BROMOPHENOLS IN RHODOMELA LARIX:

A STUDY IN CHEMICAL ECOLOGY

by

DAVID WILLIAM PHILLIPS

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Department of BOTANY

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date 30 JUNE 1980

ABSTRACT

In this study aspects of the ecology, biology and chemistry of Rhodomela larix (Turner) C. Agardh are investigated and an effort made to relate the findings to a determination of the reasons for the production of bromophenols by red algae.

Environmental factors occurring in local tidepools which might affect the production or exudation of bromophenols by this alga were examined in summer over a three month period. Measurements of algal biomass and levels of total phenols in the pools show that R. larix produces and exudes copious amounts of these compounds. The ecological implications of this rate of release are introduced.

Chemotaxonomic comparison of the morphologically different tidepool and low intertidal populations of R. larix indicates that the two are sufficiently similar to be considered the same species. Several bromophenols previously thought to be artifacts are now considered to be natural constituents of algae.

A high-performance liquid chromatographic method was developed for the separation, identification and

quantitative determination of red algal bromophenols. The method is evaluated on the basis of several chromatographic parameters, and steps in its development and improvement are discussed.

Previous studies on red algal phenols have generally overlooked quantitative considerations. This study presents data for various aspects of the quantitative analysis of the major bromophenolic substance (lanosol, 2,3-dibromo-4,5-dihydroxybenzyl alcohol) in R. larix. Temporal determination of lanosol concentrations demonstrates that the highest levels occur in winter months. Populational differences were observed and the highest levels within a single plant were found to be in the youngest regions.

Control of the exudation of bromophenols by R. larix was studied using artificial conditions which fell within the ranges of environmental conditions previously monitored. Exudation of lanosol is enhanced in light, at higher temperatures and at lower salinities. pH has little effect on exudation. The rates of exudation found in these experiments correspond to those measured in the tidepool.

Examination of the broad spectrum antibiotic activity of lanosol proves this compound to be effective even at low levels against a wide variety of organisms. The

effect is enhanced at low pH, and comparison with R. larix exudates suggests lanosol to be the major active component.

A discussion of the ecological significance of bromophenol production is presented and an argument made for the production of these compounds as antibiotic substances. Comparison with previous experiments describing the temporal antibiotic activity of another Rhodomela species supports this conclusion.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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PROEM

The ecologist's increasing awareness of the intricate and subtle chemical balance in nature and the chemist's access to modern instrumentation enabling him to detect small quantities of natural products have led to the evolution of a new and exciting branch of science called chemical ecology. Chemical ecology attempts to unravel the complex interrelationships of organisms at both chemical and biological levels. One corner of this broad field deals with the interactions of plants and other living creatures, and has been called phytochemical ecology (Harborne 1972). Whether the interaction is plant to plant, plant to animal or plant to microbe, it can be described as being antagonistic, beneficial or mutually indifferent.

The majority of phytochemical ecologists find antagonistic interactions the most interesting. The production of defensive compounds in response to predation (herbivory) and parasitism is common throughout the plant kingdom. Vuillemin (1889) first used the term "antibiosis" to describe the counteraction of one organism to the life of another, a concept which has grown to include the study of antibiotics produced by marine organisms, not to mention those thousands of terrestrial origin.

This dissertation takes a step in furthering our understanding of the role of an unusual group of "secondary" metabolites which are widespread among red algae. Aspects of the ecology, chemistry and biology of a particular red alga are approached from the standpoint of attempting to relate "form to function," form being the production of the compounds and function their raison d'être. I sincerely hope that even if the reader remains unconvinced by the final verdict, I will have at least stimulated imaginative speculation, if not the desire to intensify research into the chemical ecology of the oceans.

CHAPTER I

ASPECTS OF THE ECOLOGY OF *RHODOMELA LARIX*

INTRODUCTION

Tidepools are scattered throughout the rocky intertidal zone along much of the coast of British Columbia, Canada. Within these pools a variety of organisms can be found, including many species of red algae (Rhodophyta). Red algae generally occur in the low intertidal to subtidal regions of the seashore (Fritsch 1945, Scagel 1967), and prefer lower light intensities (Biebl 1962, Johnson et al. 1974) and/or longer periods of immersion (Doty 1946) than do many of the brown and green macrophytes. The occurrence of red algae in pools high in the littoral zone should therefore pose many different and unusual problems for these species. The environmental conditions in tidepools are quite unstable and often extreme, indicating that a variety of physiological adaptations must have occurred for such algae to survive in the tidepool habitat.

Although some work has been done on the monitoring of physical and environmental conditions in pools in other parts of the world (Klugh 1924, Johnson and Skutch 1928, Pyefinch 1943, Ganning 1971, Edelstein and McLachlan 1975), little or no such work has been done on the North Pacific coast (see Humphrey and Macy 1930). The above studies

considered factors such as dissolved oxygen, light, temperature, salinity and pH and how these might affect the distribution of algae in coastal tidepools. Only recently have factors such as the release of dissolved organic matter (Khailov and Burlakova 1969) and other chemical substances (Ragan and Jensen 1979) been considered in relation to the presence or absence of an algal species in a particular habitat. Biological factors such as inter- or intra-specific competition between plants or plants and animals have been frequently overlooked. These too could play an important role in determining the distribution of algae in tidepools.

In this context, I wish to report on some experiments performed during the summer of 1978 on two tidepools in British Columbia. Physical and chemical changes in the environmental conditions in these pools were monitored over a three month period. The algal flora in the two pools were identified and the abundance (percentage cover and standing crop) of each species was determined. In addition, an attempt was made to monitor the exudation from the algae of several classes of chemical compounds. It was hoped that this phase of the experiments would give some indication of the physiological stresses to which these algae were subjected during changing environmental conditions.

MATERIALS AND METHODS

The Study Site

The two tidepools studied were located on Bath Island, British Columbia, Canada (Figure 1a), in the Strait of Georgia. A rock ledge containing a series of tidepools surrounds the island at a height of about 4.5 m above zero tide level (MLLW, Canadian Chart Datum). The pools chosen were at 4.4 m (pool 1) and 3.7 m (pool 2) above MLLW (Figure 1b) and had maximum depths of 53 and 41 cm respectively. Levels of the pools and relevant tidal cycles were determined using the Canadian Tide and Current Tables (1978), with Point Atkinson as a reference port and Silva Bay as a secondary port. Standard surveying instruments were employed.

Measurement of Physical and Chemical Factors

Light intensity readings were made using a Li-Cor Model LI-185 quantum meter, with the probe (Model UWQ 2192) in a vertical position[1]. Readings were made at each pool's surface except in the case of depth vs. light intensity profiles.

Temperature and salinity (given as parts per thousand salinity, $^{o}/oo$) profiles, as measured with a calibrated YSI Salinometer, were determined over a 24 hour period

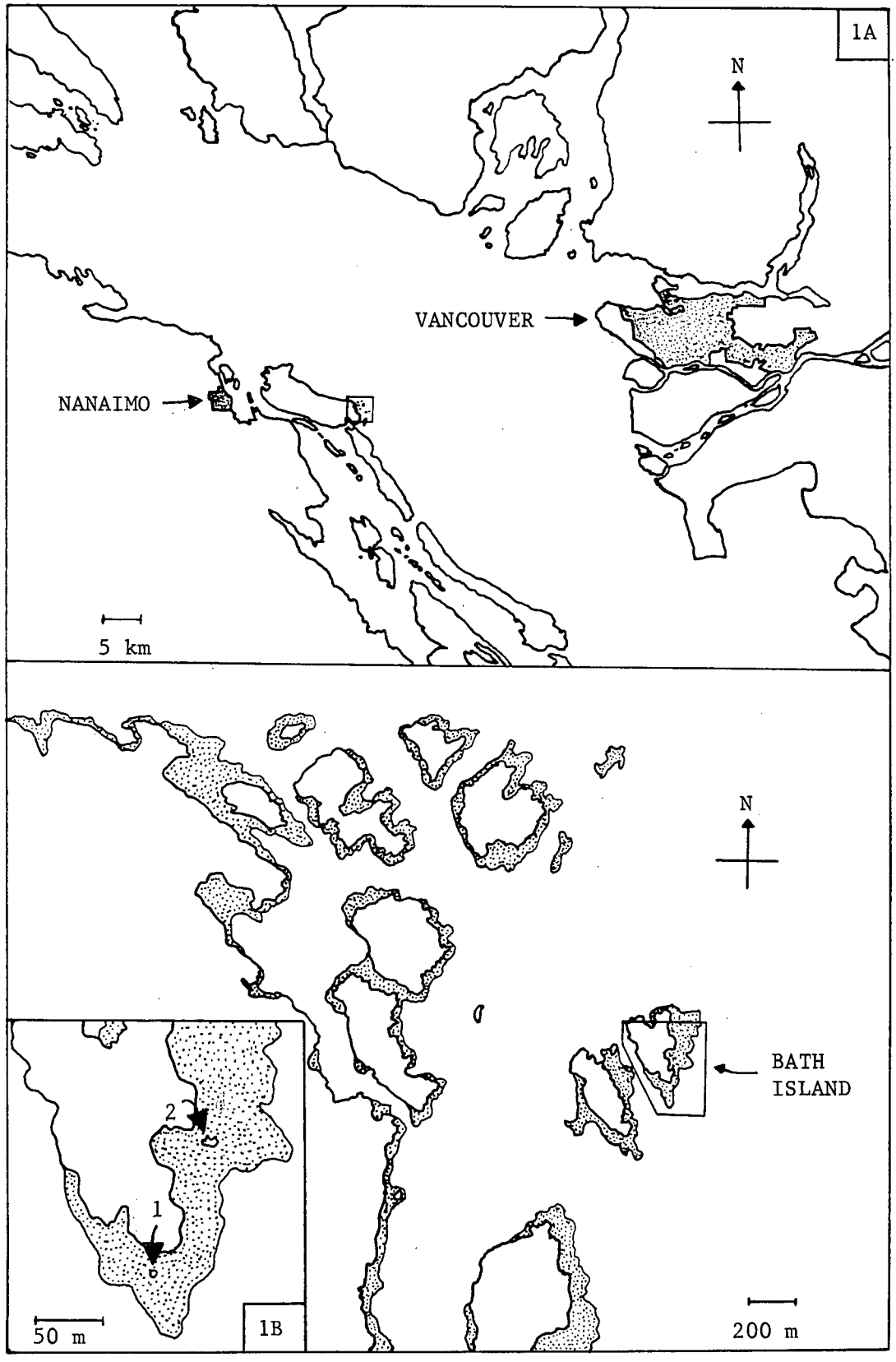


Figure 1. Map of Bath Island, British Columbia, and environs (1A), and detail (1B) showing location of the tidepools studied.

in each of three consecutive months (July, August and September, 1978).

pH was measured using a Beckman Expandmate portable pH meter equipped with a Beckman Model 39502 electrode. Standard buffers (pH 7.00 and 10.00 Amachem, Seattle) were used for calibration.

Dissolved oxygen was measured by the Winkler method (Strickland and Parsons 1968). Three replicate titrations were done on each water sample, and the average value was converted to mg O_2/1.

Quadrat Sampling Methods

The surface areas of the pools and the percentage cover of each algal species contained within them were determined using quadrat frames. A $(1.5\ m)^2$ frame divided in $(10\ cm)^2$ squares was used for pool 1, and a $(4\ m)^2$ frame divided into $(25\ cm)^2$ squares was used for pool 2. The number of squares encompassed by each pool was used to calculate surface area. The percentage cover for each algal species (all of which were identified using Widdowson 1974 or Abbott and Hollenburg 1976) was first estimated for each square and then calculated for the entire pool. Frequency, dominance and, subsequently, importance values were determined for each species according to Cox (1972). X^2 species association values were assigned to all species relative to Rhodomela larix (Turner) C. Agardh (Kershaw

1964).

Standing crop measurements were obtained for R. larix and Prionitis lyallii Harvey by scraping a series of (10 cm)^2 sections containing these two species in various combinations of percentage cover. Algal dry weights were determined after 5 days at 90°C (or until constant dry weight was achieved).

Chemical Measurements

For chemical analysis of tidepool water, 100 ml water samples were taken, frozen on Dry Ice and returned to the laboratory. Total phenols were measured using the method of Langlois (1975) on a Unicam Model SP 800 Scanning Spectrophotometer set at a constant wavelength (445 nm). Seawater was used as a blank, and varying, but known, concentrations of phloroglucinol in seawater were used for calibration and the estimation of the concentration of total phenols in the tidepool water. Attempts to measure carbohydrate (Dubois et al. 1956) and protein (Lowry et al. 1951) proved unsuccessful due to the low concentrations of these constituents in the pools (e.g. there were no apparent changes in concentration over time).

RESULTS AND DISCUSSION

The problem of monitoring physical and chemical

conditions in systems such as tidepools is complicated by the stratification of these factors which may occur within an individual pool. For example, as shown in Figure 2, pool 1 had salinity ranges of 11-28 °/oo vertically and 11-25 °/oo horizontally depending on the amount of ocean wash or precipitation entering the pool. Pool 2 showed a similar salinity stratification, but less mixing with ocean water; therefore, a low salinity, less dense layer of water predominated over the pool surface. The algae in the pools are distributed over most of the available rock surface, indicating that their tolerance to these varying salinities is broad. The degree of temperature stratification in the two pools is also shown in Figure 2. Ganning (1971) has reported similar results for the Swedish tidepools he studied. He believes that tidepool biota are confined to that habitat because of their lack of desiccation tolerance and not because of osmoregulatory problems which they might encounter. Klugh (1924), on the other hand, has stressed the importance of temperature and its fluctuations as the main limiting parameter for the tidepool organism.

