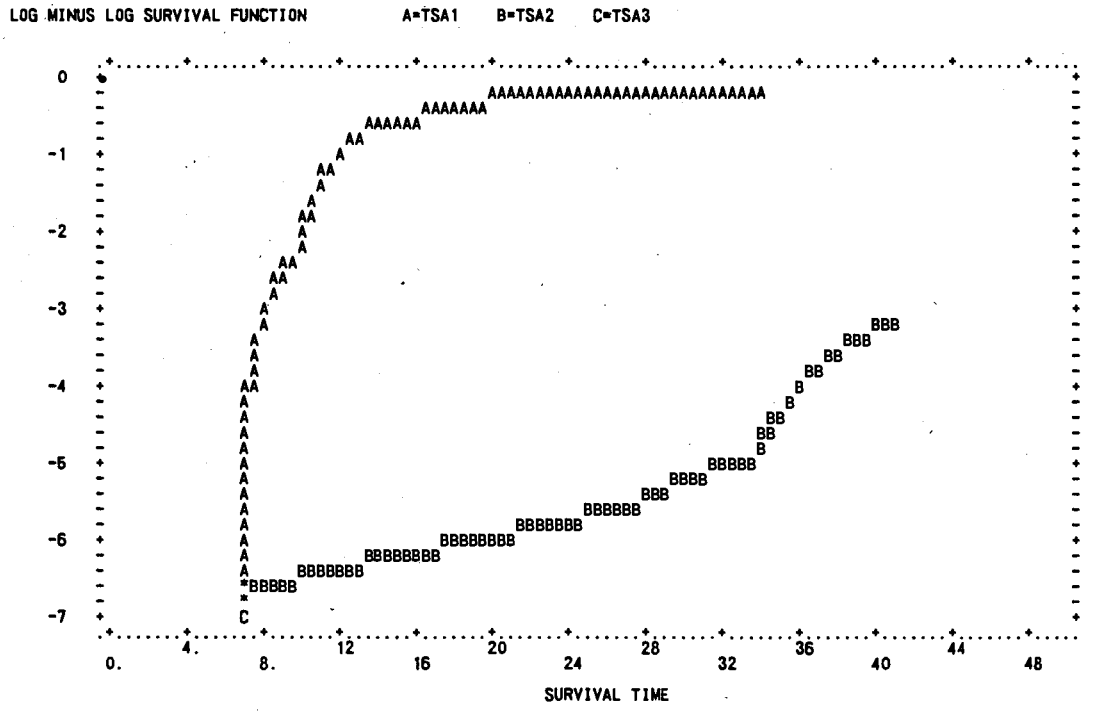


FIGURE 4.8

Checking Proportionality for SITE within DON3 in Phase I

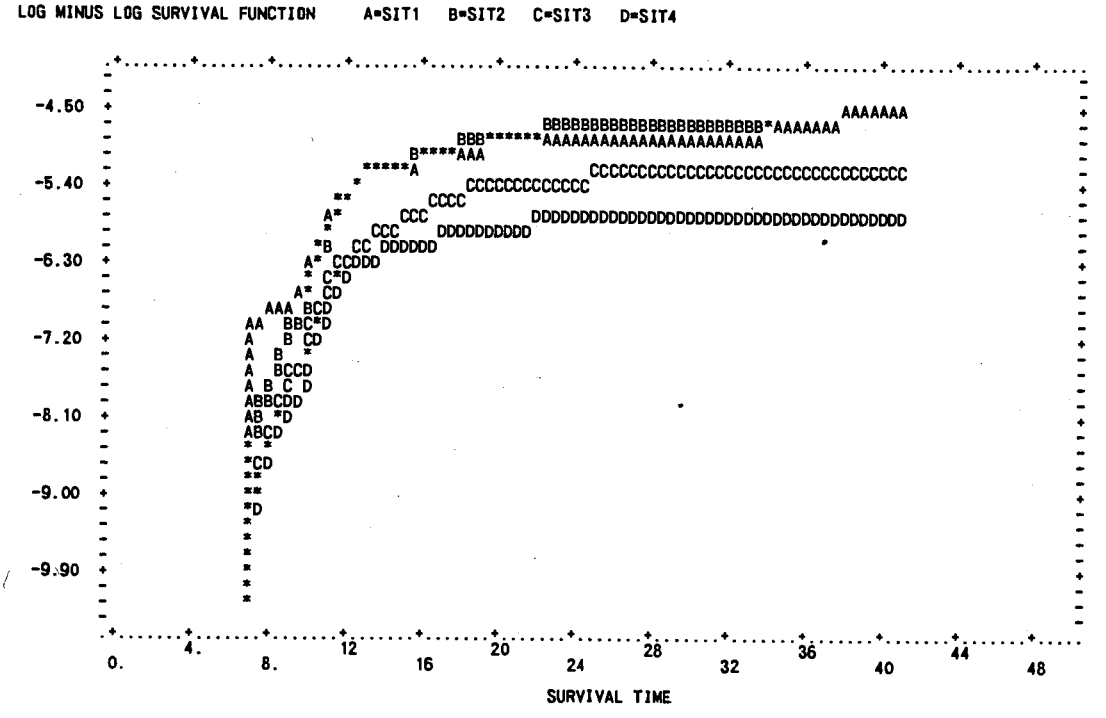


FIGURE 4.9

Checking Proportionality for PSUB within DON3 in Phase I

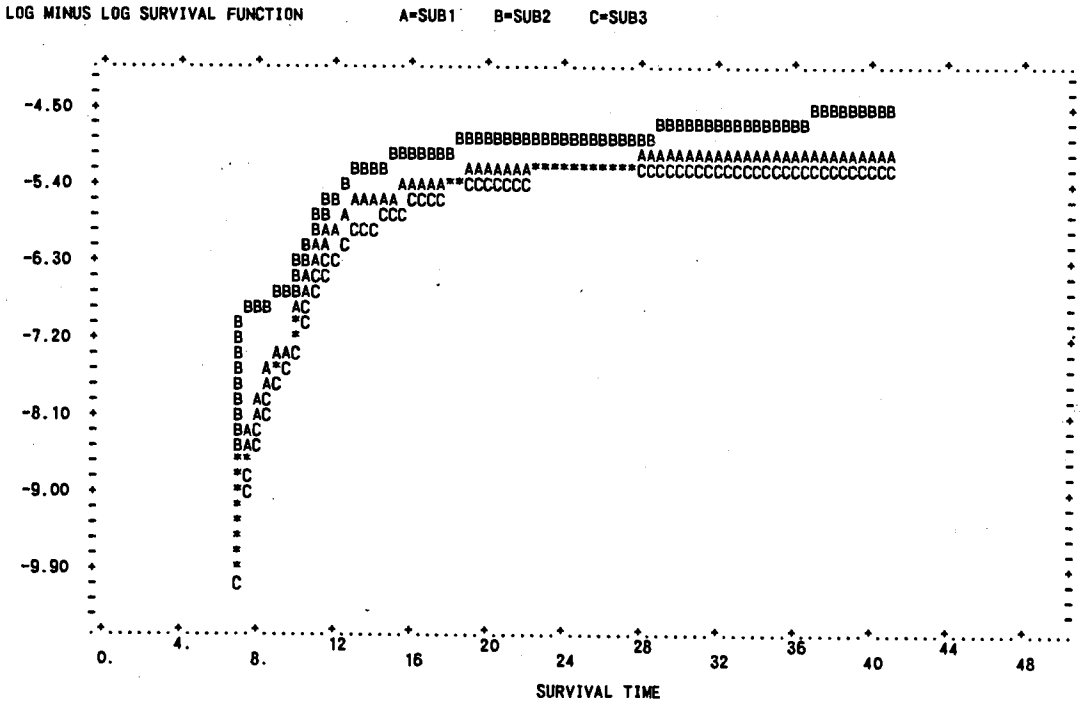


TABLE 4.2

Parameter estimates and standard errors in fitting Cox proportional hazards model in Phase I

Independent variable		Estimated value of coefficient	Standard error of estimator	Coeff./S.E.
TSAL				
TSA1	vs. TSA3	6.2526	0.3554	17.5940
TSA2	vs. TSA3	4.2384	0.3612	11.7330
SITE				
SIT2	vs. SIT1	0.7381	0.1093	6.7546
SIT3	vs. SIT1	0.5593	0.1141	4.9012
SIT4	vs. SIT1	-0.3773	0.1727	-2.1844
PSUB				
SUB1	vs. SUB3	0.4151	0.1575	2.6359
SUB2	vs. SUB3	1.1919	0.1654	7.2047

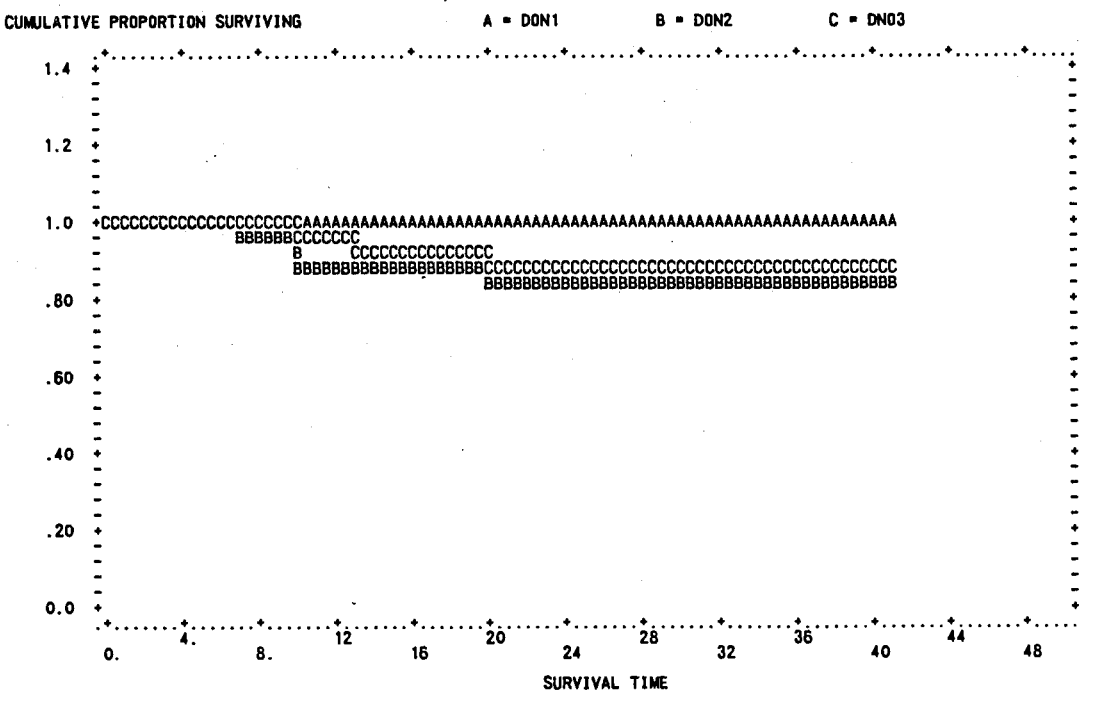
From Table 4.2, it is easily seen that, TSAL, SITE, and PSUB are all important in evaluating survival. In using stratified analysis, we cannot find out the effect of DONM on the survival. As a complement, we perform a univariate analysis on the effect of DONM. The survival curves for DON1, DON2 and DON3 (using the product limit method) are plotted in Figure 4.10. Mantel's test is performed for the null hypothesis that survival patterns of DON1 , DON2, and DON3 are the same. The test statistic gives a value of 304.13 and the degree of freedom is 2, thus the p-value is practically zero. Mantel's test is also performed to test the equality of the survival patterns of DON2 and DON3. The test statistic gives a value of 22.468 and the degree of freedom is 1, thus the p-value is also almost zero. This suggests that the survival curves of DON1, DON2 and DON3 are different.