The temperatures and salinities which appear in the graphs in Figure 3 are averages of readings taken at varying depths and locations throughout the pools. Low salinities indicate that precipitation (as land surface runoff)

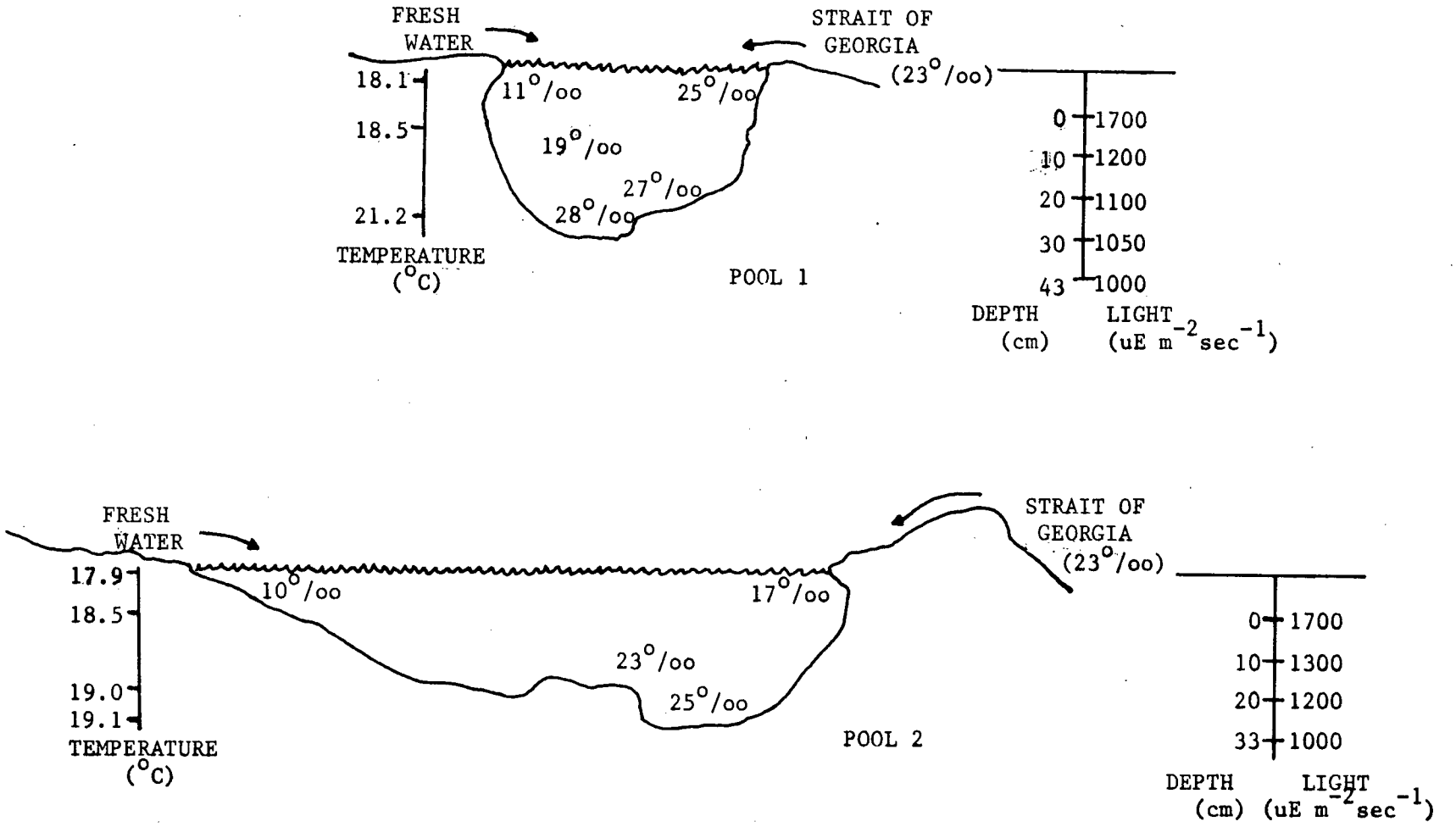


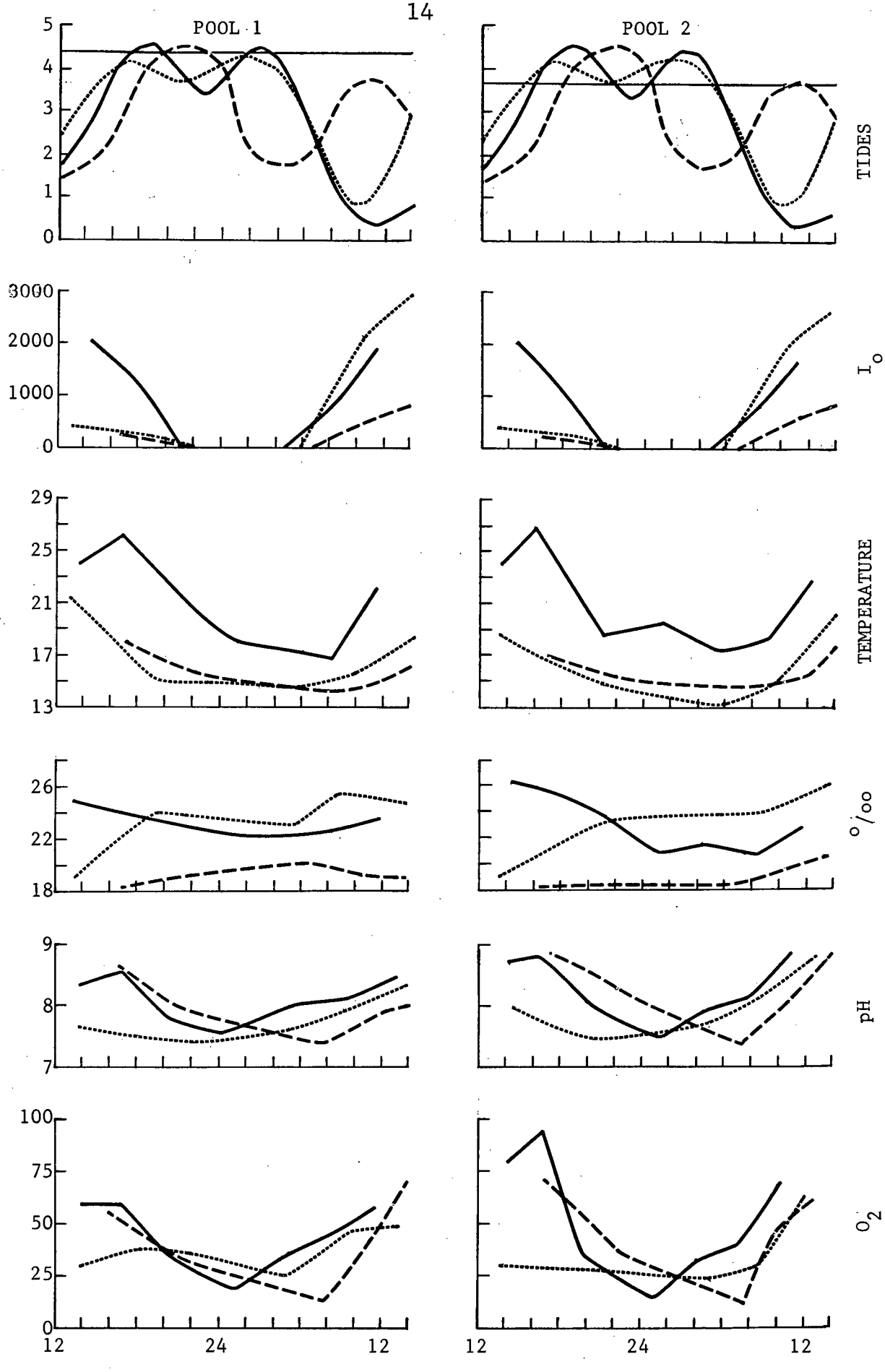
Figure 2. Diagram of pools 1 and 2 showing ranges in stratification of salinity (°/oo) and temperature and light attenuation with depth.

is occurring while the pool is exposed, and high salinities indicate the degree of evaporation occurring in the pool, reducing its volume and, in the process, concentrating the salts present. The more stable readings, of course, occur when the tide is in.

The results of the pH determinations as shown in Figure 3 clearly indicate the ratio of photosynthesis to respiration of algae in the pool. During the day active photosynthesis is taking place; oxygen is evolved and subsequently the pH of the pool goes up, not due to the increased presence of oxygen, but to a reduction in the level of dissolved CO_2 in the pool. Conversely, at night when the level of respiration is high, oxygen is consumed, CO_2 is produced and a lowering of the pool's pH occurs. Klugh (1924) and later Johnson and Skutch (1928) have suggested that the acidity of tidepools is determined by, rather than determines, the biota present. If this is true then pH alone cannot be the limiting factor for species occurrence in tidepools.

Although not immediately obvious, measurements of surface light intensities are not a wholly accurate indication of the irradiance actually received by the algae at varying depths in the pools. As much as 35% of the light striking the pool's surface is reflected off or is filtered out in the top 20 cm of pool water on a calm

Figure 3. Environmental measurements made in pool 1 and pool 2 during three consecutive months. The sampling dates were July 18-19 (———), August 14-15 (.....) and September 6-7 (------). Tide levels are in meters above MLLW. Surface light intensities (I_o) are in uE m^{-2} sec^{-1}, temperatures in ^{o}C, salinities ($^{o}/oo$) in parts per thousand of salinity and dissolved oxygen (O_2) in mg l^{-1}. The time scale is divided into two hour intervals beginning at 1200 hours on the day of the study. The level of the pool in relation to zero tide level is indicated by the fine horizontal line through the graph marked "TIDES". Readings were taken at a depth of 10 cm below the pool's surface and in the center of each pool.


POOL 1
POOL 2
TIDES
I_o
TEMPERATURE
°/oo
pH
O_2
5
4
3
2
1
0
3000
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1000
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29
25
21
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18
9
8
7
100
75
50
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0
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12

day (Figure 2). This irradiance would decrease even further with turbulence (e.g. wind or waves) on the pool surface or with an increase in dissolved organic matter in the pools. Surface insolation also controls the temperature of the water in the pools to a great extent either by heating the water directly or by heating the surrounding rock.

Dissolved oxygen in the pools is again correlated with photosynthesis and, therefore, pH. Figure 3 shows that as pH increases so does the level of dissolved oxygen. The oxygen levels of the pools were high even at the higher temperatures (warmer solutions hold less dissolved gas), indicative of the high photosynthetic rate and, hence, productivity of the algae in the pools. A levelling effect in the oxygen content of the pools would occur when they are reimmersed and the turbulence of waves releases or dilutes the excess gases present. Pyefinch (1943) states that the diversity of the flora in a rock pool is the key factor in governing the range of pH and oxygen values. This appears to be the case in the two pools studied here. The larger and more densely populated pool (see below) shows the greater rate of change in the four variables so far discussed. The contribution of phytoplankton to the productivity of the pool has not been considered since it should be minimal when compared to that of the attached flora.

Tabulation of the quadrat sampling data revealed that pool 1 had a surface area of 1.65 m^2 (encompassing 165 $(10\ cm)^2$ quadrats) and pool 2 a surface area of 10.88 m^2 (encompassing 134 $(25\ cm)^2$ quadrats). Values of percentage cover, dominance, frequency and importance for the algae in the two pools are shown in Table I. R. larix was dominant in both pools. Ceramium washingtoniense Kylin and Ralfsia crusts were more important in the higher pool than in the lower pool, and P. lyallii was more important in the lower pool than in the higher.

Standing crop measurements (as g dry weight, g d. wt.) of Rhodomela and Prionitis gave averages of 1.81 (± 0.56) g and 7.67 (± 2.53) g respectively per $(10\ cm)^2$ in both pools. Using the formulae for surface area and volume of a sphere, one can estimate the overall standing crop of Rhodomela in pool 1 (with its closely spherical shape) and attempt to correlate the production of total phenols (assuming Rhodomela to be the sole source) with pool volume. I found that there was 0.39 g d.wt./l of this alga in pool 1 and that the level of total phenols reached 0.5 mg/l day.[3] Therefore, each g d.wt. of Rhodomela exudes 1.41 mg of total phenols per day. If we assume halophenols to be antibiotic, then they should be effective at such levels.

The two pools were initially chosen because of the high percentage of R. larix in each. It was hoped that

Table I. Dominance, frequency and importance values for the algae in the two tide pools. * The first figure in each column refers to pool 1 and the second figure to pool 2.

SPECIES	COVER (%)		DOMINANCE		FREQUENCY		IMPORTANCE (%)	
Rhodomela larix	45.9*	45.1*	0.76	4.91	90.3	96.0	39.8	38.6
Ceramium washingtoniense	9.3	3.6	0.15	0.39	63.6	33.3	16.5	7.4
Prionitis lyallii	0.8	20.3	0.01	2.21	7.9	79.9	1.9	23.5
Ralfsia crusts	5.8	1.6	0.10	0.17	29.7	19.5	8.4	4.1
Bare rock	38.2	29.4	0.63	3.20	77.6	70.7	33.5	26.5

since R. larix is very high in phenolic content, the exudation of characteristic compounds from this plant could be monitored. However, the concentrations of individual phenolic components in the pool water proved to be too low for identification purposes. It was only possible to get an indication of the total phenols present in the pool water at any particular time. An increased rate of exudation of phenols and their subsequent appearance in the pool water was judged a reflection of the physiological stress placed on the plants in the pool at the time of the sampling. The change in phenol concentration in the pools as measured on July 18-19 is shown in Figure 4. The levels increase to maximum values just before reimmersion of the pool in each case, indicating the possibility that the increased production of extracellular metabolites (e.g. phenols) by the algae might be a stress response. There are numerous examples of this kind of response in the literature (Craigie and McLachlan 1964, Sieburth 1969, Sieburth and Jensen 1969).

Exudates are probably important in the ecology of tidepool organisms (Langlois 1975). Some researchers believe that exudation may be a form of waste product disposal by the algae, others that the components of exudates may act as antibiotic substances (Langlois 1975, Ragan and Jensen 1978) once in sufficient concentrations in the

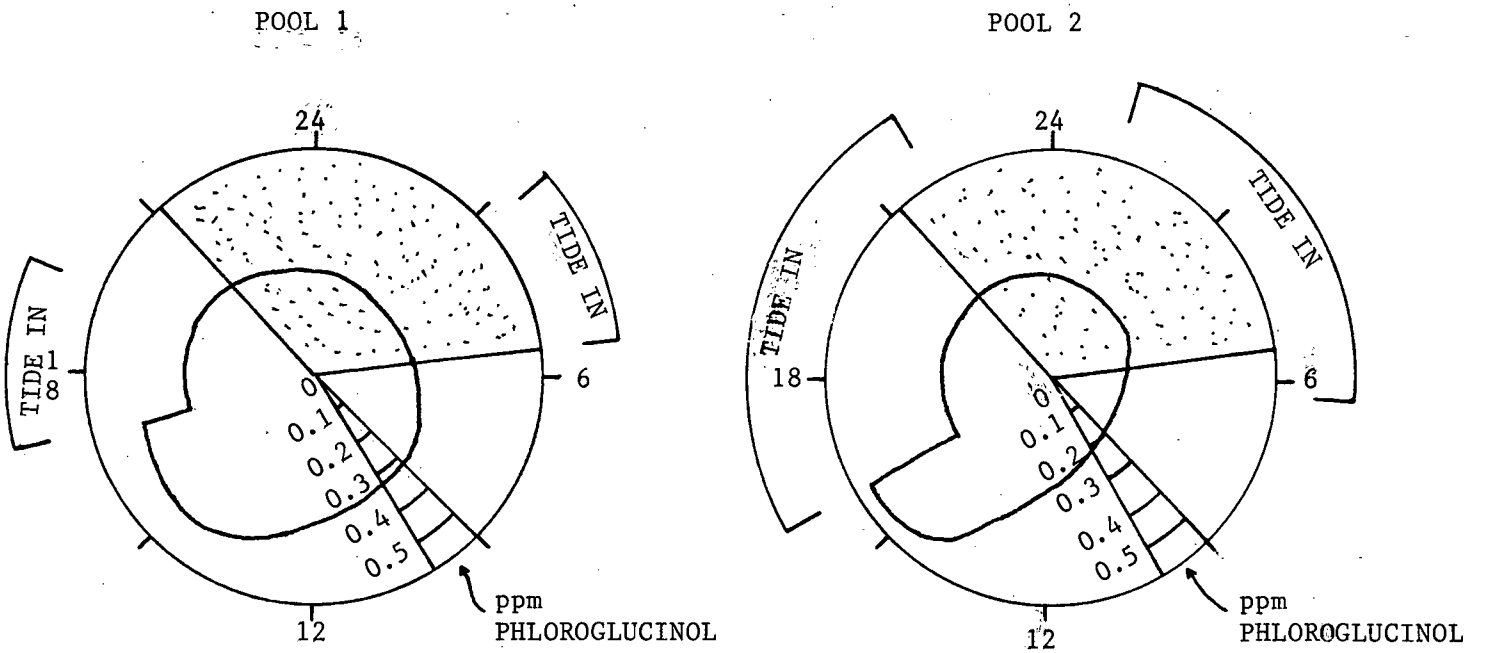

Figure 4. Total phenols in pool 1 and pool 2 over a twenty-four hour period given as parts per million of phloroglucinol. The period of dark is indicated by stippling.

tidepool. Although the antibiotic activity of algal exudates has yet to be demonstrated conclusively, there is some indication that exudates have some effects on bacteria and invertebrate life history stages (Conover and Sieburth 1966, Sieburth and Jensen 1969, Langlois 1975).

The X^2 species association data (Table II) indicate that only Rhodomela and Ceramium are closely associated in the upper pool and that no association with Rhodomela occurs in the lower pool. Ceramium is a common epiphyte of Rhodomela (personal observation 1977), hence the close association. Since Ceramium also contains halophenols (see Chapter III), it is unlikely that if these compounds show some antibiotic function in red algae, their production by Rhodomela would show no effect against epiphytization by Ceramium.

Work by Khailov and Burlakova (1969) indicates that as much as 37% of the gross productivity of an alga may be released as dissolved organic matter (DOM). Phenols must constitute a significant fraction of these exudates. Whether this release is active or passive is unknown at present; however, a large portion of the carbon budget of an alga is released into the environment. One would question such high levels of waste compound production, especially in the form of complex molecules such as

Table II. X^2 species association of algae in the two pools. The probabilities given are that the plant in question is not associated with Rhodomela larix.

SPECIES	POOL 1		POOL 2	
	X^2	P(%)	X^2	P(%)
Ceramium washingtoniense	8.77	1	0.34	59.0
Prionitis lyallii	1.52	23.2	0.50	48.8
Ralfsia crusts	2.51	14.1	0.80	78.6

halophenols or polyphenols. These compounds must, it seems, have some other function and until further evidence accumulates to the contrary, they can be considered antibiotic substances. Silva and Bittner (1979) have recently reviewed the literature on the antibiotic activity of algal metabolites.

In this chapter I have presented preliminary evidence for the exudation of phenolic compounds by R. larix found in tidepools. This habitat from all indications is a stressful one, promoting, at least in the case of Rhodomela, increased production of DOM as phenolic substances. Whether these compounds function as antibiotics remains undetermined. If antibiotic, their action against tidepool organisms could be enhanced under the increased environmental pressures inherent to this habitat.

CHAPTER II

BROMOPHENOLS OF *RHODOMELA LARIX*: CHEMOTAXONOMY OF MORPHOLOGICAL FORMS

INTRODUCTION

Algae of the division Rhodophyta are unique in the diversity and abundance of carbon-halogen compounds which they contain. The Rhodomelaceae is especially rich in these compounds in the form of either halogenated terpenoids or phenols. The bromophenols are widely distributed among genera in this family (Fenical 1975, Kurata et al. 1973, Kurata and Amiya 1975, Weinstein et al. 1975, Chevolot-Magueur et al. 1976, Saenger et al. 1976, Kurata et al. 1976, Combaut et al. 1978, Pedersen 1978, Ragan and Craigie 1978, Lundgren et al. 1979, Pedersen et al. 1979, Kurata and Amiya 1980). Their use as taxonomic markers has been suggested (Peguy 1964, Augier 1965) and debated (Fenical 1975). It is generally agreed that these compounds are useful in differentiating closely related species within a single habitat (Fenical 1975).

Rhodomela in British Columbia occurs in several variable and sometimes distinct morphological forms. Two of these forms--one common to tidepools in the upper intertidal zone, the other to the middle and lower intertidal to upper subtidal zone of the seashore--are

grouped within a single taxon, R. larix (Turner) C. Agardh.

To date, all collections of this species examined for bromophenols have come from the lower intertidal (Weinstein et al. 1975, Katsui et al. 1967). During investigations related to the present study, it became necessary to determine whether the two forms (tidepool and intertidal) should in fact be considered the same species. Since the technique of combined gas-liquid chromatography-mass spectrometry (GLC-MS) is commonly used for the separation and identification of algal halometabolites, a comparison of the tidepool and intertidal forms by this method was considered suitable for their assignment at the species level.

MATERIALS AND METHODS

Collection of Samples

Samples of the tidepool and intertidal forms of R. larix were collected in early September, 1978, on Bath Island. After removal of visible epiphytes, plants were washed in fresh water and returned to the laboratory on Dry Ice. Voucher specimens have been placed in the Phycological Herbarium at the University of British Columbia.

Extraction and Preparation of TMS Derivatives

In general the method of Pedersen (1978) was

followed. One g of each algal type was ground in a Virtis homogenizer with 25 ml 0.1 M sodium phosphate buffer (pH 5.0) and sonicated for 15 min (200 W). The extract was filtered through Whatman No. 1 paper and partitioned into ethyl acetate (3 x 50 ml). The ethyl acetate layers were combined and the solvent removed at 38°C (fraction A). The water layer was adjusted to pH 8 with 2 N NaOH and reextracted with ethyl acetate (fraction B). For a final fraction (C), the water layer was acidified to pH 2 with 1 N HCl, heated to 60°C (15 min) and extracted with ethyl acetate as before.

The dried fractions were taken up in 0.5 ml methanol and transferred to microsilation vials. Methanol was removed under nitrogen and the samples were dried over phosphorous pentoxide in a vacuum desiccator. For the preparation of trimethylsilyl (TMS) derivatives of the bromophenols, 25 ul each of acetonitrile and N,O-bis-trimethylsilyl-trifluoroacetamide containing 1% trimethylchlorosilane (Pierce Chemical Co.) as catalyst were added to each vial followed by heating to 60°C for 15 min.

Analysis

Samples were analyzed on a VG-Micromass mass spectrometer interfaced to a Pye-Unicam Series 104 gas-liquid chromatograph. A glass column of 3% SE-30 on

acid washed 80-100 mesh Chromosorb-W (Varian Assoc.) was used at a He flow of 30 ml/min. Temperature programming from 100-250°C at 50°C/min gave best results. Mass spectra were taken at 70 eV. In every case an ion at m/e 73 was excessive; therefore, only ions of greater m/e were recorded.

RESULTS AND DISCUSSION

Five bromophenols were identified in the extracts of two morphological forms of Rhodomela larix. The structures of these compounds and a typical GLC trace (intertidal fraction A) are shown in Figure 5. Fraction A contained compounds 1 (intertidal form only), 2, 3 and 4; fraction B, 2, 4 and 5; fraction C, 2 (intertidal only), 3 (tidepool only) and 4.

The following mass spectral data were obtained for the five compounds. Relative intensities (≥10% only) are given in parentheses following each m/e. Compound 1: m/e 74 (73), 75 (82), 77 (91), 147 (27), 205 (18), 221 (100), 222 (18), 223 (18), 257 (45), 259 (36), 271 (27), 345 (82), 347 (91), 360 (36), 362 (27). Compound 2: m/e 74 (100), 75 (74), 77 (21), 349 (12), 351 (24), 353 (15), 423 (53), 425 (100), 427 (59), 438 (32), 440 (56), 442 (35). Compound 3: m/e 74 (25),

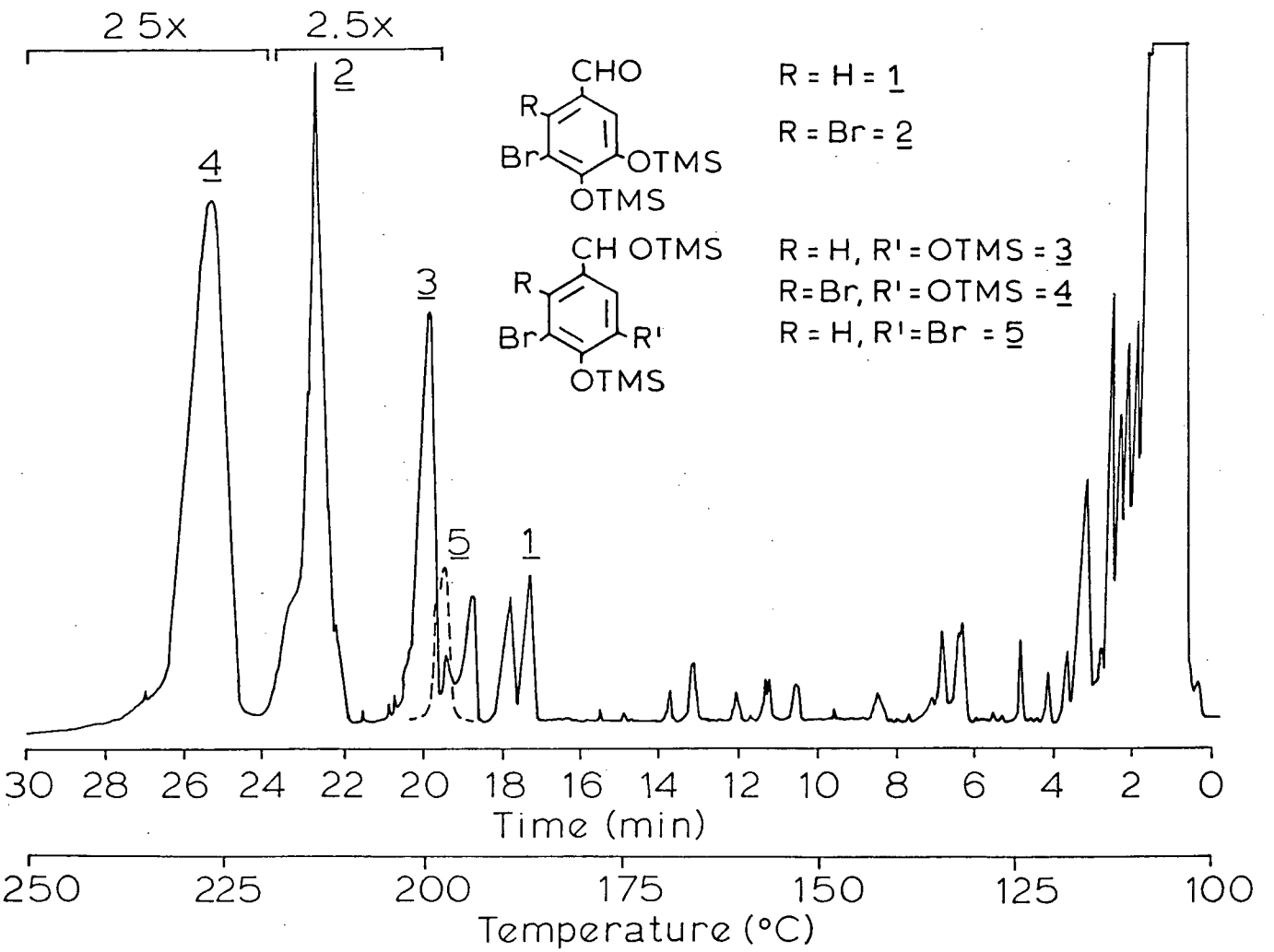

Figure 5. Temperature programmed GLC analysis of Rhodomela larix bromophenols. The relative position of compound 5 is shown by an inserted dashed line.

75 (32), 147 (27), 149 (100), 257 (84), 259 (93), 267 (11), 331 (14), 345 (20), 347 (20), 434 (34), 436 (41). Compound 4: m/e 75 (80), 147 (48), 149 (100), 335 (32), 337 (72), 339 (32), 411 (12), 423 (16), 425 (24), 427 (16), 433 (28), 435 (32), 512 (20), 514 (44), 516 (24). Compound 5: m/e 74 (28), 75 (30), 77 (12), 105 (20), 137 (13), 139 (17), 147 (100), 148 (22), 149 (12), 198 (12), 207 (13), 259 (12), 335 (22), 337 (40), 339 (23), 345 (18), 347 (23), 409 (32), 411 (65), 413 (35), 424 (10), 426 (20), 428 (12).

The two forms of Rhodomela examined here occur within about 3 m (12 ft) vertical distribution on British Columbia shores. Although their morphologies are quite distinct, it is interesting that they contain all but one of the same bromophenols.

Compound 1 occurred only in extracts of the intertidal form. If these aldehydes are to be considered artifacts of extraction procedures (Weinstein et al. 1975, Saenger et al. 1976), then the presence of this compound in one or the other of the types is inconsequential. I cannot argue against this supposition but can only question the production of such artifacts under the mild extraction procedures used here.