4.2 Analysis of Phase II Data

Phase II of the seed germination experiment began immediately after 47 days of observation in Phase I. The seeds from 5, 10, 15, 30 ppt treatment salinity levels that had not germinated in Phase I were rinsed with distilled water. Then these seeds were placed in freshwater for further observation which lasted for 61 days. As we did in the analysis of Phase I data, we analyze Phase II data by fitting a Cox model.

FIGURE 4.10

Cumulative Proportion Survival Curves for DONM in Phase I



4.2.1 Checking the Proportionality Assumption

Since the seeds are from Phase I, all the covariates in Phase II are the same as in Phase I except for the different role that TSAL plays in the analysis of Phase II data: in the analysis of Phase I data, TSAL represented the actual salinity levels at which *Carex Lyngbyei* seeds were observed to germinate; in present analysis, TSAL represents the history of salinity treatments. The code information is the same as Table 4.1 except for the levels of TSAL covariate. The new levels are 5, 10, 15, and 30 ppt. As before, the graphical method is used to check the proportionality assumption. The plots are shown in Figure 4.11 - 4.14. In Figure 4.12 , TSA3 (15 ppt) and TSA4 (30 ppt) cross

each other. TSA3's germination is faster than TSA4's germination in the first two weeks, and slower after the first two weeks. TSA3's and TSA4's germination stop by week five. This suggests that the proportionality assumption seems to be inappropriate. The other plots in Figure 4.11, 4.13, 4.14 seem to satisfy proportionality assumption reasonably well. To solve the problem of non-proportionality for covariate TSAL, one can introduce time-dependent covariates. However, it turns out that it is inappropriate to include time-dependent covariates in the model because its inclusion causes the so-called monotonicity problem (Bryson and Johnson, 1981). Another alternative is to use the stratified analysis as we did in the analyses of Phase I data. The problem of using stratified analysis is that we cannot assess the treatment effect on the survival which is our major concern in Phase II. Therefore, we consider combining TSA3 and TSA4, which violate the proportionality assumption, as one level in covariate TSAL and then redraw the graph. The graph is presented in Figure 4.15. As can be seen, there is quite an inprovement compared with Figure 4.12. Among the above three approaches, the last one seems to be reasonable, and we proceed to fit a proportional hazards model to the data using the above new coding.