Most workers who have examined marine algae for halometabolites find sufficient evidence to separate populations into distinct chemical types if not in fact

distinct species (Fenical and Norris 1975, Fenical 1976, Crews et al. 1977, Caccamese et al. 1979). For Rhodomela larix, however, it appears that the tidepool and intertidal forms should be classed within a single species, differences in bromophenol chemistry not warranting separation at this level. Similar chemical investigation of other heteromorphic species should, in the future, lend further credence to the concept of pleomorphism in marine algae.

CHAPTER III

HPLC SEPARATION OF RED ALGAL BROMOPHENOLS

INTRODUCTION

Since their initial discovery as marine natural products (Mastagli and Augier 1949), halophenols have received increasing attention. These compounds have been isolated from a number of algae representing several orders of the Rhodophyta (Fenical 1975).

Paper chromatography (Péguy 1964), thin layer chromatography (Kurata et al. 1973, Weinstein et al. 1975, Ragan and Craigie 1978, Craigie and Gruenig 1967, Glombitza and Stoffelen 1972, Stoffelen et al. 1972, Chantraine et al. 1973, Pedersen et al. 1974), column chromatography (Chevolot-Magueur et al. 1976, Kurata et al. 1976, Combaut et al. 1978, Lundgren et al. 1979, Kurata and Amiya 1980, Glombitza and Stoffelen 1972), gas liquid chromatography (GLC) (Ragan and Craigie 1978, Pedersen et al. 1979, Stoffelen et al. 1972) and combined GLC-mass spectrometry (GLC-MS) (Saenger et al. 1976, Pedersen 1978, Stoffelen et al. 1972, Pedersen et al. 1974, Pedersen and DaSilva 1973, Pedersen and Fries 1975, Pedersen and Fries 1977, also see Chapter II) have been used extensively for the separation leading to subsequent structural elucidation of this group of phenols. Perhaps the most useful information

regarding the array of halophenols within each alga so far examined has come from GLC-MS analyses. Unfortunately, such complex instruments are expensive and generally unavailable. However, recent advances in the development of high-performance liquid chromatography (HPLC) systems make this chromatographic method comparatively inexpensive and therefore more widely available for the screening of organisms for various groups of compounds. Liquid chromatography (LC) has now advanced to the point that it rivals packed column GLC in application and precision. HPLC columns packed with 5-10 um microporous beads are capable of generating up to 25,000 theoretical plates per meter. A variety of accurate and sensitive detection systems is available which, when coupled to modern LC equipment, makes the separation and detection of micro- to nanogram quantities of most compounds possible.

Because of the ease of sample preparation (derivatization as in GLC not necessary) and the resolution and sensitivity achieved by this method, I chose to apply it to the determination of bromophenols from three common species of red algae in British Columbia. Rhodomela larix (Turner) C. Agardh, having received the scrutiny of several previous chemical investigations (Weinstein et al. 1975, Katsui et al. 1967) was chosen as a model organism on which to test the effectiveness of the HPLC

method.

MATERIALS AND METHODS

Chemicals

Standards of 2,3-dibromo-4,5-dihydroxybenzyl alcohol (2) (lanosol), 3-bromo-4,5-dihydroxybenzaldehyde (3), 3,5-dibromo-4-hydroxybenzyl alcohol (5), 3,5-dibromo-4-hydroxybenzoic acid (6) and 3,5-dibromo-4-hydroxybenzyl methyl ether (14) were kindly provided by Dr. J. S. Craigie (Atlantic Regional Laboratory, Halifax, Nova Scotia). Tetramethylammonium chloride (TMA) and 3,4-dihydroxybenzaldehyde were purchased from Aldrich Chemical Co. and HPLC grade acetonitrile from Fisher Scientific. All other chemicals and solvents were from various sources and were of the highest quality obtainable.

Synthesis of Other Bromophenols

The following syntheses were performed on a microscale to provide additional standards for comparison with crude extracts. Purification of the products was not attempted due to the small quantities involved. The reaction procedures utilized are well known and for the most part produce a single product.

3-Bromo-4,5-dihydroxybenzyl alcohol (1). Approximately 1.0 mg (30 uM) sodium borohydride was slowly added to a small amount of slightly cooled methanol containing

2.0 mg (1.0 uM) of 3. When the reaction had ceased, 0.1 ml water was added to hydrolyze the hydroborane and free the alcohol.

3-Bromo-4,5-dihydroxybenzyl methyl ether (4). To 0.3 ml of the reaction mixture from 1 above, two drops of 2 N hydrochloric acid (HCl) were added. After heating for one hr on a steam bath, a mixture of unreacted 1 and its benzyl methyl ether was found.

2,3-Dibromo-4,5-dihydroxybenzaldehyde (7). One mg (0.4 uM) of 2 was dissolved in 0.3 ml dimethyl sulfoxide and 0.2 ml acetic anhydride and heated on a steam bath for 15 min (Albright and Goldman 1965) to yield a single product.

2,3-Dibromo-4,5-dihydroxybenzyl methyl ether (8). Two drops of 2 N HCl were added to 1.0 mg (0.4 uM) 2 in 0.5 ml methanol. Heating on a steam bath for one hr produced 8.

3,6-Dibromo-4,5-dihydroxybenzaldehyde (9). The detailed procedure for the preparation of this compound has been given by Lundgren et al. 1979. Freshly sublimed 3,4-dihydroxybenzaldehyde (1.8 g, 0.13 M) was used as starting material. Concentration of the reaction solvent produced white crystals which were recrystallized from ethanol (yield = 0.38 g, 21%), mp 178-180°C. Infrared and proton magnetic resonance spectra agreed with those reported in the above references.

3,5-Dibromo-4-hydroxybenzaldehyde (10). One mg (0.5 uM)

of 5 was worked up as in 7 above. However, this reaction produced three products as monitored by HPLC. In addition to 10 the other products are thought to be the hemiacetal (12) and acetal (13) of the starting material. Aqueous acid addition and continued heating caused total conversion to the aldehyde. An alternative oxidation procedure which yielded the aldehyde as sole product consisted of heating for 15 min 1.0 mg of the alcohol (5) and 2 mg manganese dioxide in 0.5 ml methanol.

2,3-Dibromo-4,5-dihydroxybenzyl ethyl ether (11). The procedure was the same as in the preparation of 8 except that methanol was replaced by ethanol.

Chromatography

HPLC was performed on a Varian Model 5000 LC linked to a Variscan 634 S Spectrophotometer set at a constant wavelength of 280 nm. The elution solvent consisted of 40% acetonitrile (in glass distilled water) containing 10 mM each of TMA and dibasic sodium phosphate (buffer). The pH of the solvent was adjusted to 3.2-3.5 with concentrated HCl.

Initially, gradient elution with 80% acetonitrile and water (both containing additives) was used to determine optimal solvent strength for best resolution of the standards in the shortest time. Once established, isocratic elution with the 40% acetonitrile solvent proved the most

useful and economical.

Octadecylsilane columns of Micropak MCH-10 (Varian) were used for separations in the reverse phase mode. All compounds analyzed as standards were used in concentrations of about 1 mg per ml in methanol to give roughly equivalent detector responses. Injection volumes were typically 1-10 ul.

Evaluation of the Chromatographic Method

Three modified solvent systems and a mixture of compounds 5 and 6 were used (in addition to the solvent system described above) for the evaluation of column selectivity and efficiency: A, acetonitrile and water (4:6) only; B, A plus 10 mM buffer (pH 3.2); C, A plus 10 mM TMA; D, 40% acetonitrile solvent containing both additives. Each system used independently gave an indication of the effectiveness of an individual component to the method as a whole. From these measurements four chromatographic parameters were calculated: capacity factor (k'), relative retention (α), number of theoretical plates (n) and resolution (R). Formulae used in these calculations are as follows (Snyder and Kirkland 1974):

$$k' = \frac{t_r - t_o}{t_o}$$

$$\alpha = \frac{k'_2}{k'_1} \qquad n = 5.554\left(\frac{t_r}{w_{\frac{1}{2}}}\right)^2$$

$$R = \sqrt{\frac{n}{4}} \quad \left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{k' + 1}\right)$$

(a) (b) (c)

where t_r= retention time in min, $w_{\frac{1}{2}}$= peak width at half height, t_o= retention time of a nonretained solute and k'_1 and k'_2= capacity factors for two compounds separated (1 eluting before 2). The terms in the resolution equation indicated by the bracketed letters (a), (b) and (c) represent column efficiency, selectivity and capacity respectively.

Collection and Extraction of Algae

Rhodomela larix. Approximately 2 kg fresh weight of alga were collected in the subtidal zone off Bath Island. Visible epiphytes having been removed, the plants were washed in fresh water and immediately frozen at -80°C. Following lyophilization, the algae were ground in a Wiley mill to pass through a 2 mm mesh screen (yield = 447 g d.wt.). Soxhlet extraction of the dried

material using a series of solvents (light petroleum ether, chloroform, ethyl acetate and methanol) gave four fractions. Each fraction was reduced in volume *in vacuo* ($\leq 40^{o}C$) to about 50 ml. The large amount of solid (42.9 g) deposited on concentration of the methanol extract was removed by filtration and identified as the dipotassium sulfate salt of lanosol (Weinstein *et al.* 1975). One ml of each of the four fractions was taken to dryness, redissolved in 1.0 ml methanol and analyzed by HPLC for bromophenols.

Ceramium washingtoniense Kylin and *Prionitis lyallii* Harvey. Ten g of each alga were ground in a Waring blender in boiling 80% methanol and refluxed for one hr on a steam bath. The extracts were acidified to pH 2 with 1 N HCl, warmed to $60^{o}C$ for 15 min and filtered. The methanol was removed *in vacuo* and the remaining water extracted three times with ethyl acetate. The ethyl acetate layers were combined and the solvent removed *in vacuo*. The residues were taken up in 5.0 ml methanol and used for HPLC analyses.

RESULTS AND DISCUSSION

Separation of Standard Compounds

Figure 6 illustrates the separation of 14 bromophenol

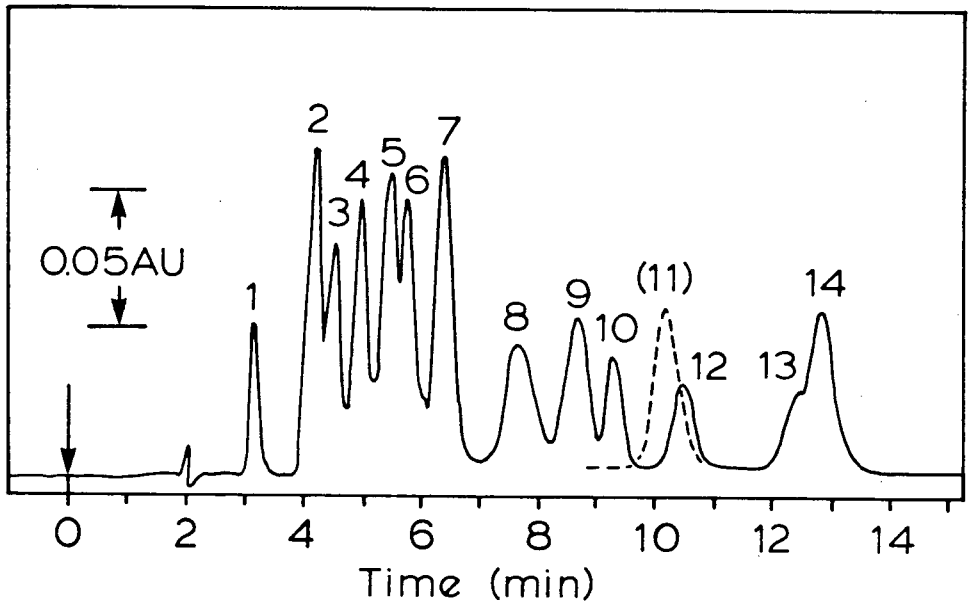

Figure 6. Reverse phase HPLC separation of fourteen bromophenol standards. The relative position of compound 11 is shown by a dashed line.

Table III. Retention times, capacity factors and relative retentions of standards of bromophenols separated by reverse phase HPLC. t_o = 1.8 min.

	COMPOUND	t_R (min)	k'	α
1)	3-bromo-4,5-dihydroxybenzyl alcohol	3.1	0.72	1.82
2)	2,3-dibromo-4,5-dihydroxy-benzyl alcohol	4.15	1.13	1.17
3)	3-bromo-4,5-dihydroxybenz-aldehyde	4.55	1.53	1.12
4)	3-bromo-4,5-dihydroxybenzyl methyl ether	4.9	1.72	1.16
5)	3,5-dibromo-4-hydroxybenzyl alcohol	5.4	2.0	1.11
6)	3,5-dibromo-4-hydroxybenzoic acid	5.8	2.22	1.13
7)	2,3-dibromo-4,5-dihydroxy-benzaldehyde	6.3	2.5	1.31
8)	2,3-dibromo-4,5-dihydroxy-benzyl methyl ether	7.7	3.28	1.15
9)	2,5-dibromo-3,4-dihydroxy-benzaldehyde	8.6	3.78	1.10
10)	3,5-dibromo-4-hydroxybenz-aldehyde	9.25	4.14	1.13
11)	2,3-dibromo-4,5-dihydroxy-benzyl ethyl ether	10.0	4.67	1.02
12)	Hemiacetal of 7 above	10.4	4.78	1.27
13)	Acetal of 7 above	12.7	6.06	1.02
14)	3,5-dibromo-4-hydroxybenzyl methyl ether	12.9	6.17	

standards at a flow rate of 1 ml/min (pressure 1120 psi). Retention times, capacity factors and relative retention data for these compounds are given in Table III.

The elution order of the compounds is, as expected, the reverse of that of a normal phase separation. The more polar alcohols and acids elute first, followed by aldehydes and ethers. o-Dihydroxy functions increase polarity while an o-dibromo function reduces polarity. Methyl and ethyl ethers are rendered quite nonpolar compared to their free hydroxyl counterparts. The ethyl ether of lanosol (11) is included to show the effect of extended chain length on retention time. Also of interest is the decreased polarity of the p- (9) versus the o-dibromoaldehyde (7).

Evaluation of the Chromatographic Method

Initial attempts at the separation of bromophenols by reverse phase LC met with little success: lanosol was irreversibly retained on the column, acids showed no retention and aldehydes gave long retention times with very broad and tailing peaks. Some authors have noted that repeated injections of an irreversibly retained compound eventually give results (van Sumere et al. 1979); however, the level of the compound retained is probably increased to the point that all column reactive sites are filled before elution occurs. Such gross contamination

must be considered undesirable since it inevitably shortens column life, not to mention its effect on efficiency.