FIGURE 4.11

Checking Proportionality for DONM in Phase II

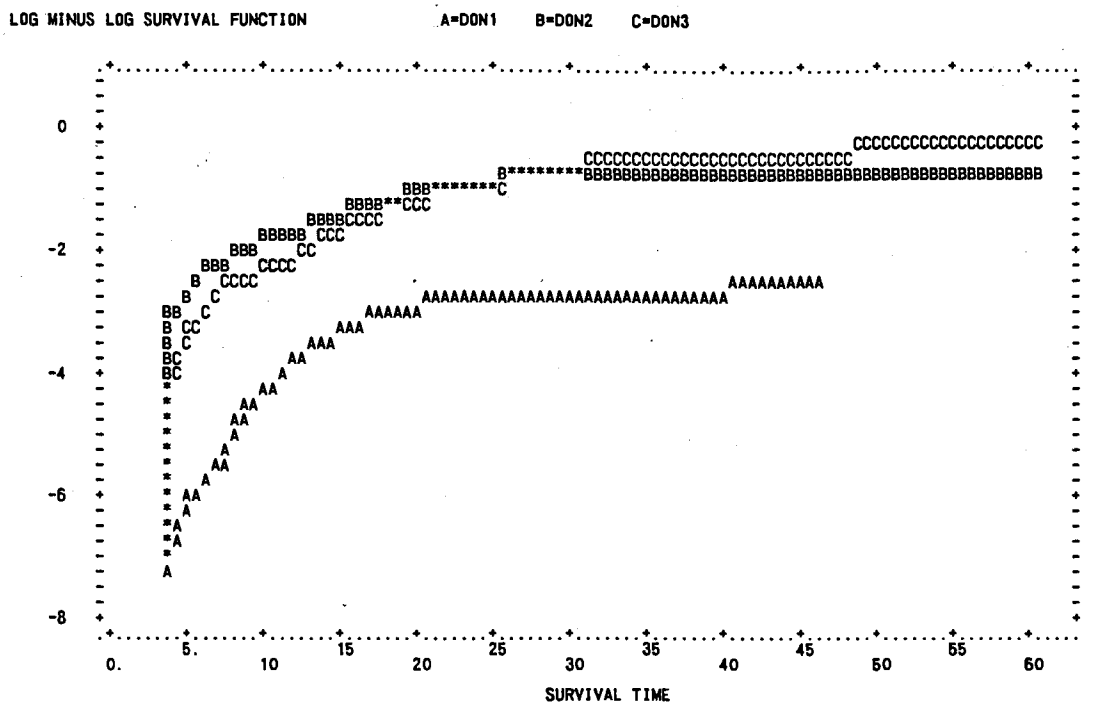


FIGURE 4.12

Checking Proportionality for TSAL in Phase II

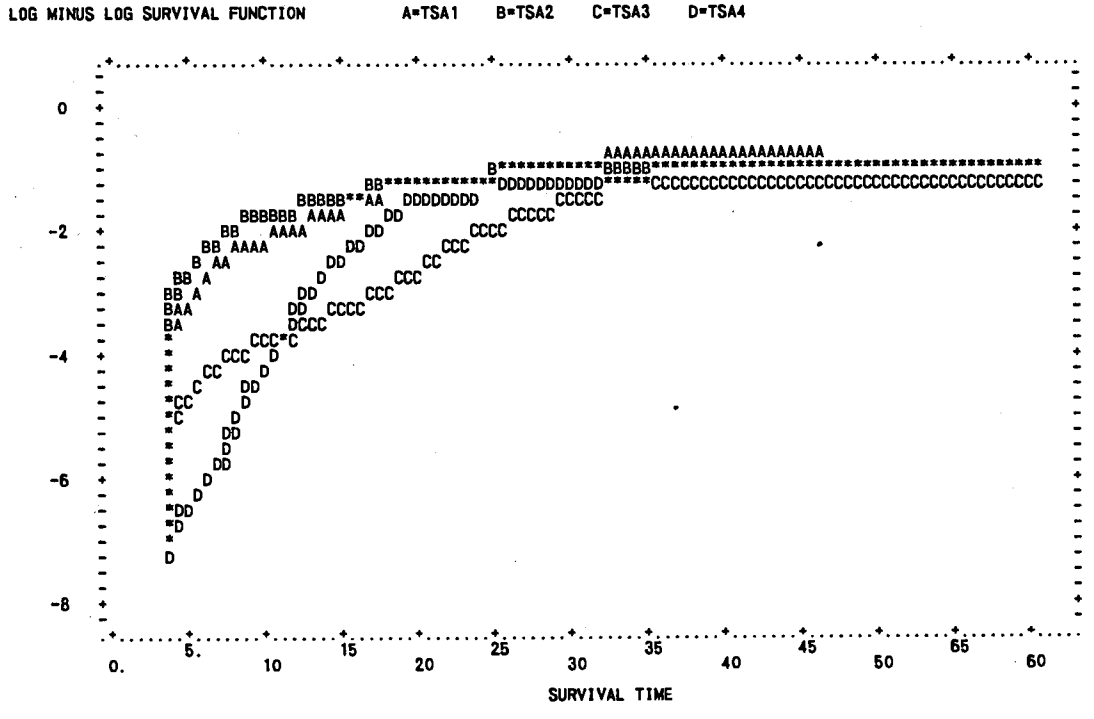


FIGURE 4.13

Checking Proportionality for SITE in Phase II

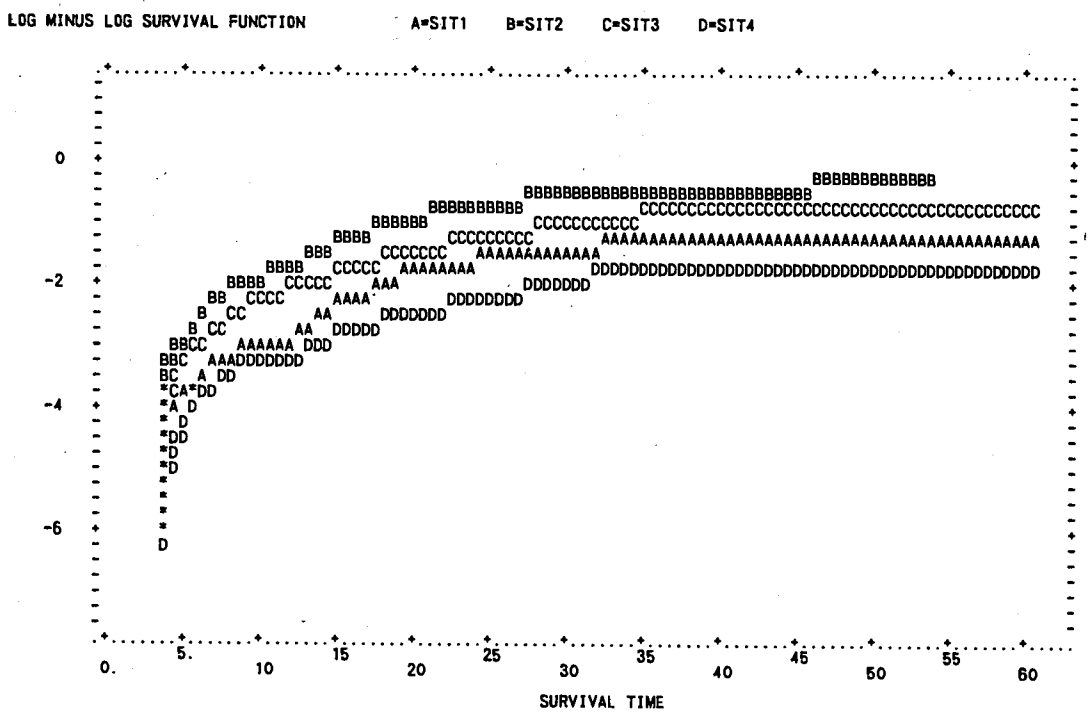


FIGURE 4.14

Checking Proportionality for PSUB in Phase II

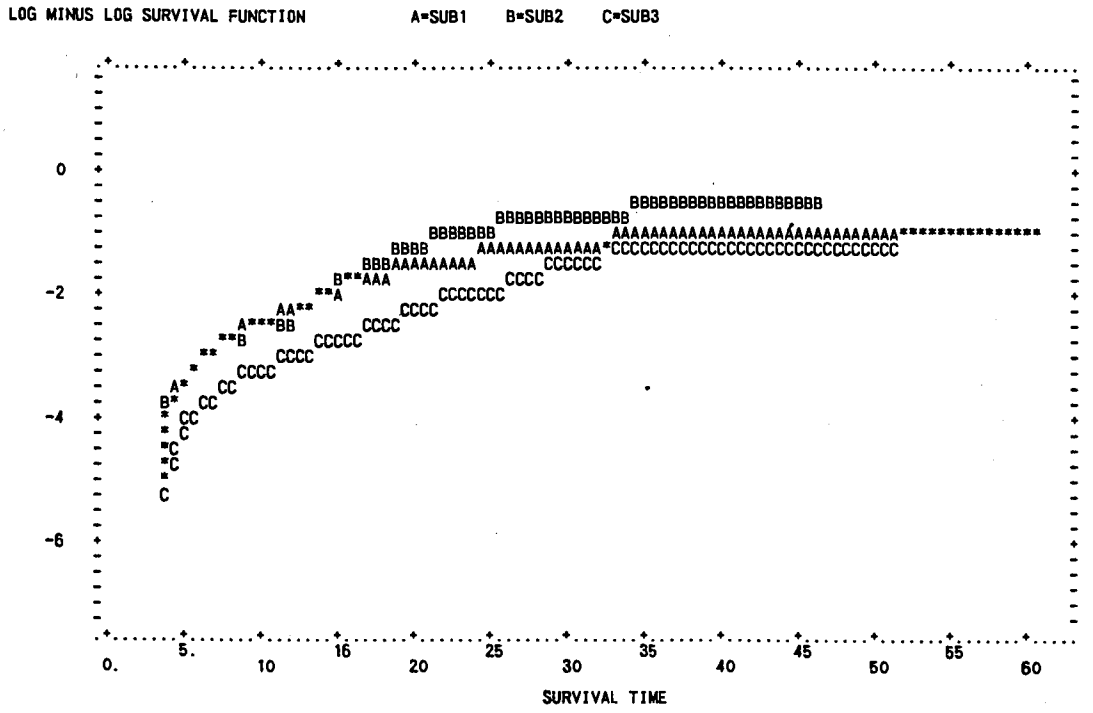
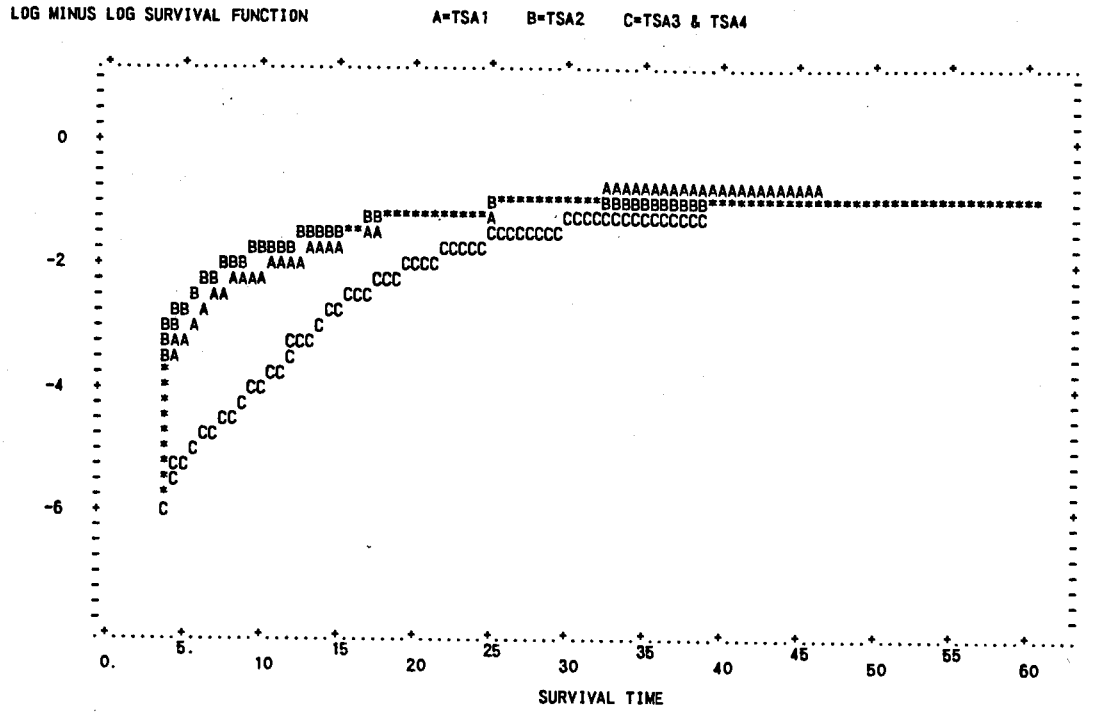