In an effort to reduce these problems, suitable solvent modifiers were sought which would improve selectivity and efficiency while maintaining capacity. Since most phenols are quite acidic ($k_a = 10^{-10}$) and are easily converted to phenoxide anions in solution, the first method chosen to improve retention capability was ion suppression. This technique is commonly used for the LC of phenols (van Sumere et al. 1979) as it generally improves peak symmetry by suppressing tailing.

For many compounds, however, there is still the problem of irreversible retention, which can be overcome simply and effectively by adding a phase-altering reagent to the solvent. TMA has been used for this purpose (Burce, personal communication). This modifier selectively bonds to unblocked silanol groups in the column packing material. Compounds interacting with the stationary phase on a reverse phase column are then exposed to a completely nonpolar environment. The possibility of hydrogen bonding to silanol functions in the packing, as I believe occurs in the case of lanosol, is therefore negated.

The combination of ion suppression and TMA addition improves separation capability considerably. The results

of the evaluation of each component taken separately and then in combination are shown in Table IV. As noted from this table, TMA triples column selectivity and also increases column efficiency in the separation of compounds 5 and 6, a closely related alcohol and acid. Selectivity is reduced by addition of the buffer, although in the dual component system efficiency is still maintained at a high level. Selectivity can easily be increased by reducing solvent strength or flow rate.

Analysis of Rhodomela Extracts

The test of a chromatographic system comes when it is applied to the separation of a complex mixture such as a crude plant extract. The separations achieved for the four fractions of the Soxhlet extract of R. larix are shown in Figures 7 and 8. Most of the standard compounds appear in trace amounts in the petroleum ether fraction. Lanosol is the major component in the chloroform fraction, and the aldehyde (7) in the ethyl acetate fraction. Methyl ethers (4, 8, 14) appear only in the methanol extract.

In previous studies the aldehydes and methyl ethers of lanosol and related compounds have been considered to be artifacts of isolation procedures (Weinstein et al. 1975, Saenger et al. 1976). There is little doubt that ethyl and methyl ethers fall into this category; however,

Table IV. Resolution as a function of column efficency, selectivity and capacity using different solvent systems in the separation of 3,5-dibromo-4-hydroxybenzyl alcohol and the corresponding acid. Abbreviations used are given in the text.

SOLVENT SYSTEM	EFFICIENCY	SELECTIVITY	CAPACITY	R
A) Acetonitrile:water ::4:6	11.8	0	-	0
B) A plus 10mM buffer	8.72	0.28	0.46	1.12
C) A plus 10mM TMA	9.22	0.88	0.44	3.61
D) B plus 10mM TMA	8.96	0.26	0.45	1.05

Figure 7. HPLC analysis of the petroleum ether and chloroform fractions from the large scale (Soxhlet) extraction of Rhodomela larix. The assignment of peaks in parenthesis is uncertain.

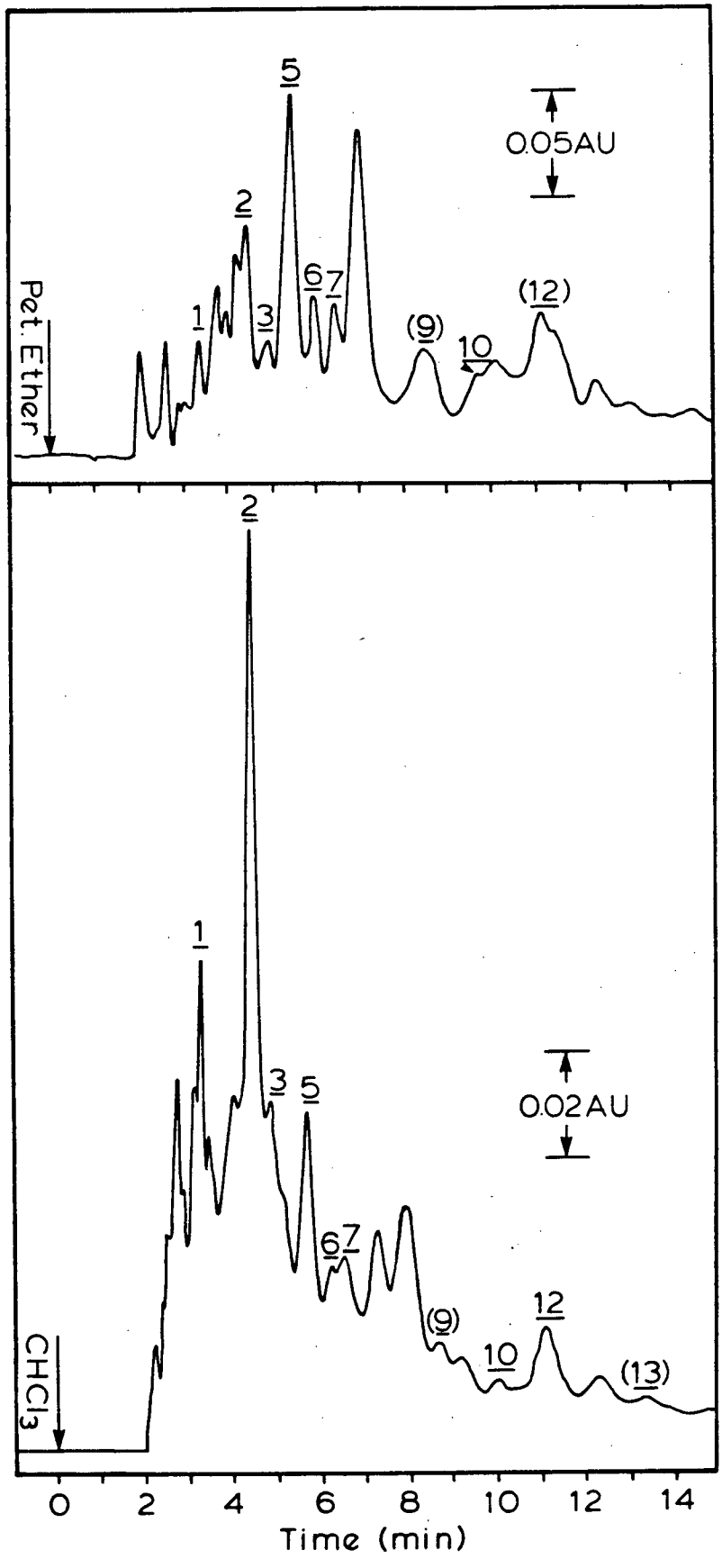
5
2
0.05AU
6 7
1
3
(9)
10
(12)
Pet. Ether
2
1
3
5
0.02AU
6 7
(9)
12
10
(13)
CHCl3
0
2
4
6
8
10
12
14
Time (min)

Figure 7

Figure 8. HPLC analysis of the ethyl acetate and methanol fractions from the large scale (Soxhlet) extraction of Rhodomela larix.

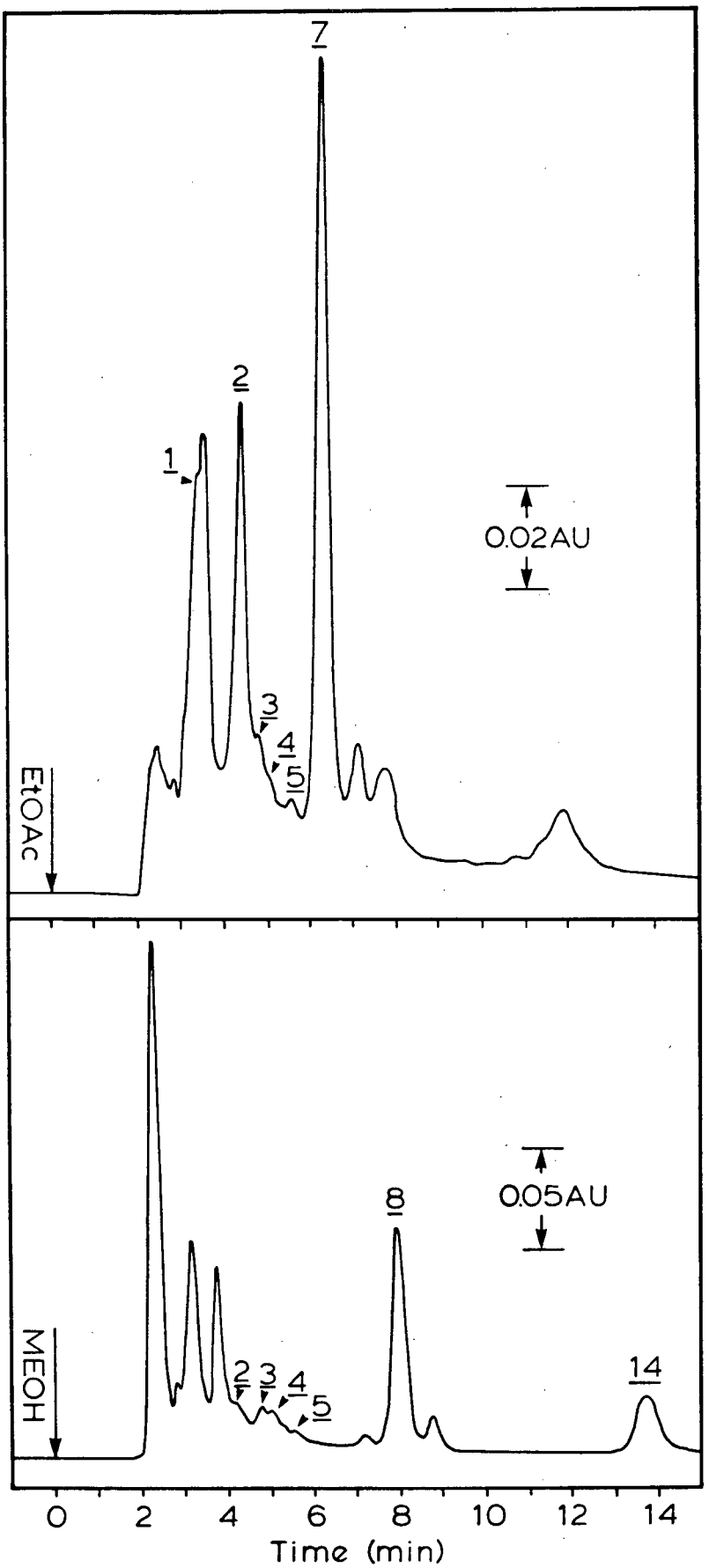
7
2
1
0.02AU
3
4
5
EtOAc
8
0.05AU
MEOH
2
3
4
5
14
0
2
4
6
8
10
12
14
Time (min)

Figure 8

the presence of aldehyde 7 throughout all four fractions causes me to believe that such compounds are natural constituents of the algae. Even the most mild extraction procedures (Pedersen 1978, see also Chapter II) show the presence of these compounds. The possibility of their existence in other species examined so far should, therefore, be reevaluated.

Chromatographic Analysis of Other Red Algae

In this study two other algal species, Ceramium washingtoniense and Prionitis lyallii, were examined for the presence of bromophenols. The LC traces for the extracts of these two species appear in Figure 9. Both algae contain bromophenols, Ceramium containing only one major compound (2). The identity of lanosol in this species was verified by cochromatography with the authentic standard at different solvent strengths and flow rates.

Prionitis, like Rhodomela, contains a variety of bromophenols, the most abundant being lanosol and the m-dibromo acid (6) and alcohol (5). A more careful extraction of both Prionitis and Ceramium may lead to the identification of an even wider array of phenolic compounds.

In conclusion, HPLC is a rapid and sensitive method for the determination of bromophenolic compounds in algae. Column selectivity and efficiency in reverse phase

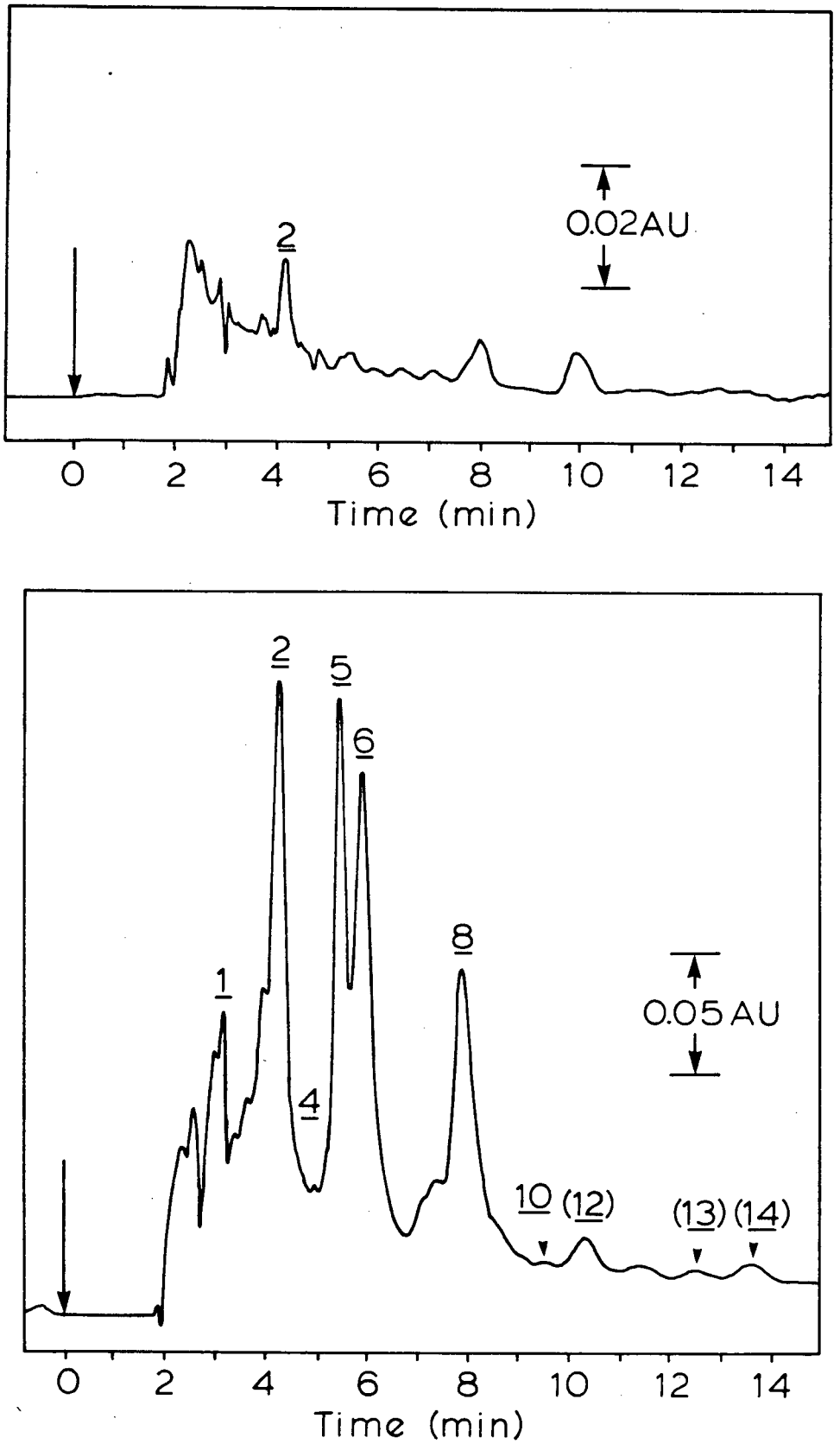

Figure 9. Reverse phase separation of bromophenols extracted from Prionitis lyallii (bottom) and Ceramium washingtoniense (top). Compounds in parenthesis are tentatively identified.

separations can be improved by the addition of modifiers which alter the nature of the stationary phase or suppress ionization when, for example, the separation of acidic metabolites is necessary. Isocratic elution with solvents containing modifiers produces adequate separation of a wide variety of bromophenols containing many types of functional groups. Under these conditions the need for chromatographs equipped with complex gradient forming devices is eliminated.