FIGURE 4.15

Checking Proportionality for TSAL

when TSA3 and TSA4 are combined as one level in Phase II



4.2.2 Fitting Cox Proportional Hazards Model

The Cox proportional hazards model (1) is fitted to the data. Table 4.3 gives the results of the estimated parameters and the standard errors. From the results of Table 4.3, we find out that all the covariates are significantly related to the survival.

TABLE 4.3

Parameter estimates and standard errors in fitting Cox proportional hazards model in Phase II

Independent variable	Estimated value of coefficient	Standard error of estimator	Coeff./S.E.
DONM			
DON2 vs. DON1	2.0136	0.1022	19.7017
DON3 vs. DON1	2.1149	0.1052	20.1120
TSAL[1]			
TSA1 vs. TSA3	0.4099	0.0461	8.8885
TSA2 vs. TSA3	0.2896	0.0460	6.2947
SITE			
SIT2 vs. SIT1	0.9082	0.0720	12.6072
SIT3 vs. SIT1	0.4969	0.0787	6.3164
SIT4 vs. SIT1	-0.4227	0.1034	-4.0902
PSUB			
SUB1 vs. SUB3	0.2069	0.0945	2.1898
SUB2 vs. SUB3	0.6681	0.1049	6.3663

---

[1]TSA1, TSA2, TSA3 represent 5 ppt, 10 ppt, 15 and 30 ppt respectively.

CHAPTER V

CONCLUSIONS

Upon recognizing the applicability of survival analysis by treating the germination of a seed as the "death" of the seed, the data from the germination experiment of *Carex Lyngbyei* seeds were analyzed by fitting the Cox proportional hazards model.

Based on the results obtained in Chapter 4, the environmental differences among the Squamish, Skagit and Nanaimo deltas certainly influence the germination of *Carex Lyngbyei* seeds. These results indicate that environmental differences have produced intraspecific variation between the Carex populations from these three deltas (Smythe, 1987). In phase I, seeds from Skagit (intermediate) have higher germination than seeds from Squamish (fresh) and seeds from Squamish have higher germination than seeds from Nanaimo (saline)(see Figure 4.1). Mantel's test also showed that this difference is statistically significant. In phase II, similar results (see Table 4.3) were obtained except that the difference in germination process between Skagit and Squamish is not statistically significant. In both phases, seeds from Nanaimo have the least germination. This may be due to the fact that the Nanaimo plants grow in the most stressed of the environments examined (i.e. the highest salinity level). It therefore appears that seeds taken from high salinity conditions will result in low viability.

The factor which almost always and everywhere affects the germination of *Carex Lyngbyei* seeds is the salinity level of the environment. As can be seen in Table 4.2, the seed germination process has been greatly reduced as the level of salinity increases and increased as the level of salinity decreases. This factor is seen to have a direct effect on seed germination in the analysis of phase I data. Also it has an indirect effect on seed germination as shown in the analysis of phase II data (see Table 4.3) by forcing the seeds to lie dormant, therefore delaying the germination process. In general, seeds from 5, 10 ppt will recover faster from their dormancy than seeds from 15 and 30 ppt. However, in Figure 4.12 one can see that seeds from 30 ppt have more germination than seeds from 15 ppt after 2 weeks of dormancy period. The probable explanation is that seeds from high salinity levels that have gone through dormancy need a longer time to recover than seeds from lower salinity levels.

The parental *Carex Lyngbyei* of the seeds used in this experiment grew up in water of various depths and this "history" factor has a significant effect on a new generation's germination. In the process of segregating the seeds by different degrees of submergence we found that seed germination is highest in the 50 - 70 % range, and lowest in the range of 71 - 100 % (see Table 4.2 and 4.3). Moreover, the results from both phases are similar. One of the prominent disabilities for long duration of submergence (71 - 100 %) for the seeds is lack of direct sunlight which is necessary for photosynthesis. As such,

this may be the cause of low seed germination in this range. In the 0 - 30 % range, seed germination is only intermediate, hence, we can deduce that in 50 - 70 % range, the condition is best suited for *Carex Lyngbyei* seed germination. However, this result did not take the differences in source (saline and fresh water) into consideration.

In addition, seeds taken from four different places high-saline, low-fresh, high-fresh, low-saline; were found to produce different number of germinations. In ascending order, they are low-saline, high-fresh, low-fresh, and high-saline (see Table 4.2 and 4.3). In the saline category, the results appeared as expected, i.e., high-saline is preferred to low-saline. High salinity level is detrimental to *Carex Lyngbyei* plant, therefore at a lower plain, the *Carex Lyngbyei* plant is more submerged in water and thus has to maintain more resistance against salinity which will greatly reduce its vigour for reproduction. On the other hand, fresh water seems to have different effect on the *Carex Lyngbyei* plant. From the result, low-fresh has more seed germination than high-fresh, probably due to the different properties of saline and fresh water. In general, fresh water should produce better germination for *Carex Lyngbyei* plant than saline water, but the results above have given us an unexpected consequence, i.e., high-saline has more seed germination than high-fresh and low-fresh. This raised our suspicion on the sufficiency on the variable, SITE. The collection of data from each site of the three deltas give rise to the differences in

salinity level which at this point is an important variable to take into consideration.

When consolidating the analyses of the covariates, PSUB and SITE, we find similarities between the high-low levels in saline and fresh water of the SITE covariate and the level of submergence. As such, it may be appropriate to categorize the SITE covariate to fresh and saline water levels only instead of the previous addition of high and low levels. This will be helpful in our analysis since the differences in parental source of the *Carex Lyngbyei* plant is a considerable factor in seed germination. Moreover, it further adds to our information on PSUB which did not take saline-fresh level into consideration as mentioned above by adding the interaction term (PSUB and the new SITE) into our analysis to distinguish the corresponding high-low levels of fresh and saline water.

From the data in Appendix I we found that there is overdispersion among plates, such that, as shown in row 14-17 on page 46 seeds from Skagit high-fresh when placed in four replicate dishes resulted in great differences in seed germination from as small as 1 to as large as 27. This phenomenon of variation may have been caused by unknown lapses in experimental procedure, perhaps involving salinity levels or impurities. As a result, our analysis may be distorted by this replicate effect. However, in our experimental analysis, we did not take this effect into consideration because of its difficulty in using coding in the BMDP (P2L) package.

In addition to our application of Cox model in this data analysis, another widely known method of modeling survival data is the Weibull model. The Weibull model was originally implemented by Aitkin & Clayton (1980) in the GLIM package. This procedure is also described in Mccullagh and Nelder (1983). It was applied to the phase II data with the same covariate structure as that which we applied with the Cox model (see Table 5.1). We found that the estimated coefficients do not differ greatly. Thus the Weibull model is an appropriate application for this data set. This was not a surprise, because it was found, when applying the Cox model, that a plot of $\ln\text{-}\ln\hat{S}_0(t)$ against $\ln(t)$ appeared to be a straight line.

We would like to summarize our suggestions for gathering similar data in such a way as to allow us to focus more clearly on scientific questions such as:

1. Is the difference in seed germination from the different sites of donor marsh due to its different salinity level or slight intraspecies genetic variability?
2. Instead of using the average of measurements as a covariate, is there an improved method to incorporate the actual data that was measured over a period of time?

Recording of salinity levels in the course of experiment is one of the suggestions for the first questions above. This information may further help to improve our experimental analysis on differences in seed germination.

TABLE 5.1

Parameter estimates and standard errors in fitting Weibull proportional hazards model in Phase II

Independent variable	Estimated value of coefficient	Standard error of estimator	Coeff./S.E.
DONM			
DON2 vs. DON1	2.1000	0.1015	20.6897
DON3 vs. DON1	2.2710	0.1044	21.7529
TSAL			
TSA1 vs. TSA3	0.4168	0.0527	7.9089
TSA2 vs. TSA3	0.1897	0.0461	4.1150
SITE			
SIT2 vs. SIT1	0.9951	0.0719	13.8401
SIT3 vs. SIT1	0.4691	0.0700	6.7014
SIT4 vs. SIT1	-0.4749	0.0769	-6.1756
PSUB			
SUB1 vs. SUB3	0.2109	0.0955	2.2084
SUB2 vs. SUB3	0.6945	0.0946	7.3414

For a "history" factor such as average annual submergence, it was actually measured over a period of time, but when fitting a survival model one usually uses some kind of average of the measurements to create one covariate and put it into the model. It would be very nice to be able to fit the actual measurements of the "history" over a period of time into a survival model. We would like to conclude this project by writing down the following title for someone's thesis: Survival Analysis With Time Series Covariates.

APPENDIX I

The raw data from the germination experiment are presented below. The variables represented in the data files are as follows:

Column 1:
Donor marsh
1 = Nanaimo River delta
2 = Skagit River delta
3 = Squamish River delta

Column 2:
Donor site elevation
1 = high
2 = low

Column 3:
Donor site salinity
1 = relatively saline
2 = relatively fresh

Column 4:
Replicate number

Column 5:
Treatment number
1 = Phase I, 0 ppt salt
2 = Phase I, 5 ppt salt
3 = Phase I, 10 ppt salt
4 = Phase I, 15 ppt salt
5 = Phase I, 30 ppt salt
6 = Phase II, after immersion in 5 ppt salt
7 = Phase II, after immersion in 10 ppt salt
8 = Phase II, after immersion in 15 ppt salt
9 = Phase II, after immersion in 30 ppt salt

Column 6:
Percent annual submergence

Column 7-17:
Number of seeds germinated in a given periods where the periods in phase I are 4, 7, 10, 13, 20, 27, 34, and 41 days and phase II are 4, 8, 12, 17, 20, 26, 33, 40, 47, 54 and 61 days.