CHAPTER IV

TEMPORAL, INTERPOPULATIONAL AND INTRATHALLIAL MEASUREMENT OF LANOSOL LEVELS IN *RHODOMELA LARIX*

INTRODUCTION

Fenical (1975) has suggested that red algal halometabolites, rather than being involved in primary metabolic pathways, function in an exocrine system providing the alga containing them with a selective environmental advantage. The nature of this advantage, if it exists, is unclear; however, excretion of dissolved organic matter (DOM) by algae is well documented (Hellebust 1974, Wangersky 1978, Ragan and Jensen 1979). Such materials may only be waste products of metabolism, but they may also be actively produced as antibiotic substances (Langlois 1975, Ragan and Jensen 1978).

The study of variations in the temporal abundance of bromophenols in red algae has received little attention. Our present knowledge consists only of quantitative data derived from algal extractions which, unless very carefully done, must invariably lead to underestimation of compounds in the plants. Table V summarizes the available literature in this area and illustrates the great variability in the data even for multiple extracts of a single species.

As a result, the physiological and ecological

Table V. A summary of data available on quantitative aspects of bromophenols in red algae. All estimates are based on the abundance of the naturally occurring phenol, not on derivatives which may have been prepared in isolation procedures. The compounds listed are as follows: 1) 3,5-dibromo-4-hydroxyphenyl acetic acid, 2) 3,5-dibromo-4-hydroxyphenyl pyruvic acid, 3) perdesmethylcyclotribromoveratrylene, 4) 2,3-dibromo-4,5-dihydroxybenzyl methyl ether, 5) 2,3,2',3'-tetrabromo-4,5,4',5'-tetrahydroxydiphenyl methane, 6) 2,3-dibromo-4,5-dihydroxybenzyl alcohol, 7) 2,3-dibromo-5-hydroxybenzyl-1',4-disulfate (potassium), 8) 3,5-dibromo-4-hydroxybenzyl alcohol, 9) 2,3-dibromo-4,5-dihydroxybenzaldehyde, 10) 2,3-dibromo-4,5-dihydroxybenzyl ethyl ether, 11) 3-bromo-4,5-dihydroxybenzaldehyde, 12) 3,3'-dibromo-4,4',5,5'-tetrahydroxybibenzyl, 13) 3-bromo-4,5-dihydroxybenzyl methyl ether, 14) 3,5-dibromo-4-hydroxybenzyl methyl ether, 15) 2,4-dibromo-1,3,5-trihydroxybenzene, 16) 5,6,3',5'-tetrabromo-3,4,2',4',6'-pentahydroxydiphenyl methane, 17) bis(2,3,6-tribromo-4,5-dihydroxybenzyl) ether. * = estimated abundance. ! = tentative identification.

ALGA	COMPOUND	% d.wt.. (% w.wt.)	REFERENCE
Halopytis incurvus	1	(0.003)	Chantraine et al. 1973
	2	(0.002)	
H. pinastroides	1	(0.003)	Combaut et al. 1978
	2	(0.003)	
	3	(0.003)	
Odonthalia corymbifera	4	(0.01)	Kurata et al. 1973
	5!	(0.004)	
	6	(0.008)	
	7	(2.0)	
O. dentata	6	0.5-2.0*	Craigie and Gruenig 1967
	8	0.1, (0.024)	ibid., Glombitza and Stoffelen 1972
Polysiphonia brodiaei	8	0.5, 0.005	Pedersen et al. 1974, Lundgren et al. 1979
	9	0.08	Lundgren et al. 1979

Table V. continued.

	10	0.006	
	5	0.005	
P. lanosa	6	1-5, 2-3	Hodgkin *et al.* 1966 Ragan and Craigie 1978
	7	1.0	Glombitza and Stoffelen 1972
P. morrowii	11	0.07	Saito and Ando 1955
P. urceolata	11	0.08	Kurata *et al.* 1976
	12	0.005	
	13	0.02	
	14	0.03	
Rhodomela confervoides	6	0.02-0.06*, (0.003)	Craigie and Gruenig 1967, Glombitza and Stoffelen 1972
	8	0.003	Craigie and Gruenig 1967
R. larix	4	0.005, (0.009)	Katsui *et al.* 1967, Weinstein *et al.* 1975
	9	0.03	Katsui *et al.* 1967
	7	(0.04)	Weinstein *et al.* 1975
R. subfusca	4	0.06	Kurata and Amiya 1975
	7	9.2	
	9	0.02	
Rytiphlea tinctoria	6	(0.009)	Chevolot-Maguer *et al.* 1976
	10	(0.009)	
	15	(0.009)	
	16	(0.004)	
Symphyocladia latiscula	17	(0.01)	Kurata and Amiya 1980

significance of bromophenols in red algae is unknown. Bromophenols have been shown to be effective antibiotics (Silva and Bittner 1979), but no information is available on quantitative aspects of their occurrence, which might be helpful in assessing their importance to the plant. Ragan and Jensen (1978) have shown that a knowledge of seasonal variation in concentrations of polyphenols in brown algae can provide useful information regarding their significance. For this reason I chose to examine the red alga Rhodomela larix (Turner) C. Agardh for changes in the levels of lanosol, the major bromophenol in this species (see Chapter III), over a one year period. Also examined were differences in bromophenol content among three distinct populations and within a single plant (intrathallial). It was hoped that an extensive quantitative chemical examination of this alga would lead to a clearer understanding of the raison d'être of bromophenols in red algae.

MATERIALS AND METHODS

Collection of Algal Samples

Unless otherwise indicated, all algal samples were collected in the lower intertidal to upper subtidal zone off Bath Island during the highest tide of the month.

The plants for each sample were randomly selected over a large area, cleaned of epiphytes and returned to the lab in plastic bags over ice. Algae were then used immediately or frozen (-20°C). Other samples came either from tide-pools on Bath Island or from the low intertidal zone near Bamfield, British Columbia.

Extraction Procedures

All extractions were done in triplicate; dry weights were taken in duplicate, the samples having been dried for one week at 90°C. One g of each algal sample was ground in a Virtis microhomogenizer in boiling 80% methanol. Following one hour reflux on a steam bath, particulate matter was filtered off and the methanol removed *in vacuo*. The residual water layer was acidified to pH 2.0 with 1.0 N HCl, heated to 60°C for 15 min (to hydrolyze ester sulphates) and then continuously extracted with ethyl acetate (4 hrs). The ethyl acetate was removed and the residue taken up in 5.0 ml methanol for subsequent chromatographic analysis.

Collections from the other two locations (see above) were worked up in the same way for relative comparison of phenol content among populations.

For measurement of lanosol concentration in different parts of the plant, 1.0 g each of growing tips, branches with many laterals, branches with few laterals

and holdfasts was excised and extracted as before.

Chromatographic Methods

Quantitative determination of lanosol was performed by HPLC using the chromatographic system and eluants described in Chapter III. In this case, however, a wavelength of 292 nm was used since this is the maximum absorption of lanosol (Weinstein et al. 1975). The chromatograph was fitted with a 10 ul calibrated loop. Injections of this volume were always used to minimize potential error in this part of the quantification procedure. The flow rate was 1 ml/min.

Lanosol was identified by comparison with the authentic standard. Calibration curves for lanosol in methanol were made by measuring peak areas by the width at half height method and converting to mg/ml of lanosol and subsequently to mg/g dry weight as quantities in the alga.

The life history data for algae in these samples were provided by Ulla Visscher (personal communication).

RESULTS AND DISCUSSION

Figure 10 shows a typical HPLC separation of the bromophenols from R. larix. Lanosol was chosen as an indicator of the level of total bromophenols in the plant.

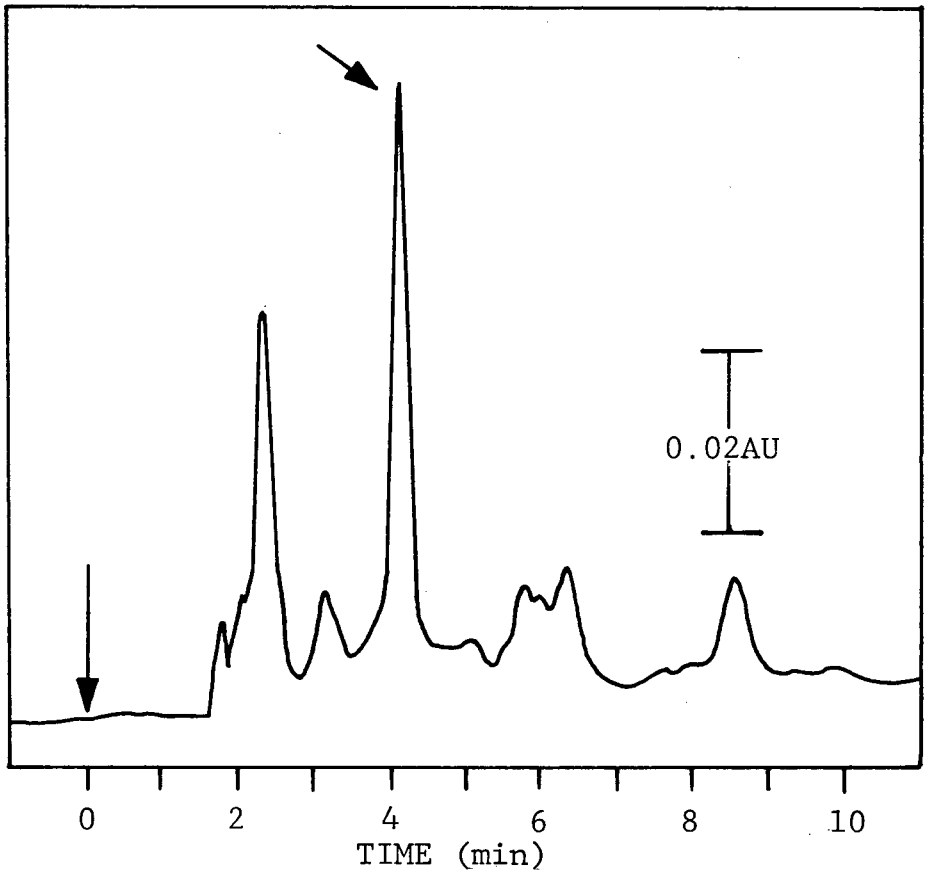

Figure 10. A typical HPLC separation of Rhodomela larix bromophenols used for the quantitative determination of lanosol (indicated by the arrow). The chromatographic conditions are listed in the text.

The peak corresponding to lanosol is well separated and can easily and accurately be quantified by this method.

The yearly range of lanosol levels in R. larix is 1.2 to 3.8% on a dry weight basis (Figure 11). The levels in winter are essentially three times those of the summer months. Ragan and Jensen (1978) found similar maxima and minima in the brown algae which they examined for polyphenols.

The results of the interpopulational comparisons are shown in Table VI. The wide range of lanosol levels among the three populations examined here is typical of the variation observed for this species (Table V). The tidepool form had higher lanosol levels in the summer months than did either the subtidal Bath Island or Bamfield populations.

Ragan and Jensen (1978) have pointed out that the higher levels of polyphenols in brown algae occur not during the period of maximum potential epiphytization (spring and summer) but during the fall and winter months when colonization by the smaller marine algae is at a minimum. For Rhodomela the same pattern was observed. Both tidepool and subtidal plants are typically epiphytized in the early spring increasing to a maximum epiphyte cover by early to mid summer. Thereafter the diversity of Rhodomela epiflora decreases until in winter none is

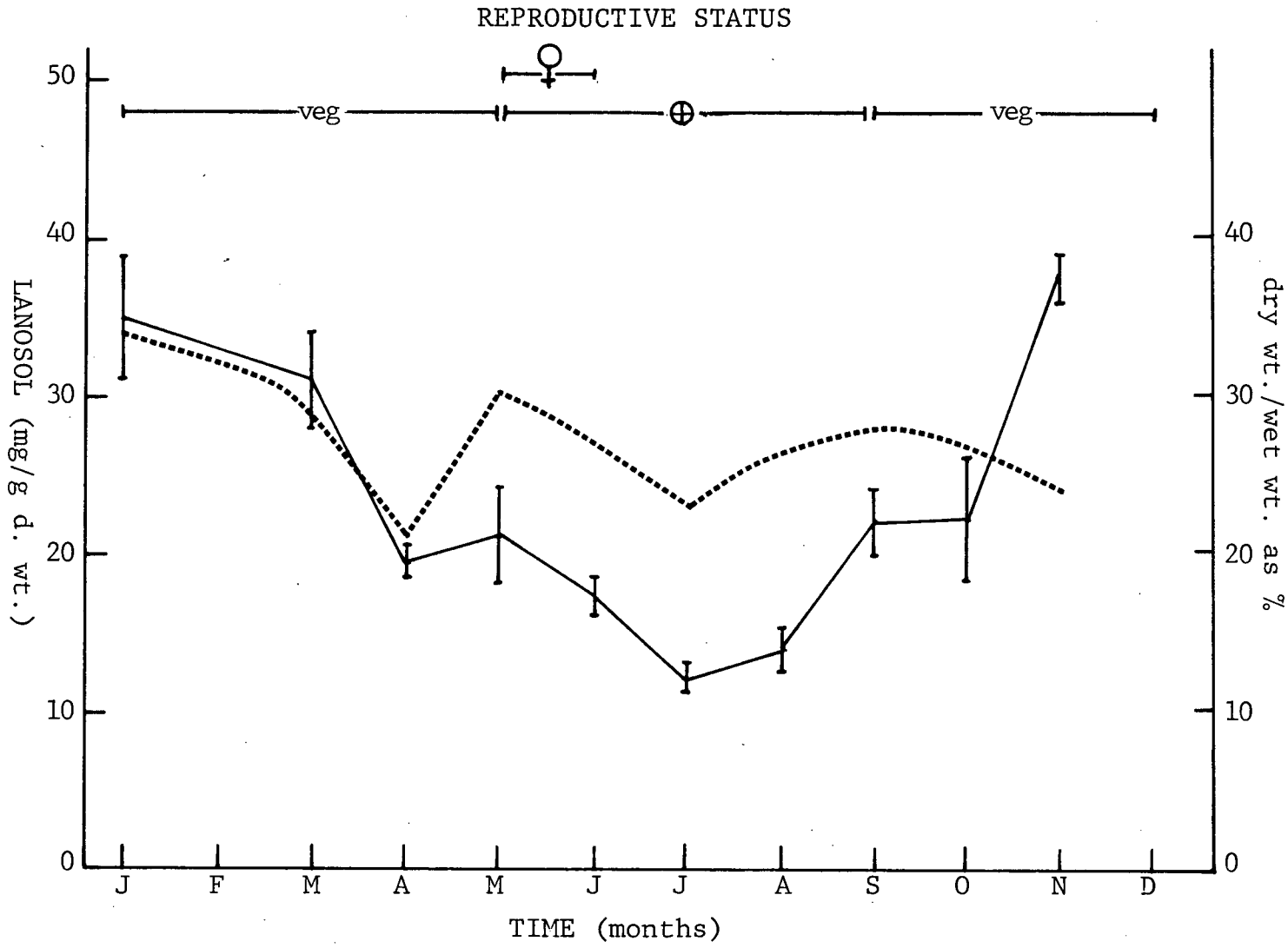

Figure 11. Seasonal variation of lanosol content in Rhodomela larix (solid line) and change in the dry to wet weight ratio over the period of one year (dashed line). The deviation from the mean for all samples is indicated by vertical bars and the reproductive state of the plants by horizontal bars (⊕ = tetrasporic plants, ♀ = carposporic plants, veg = vegetative plants).

Table VI. Quantitative comparison of lanosol concentrations in three distinct populations of Rhodomela larix (collected in August 1978).

POPULATION	LANOSOL (mg/g d.wt.)	REPRODUCTIVE CONDITION
Bamfield (low intertidal)	11.8 (± 0.30)	tetrasporic
Bath Is. (low inter- to high subtidal)	13.9 (± 0.17)	vegetative and tetrasporic
Tidepool (Bath Is.)	21.7 (± 0.55)	vegetative

observed.

The data for intrathallial variation in lanosol content (Figure 12) show maximal levels of the compound in the youngest and most rapidly growing portions of the algal thallus. Rhodomela is almost invariably epiphytized in the older regions of the thallus as are many other species of marine algae (Ballantine 1979). This could be a result of the lower concentrations of lanosol in these portions, as contrasted to the growing tips. Leaching out or exudation of the compounds during a period of less active growth (summer) would lead to lower levels of the compounds for the entire plant. This could explain the rapid drop in lanosol content as summer proceeds. Higher levels of insolation and higher water temperatures, or a combination of factors might affect the levels or rate of change in the levels of lanosol in the plants. Further study correlating the period of most active growth with changes in lanosol levels should provide a better understanding of the role of lanosol in epiphyte control.

Lanosol has been shown to be an effective antibacterial and antifungal agent (Silva and Bittner 1979). Again, as with brown algal phenols (Ragan and Jensen 1978), the ecological significance of bromophenols to red algae may be in the control of pathogens rather than epiphytes.

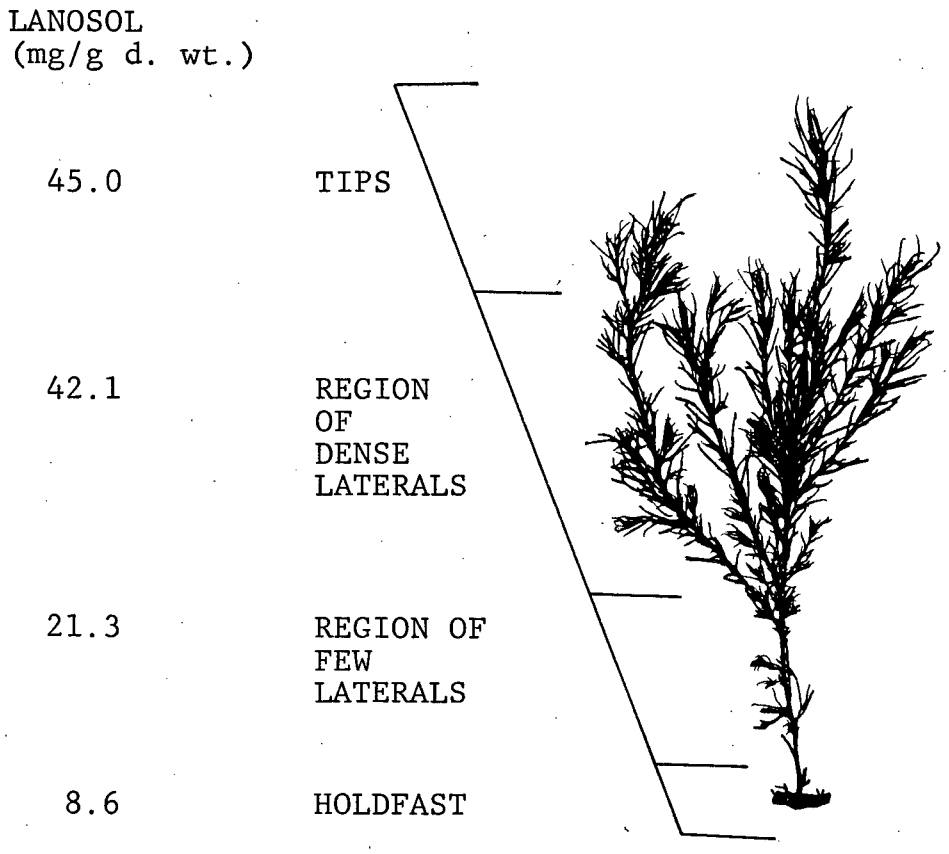

Figure 12. Lanosol content in different parts of the Rhodomela larix thallus (collected from tidepools on Bath Is., B.C. in September, 1979).

The control of herbivory by these compounds is also not excluded. The author has shown (Chapter VI) that lanosol and, at much higher concentrations, its dipotassium sulfate salt are effective as repellants against tidepool snails. This effect could extend to other potential predators as well.

The possibility that these compounds are waste products of metabolism (Pedersen et al. 1979) seems highly unlikely. Biochemical evolutionary trends do not generally lead to the formation of by-products more complex and potentially more toxic than the starting materials from which they are synthesized (Swain 1977). Neither should bromophenols be considered storage products since their minimum accumulations occur during the period when environmental conditions would favor increased photosynthesis and, subsequently, increased food storage.

From the results of these experiments one can conclude that the scope of possible functions for bromophenols has been considerably narrowed. Until a more careful assessment of the effectiveness of lanosol as antibiotic, antiepiphyte or antiherbivore agent is made, the significance of bromophenols in the ecology of red algae will remain unclear. Work done along these lines will be presented in Chapter VI.

CHAPTER V

EXUDATION OF BROMOPHENOLS BY *RHODOMELA LARIX*

INTRODUCTION

The exudation of tannins, polyphenols and other reducing substances ("Gelbstoff") by brown algae has long been recognized (Wangersky 1978). Ragan and Jensen (1979) have recently examined the control of exudation in Ascophyllum nodosum and shown that this alga produces polyphenols whose rate of release is enhanced in light. When such algae occur in tidepools, the accumulation of their yellow brown exudates is known to be effective in antibiosis against many groups of organisms, ranging from bacteria to planktonic and sessile marine animals (Conover and Sieburth 1966, Sieburth and Jensen 1969, Langlois 1975).

Less studied are the exudates of marine red algae, which are known (Khailov and Burlakova 1969, Langlois 1975) to release copious amounts of dissolved organic matter (DOM). These exudates have not been chemically characterized, but only estimates of total phenols or carbohydrates (or of both as DOM) have been made (Khailov and Burlakova 1969, Langlois 1975). Red algae contain high levels of carbon-halogen compounds (Fenical 1975), many of which are antibiotic (Silva and Bittner 1979). If it is these compounds that are exuded, then their presence in ocean waters

in significant concentrations raises interesting ecological questions as to the advantage afforded the alga producing them (Pedersen et al. 1974, Fenical 1975). Such compounds may function in controlling epiphytization, herbivory or fouling of marine algae by other algae, animals or microorganisms.

In the marine environment, physical and chemical factors such as pH, salinity and temperature play an important part in the physiology of macrophytes (Biebl 1962, Soeder and Stengel 1974) and should also affect exudation processes such as the rate of release of DOM as polyphenols and as other substances. In this study I examined the effects of selected environmental modifications on the rate and quality of exudation by Rhodomela larix (Turner) C. Agardh, obtained from high intertidal tidepools in British Columbia. During the day an increasing amount of yellow to reddish brown discoloration becomes apparent in coastal pools containing this species. Much of this coloration is attributable to phenolic compounds (see Chapter I); however, earlier attempts at the identification of the individual components of the exudates were unsuccessful.

I have now reexamined the exudates of R. larix in controlled laboratory experiments and am able not only to control exudation within normal ranges of environmental

factors, but also to identify and quantify separately the major phenolic component of exudates.

MATERIALS AND METHODS

Collection and Maintenance of Algal Specimens

Algae were collected from tidepools in the high intertidal or from the upper subtidal zone of Bath Island. Plants were cleaned of epiphytes, washed in fresh sea water and immediately returned to the laboratory wrapped in newspaper over ice. In the lab the plants were washed in filtered sea water (0.45 um), rechecked for epiphytes and maintained in 2.5 l "low form" culture flasks on gyro-rotary shakers (60 rpm) for 48 hrs under medium light (330 uE. $m^{-2}sec^{-1}$, light cycle = 12:12) at 15°C. After this "adjustment" period the algae were used in exudation experiments.

Experiments Involving Measurement of Total Phenols

Ten grams of each algal type (subtidal and tidepool collections) were washed in 0.22 um filtered sea water and transferred (in duplicate) to 100 ml of the same in 250 ml Erlenmeyer flasks. An additional pair of flasks was set up as a dark control by wrapping them in several layers of aluminum foil to exclude light. The flasks were returned to the shaker and the temperature raised to 20°C. Total

phenols were measured at 0, 4, 8 and 24 hrs according to the method of Langlois (1975). Optical density was read on a Pye Unicam Model SP-8500 UV-VIS Spectrophotometer and converted to phenol concentrations as ppm of phloroglucinol.

Exudation under Different Environmental Conditions

Three Controlled Environments (Model E15) growth chambers were used at temperatures of 10, 20 and 30°C. Light conditions were set so that a range of from 0-1000 uE $m^{-2}sec^{-1}$ could be achieved in the 20°C chamber. For attenuation to the lower light intensities, multiple layers of cheesecloth were placed over foil-lined boxes. Light was measured with a Li-Cor LI- 185 Quantum meter fitted with a Model UWQ 2192 probe. The light source in each chamber was a bank of 24 Duro-test Vita lights (72T12) which were used at full output for the higher light intensity. Two hundred and fifty ml exudation flasks containing 100 ml filtered sea water were set up as before. For a series of increasing salinities (5, 15, 25 and 35 °/oo, or parts per thousand of salinity) and pHs (6, 7, 8 and 9), filtered sea water was either diluted with glass distilled water (or partially evaporated for 35 °/oo) or the pH was adjusted with dilute HCl or NaOH. Into each flask (in duplicate) for each set of conditions to be tested, 10.0 g of freshly washed and gently blotted

tidepool R. larix were added and the flasks placed in the growth chambers under the appropriate conditions. Dissolved oxygen levels were monitored initially and at the end of the experimental period (8 hrs) as an indication of algal viability. Five replicate dry weight (after drying for 1 wk at 90°C) determinations were made for 10.0 g samples washed and blotted as above and the values obtained averaged for the estimation of the initial dry weights of experimental samples.

Extraction and Chromatography of Exudates

After 8 hrs each flask was removed from the growth chamber, the algae filtered off and the medium acidified to pH 2 with 1 N HCl and warmed 15 min on a steam bath. The water was then extracted with ethyl acetate (3x100 ml), and the ethyl acetate layers were combined and dried over anhydrous sodium sulfate. After the solvent was removed in vacuo (38°C), the residue was taken up in 0.5 ml methanol and used for chromatographic analysis.

RESULTS AND DISCUSSION

A definite increase in Brentamine reactive substances is observed when Rhodomela larix is exposed to moderate irradiation and temperatures in a controlled laboratory environment (Table VII). This release occurs

Table VII. Exudation measured as total phenols over a twenty-four hour period by tidepool and subtidal (both in the light and dark) forms of Rhodomela larix. Quantities given are as parts per million of phloroglucinol.

COLLECTION	TIME (hours)		
	4	8	24
Tidepool form	5.7	6.4	46.5
Subtidal form (in light)	21.5	24.0	63.1
Subtidal form (in dark)	6.3	6.5	47.3

from algal thalli which have been given time to adjust to the "shock" of being transferred from their natural habitat to the culture chamber. The rate of release is rather constant whether the algae are kept in the light or dark. Since the tidepool form of R. larix releases less total phenols, it seems better adapted to longer and more intense periods of insolation than does the subtidal form. This would appear to be typical of many physiological responses examined in marine micro- and macrophytes (Biebl 1962, Soeder and Stengel 1974). Increased "stress" promotes a greater response and in the case of the subtidal form, which is exposed to lower light intensities and cooler water temperatures than its high intertidal counterpart for most of the year, such stress increases the rate of exudation.