1	1	1	1	1	10	0	0	6	1	0	0	0	0			
1	1	1	2	1	10	0	0	0	0	0	0	0	0			
1	1	2	1	1	0	0	0	2	0	3	0	0	0			
1	1	2	2	1	0	0	0	0	1	1	0	0	0			
1	1	2	3	1	0	0	0	0	2	0	1	1	0			
1	1	2	4	1	0	0	0	0	0	1	0	0	1			
1	2	1	1	1	35	0	0	0	0	0	0	0	0			
1	2	1	2	1	35	0	0	0	0	0	0	0	0			
1	1	1	1	2	10	0	0	0	0	0	0	0	2			
1	1	1	2	2	10	0	0	0	0	0	0	0	0			
1	1	2	1	2	0	0	0	0	0	0	1	0	0			
1	1	2	2	2	0	0	0	0	0	0	0	0	0			
1	1	2	3	2	0	0	0	0	0	0	0	0	0			
1	1	2	4	2	0	0	0	0	0	0	0	0	0			
1	2	1	1	2	35	0	0	0	0	0	0	0	0			
1	2	1	2	2	35	0	0	0	0	0	0	0	0			
1	1	1	1	3	10	0	0	0	0	0	0	0	0			
1	1	1	2	3	10	0	0	0	0	0	0	0	0			
1	1	2	1	3	0	0	0	0	0	0	0	0	0			
1	1	2	2	3	0	0	0	0	0	0	0	0	0			
1	1	2	3	3	0	0	0	0	0	0	0	0	0			
1	1	2	4	3	0	0	0	0	0	0	0	0	0			
1	2	1	1	3	35	0	0	0	0	0	0	0	0			
1	2	1	2	3	35	0	0	0	0	0	0	0	0			
1	1	1	1	4	10	0	0	0	0	0	0	0	0			
1	1	1	2	4	10	0	0	0	0	0	0	0	0			
1	1	2	1	4	0	0	0	0	0	0	0	0	0			
1	1	2	2	4	0	0	0	0	0	0	0	0	0			
1	1	2	3	4	0	0	0	0	0	0	0	0	0			
1	1	2	4	4	0	0	0	0	0	0	0	0	0			
1	2	1	1	4	35	0	0	0	0	0	0	0	0			
1	2	1	2	4	35	0	0	0	0	0	0	0	0			
1	1	1	1	5	10	0	0	0	0	0	0	0	0			
1	1	1	2	5	10	0	0	0	0	0	0	0	0			
1	1	2	1	5	0	0	0	0	0	0	0	0	0			
1	1	2	2	5	0	0	0	0	0	0	0	0	0			
1	1	2	3	5	0	0	0	0	0	0	0	0	0			
1	1	2	4	5	0	0	0	0	0	0	0	0	0			
1	2	1	1	5	35	0	0	0	0	0	0	0	0			
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1	1	2	2	6	0	1	1	0	2	0	0	0	0	2	0	0
1	1	2	3	6	0	0	0	0	0	0	0	0	0	0	0	0
1	1	2	4	6	0	0	0	2	0	1	0	0	0	1	0	0
1	2	1	1	6	35	0	0	0	0	0	0	0	0	0	0	0
1	2	1	2	6	35	0	0	0	0	0	0	0	0	0	0	0
1	1	1	1	7	10	0	3	2	0	0	0	0	0	0	0	0
1	1	1	2	7	10	0	0	4	1	0	0	0	0	0	0	0
1	1	2	1	7	0	0	0	3	2	0	1	1	1	0	0	0
1	1	2	2	7	0	0	0	5	1	0	1	0	0	0	0	0
1	1	2	3	7	0	0	0	3	1	0	0	0	0	0	0	0

1	1	2	4	7	0	0	0	0	6	0	1	0	0	0	0	0
1	2	1	1	7	35	0	0	0	0	0	0	0	0	0	0	0
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3	2	2	3	8	75	0	0	0	0	1	8	16	7	3	1	0
3	2	2	4	8	75	0	0	0	0	0	0	10	3	3	2	0
3	1	1	1	9	10	0	0	1	16	8	5	7	0	3	0	0
3	1	1	2	9	10	1	3	6	20	8	4	2	1	0	0	0
3	1	1	3	9	10	0	0	2	34	6	2	0	1	0	0	0
3	1	1	4	9	10	0	0	0	3	7	0	1	18	5	0	0
3	1	2	1	9	60	0	0	0	10	8	8	1	0	0	0	0
3	1	2	2	9	60	0	0	0	9	2	6	2	3	0	0	0
3	1	2	3	9	60	0	0	0	8	19	5	2	0	0	0	0
3	1	2	4	9	60	0	0	0	0	6	0	1	0	2	0	0
3	2	1	1	9	20	1	0	0	1	0	2	7	1	0	0	1
3	2	1	2	9	20	0	0	1	11	2	1	1	1	0	0	0

3	2	1	3	9	20	0	0	0	6	5	3	2	2	0	0	0
3	2	1	4	9	20	0	0	0	2	0	0	0	0	0	0	0
3	2	2	1	9	75	0	0	0	6	5	9	0	1	0	0	0
3	2	2	2	9	75	0	0	0	4	3	7	1	1	2	1	0
3	2	2	3	9	75	0	0	0	1	8	7	8	3	0	1	0
3	2	2	4	9	75	0	0	0	6	4	6	4	0	1	0	0

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