HPLC analysis of the individual phenols in the 24 hr exudate shows lanosol to be the major component (Figure 13). Pedersen et al. (1974) have also found this compound in sea water of the Polysiphonia brodiaei zone on Swedish shores. Therefore, it appears likely that exudation of bromophenols is a widespread phenomenon, even for algae still in their natural habitats.

The experimental conditions and results given as mg/ml of lanosol exuded are given in Table VIII. As temperature increases so does lanosol exudation and

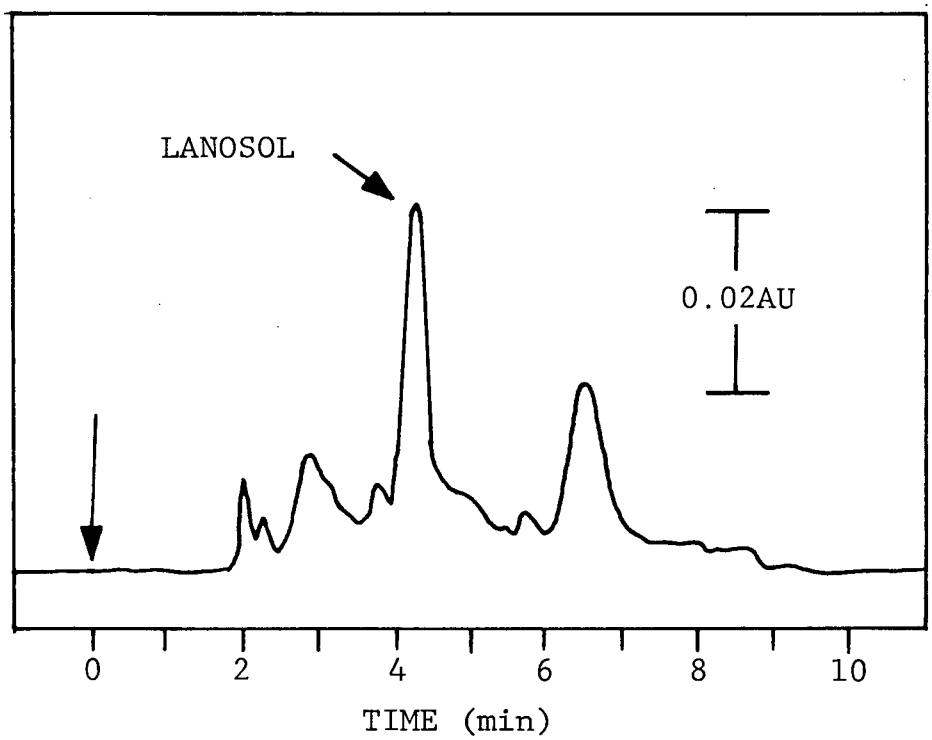

Figure 13. HPLC of the twenty-four hour exudate of the subtidal form of Rhodomela larix kept in the light for 24 hrs (see Table VII and p. 70).

Table VIII. Quantitative determination of exuded lanosol and other observations under varying experimental conditions. Unless specified, temperature = 20°C, °/oo = 25, light intensity = 330 uE $m^{-2}sec^{-1}$ and pH = 8. The initial oxygen concentration was taken as 0 ppm and the mean initial dry weight was 1.85 g. (*) = replicate at 1000 uE $m^{-2}sec^{-1}$ to determine ratio of lanosol to lanosalt exuded over the experimental period.

CONDITION		FINAL pH	FINAL dry wt.	OXYGEN (ppm)	LANOSOL (mg/ml)
TEMP.	10°C	8.0	1.78	7.02	0.02
	20°C	8.0	1.90	6.51	0.05
	30°C	4.5	1.41	1.59	0.56
SALINITY	5 °/oo	5.4	1.33	5.28	0.61
	15°/oo	8.6	1.83	5.98	0.04
	25°/oo	8.6	1.80	7.13	0.04
	35°/oo	8.5	1.85	6.95	0.03
LIGHT	0	6.7	1.66	1.66	0.04
	100	7.8	1.93	6.35	0.03
	330	8.0	1.90	6.51	0.05
	1000	4.9	1.51	5.25	3.0
(*)	1000($-H^+$)	4.6	1.35	5.15	3.3
	1000($+H^+$)	-	-	-	0.17
pH	6	7.9	1.79	5.19	0.03
	7	7.4	1.77	6.25	0.05
	8	8.9	1.78	6.51	0.04
	9	8.9	1.98	6.86	0.04

discoloration of the medium (Figure 14)[4]. An opposite effect is seen with regard to increasing salinity. Lower salinities produced the highest levels of pigmentation and lanosol in the medium (Figure 14). Saenger (1970) has previously extracted the red pigment floridorubin from several species of red algae by placing them in distilled water for extended periods. This pigment upon hydrolysis yields a variety of halophenols including lanosol (Saenger et al. 1976). Varying pH had little effect on exudation, while higher light intensities increased exudation (Figure 15) without apparent damage to the algae (see oxygen evolution data). The highest temperatures, on the other hand, were damaging (respiration exceeding photosynthesis). The greater rates of release for algae under these conditions were probably due to the leaching out of lanosol from dead or dying cells.

The initial pH of all media, except those where this factor was altered, was 8.0. In every case where there was a large release of lanosol into the water surrounding the algae, the pH dropped well below 5.0 due to an accumulation of the acidic phenolics (k_a phenol = 10^{-10}) in the medium. However, had the phenols been exuded as sulfate esters, the observed pH change would not have been as great. As a means of

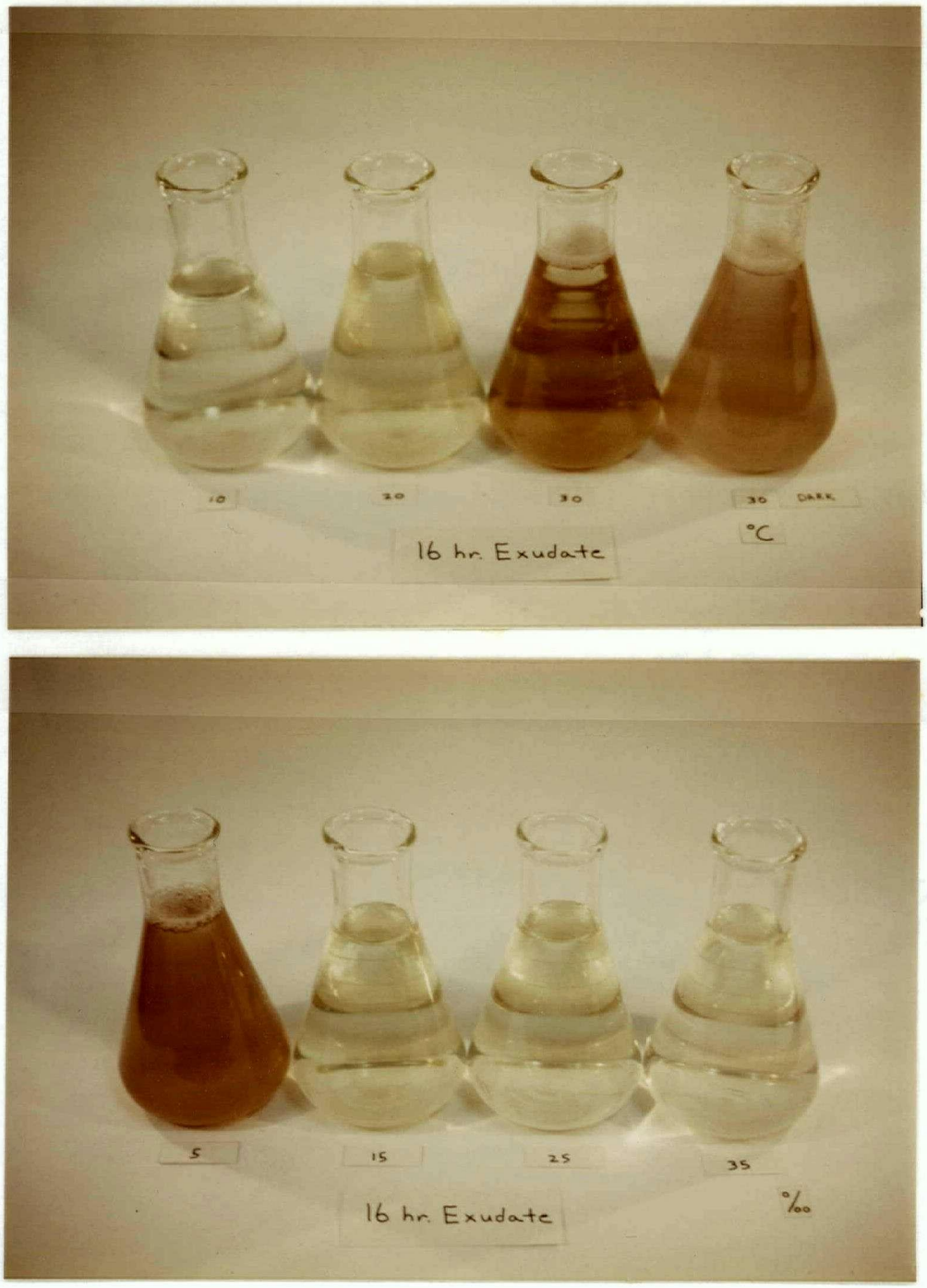

Figure 14. Effect of temperature and salinity on the production of colored matter by Rhodomela larix during an eight hour exudation experiment. The algae have been removed from the media to improve clarity of the colors. (see Table VIII for further details of conditions)

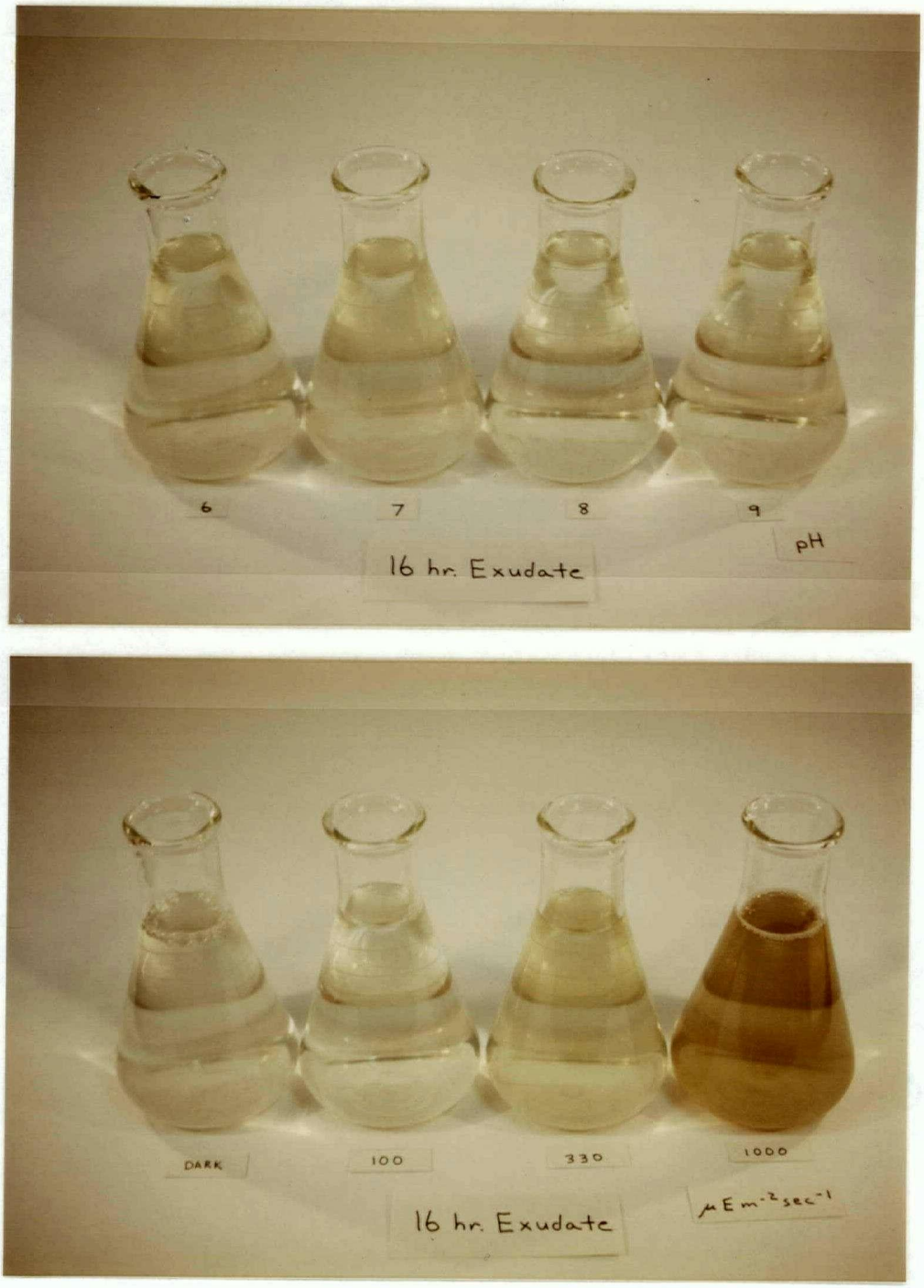

Figure 15. Effect of pH and light on the production of colored matter by Rhodomela larix during an eight hour exudation experiment. The algae have been removed from the media to improve clarity of the colors. (see Table VIII for further details of conditions)

determining the chemical nature of exuded lanosol, a replicate, illuminated at 1000 uE $m^{-2}sec^{-1}$, was used and extracted without being previously acidified. The medium was then reextracted after acidification. The additional lanosol removed from the acidified fraction constituted only 5.2% of that obtained upon unacidified extraction (Table VIII). As a result, I conclude that lanosol is exuded as the free phenol.

Another question which arises is whether sulfate esterified lanosol is broken down by bacteria or other microorganisms after exudation into the medium. In order to answer this question, 1 mg of the dipotassium sulfate salt of lanosol (see Chapter III) was added to 10 ml "dirty" sea water obtained directly from the tidepool from which R. larix was originally collected. After 24 hrs at 20°C under high light, no lanosol could be detected even at the highest detector sensitivities.

Conversion of the data in Table VIII into mg lanosol exuded/g dry weight hour gives the comparative rates of exudation illustrated in Figure 16. All of the physical and chemical modifications used in these experiments fall within ranges normally occurring in the tidepools in summer months (see Chapter I), and within this set of conditions the exudation rates for this species range from about 1-150 ug/g d.wt.hr.

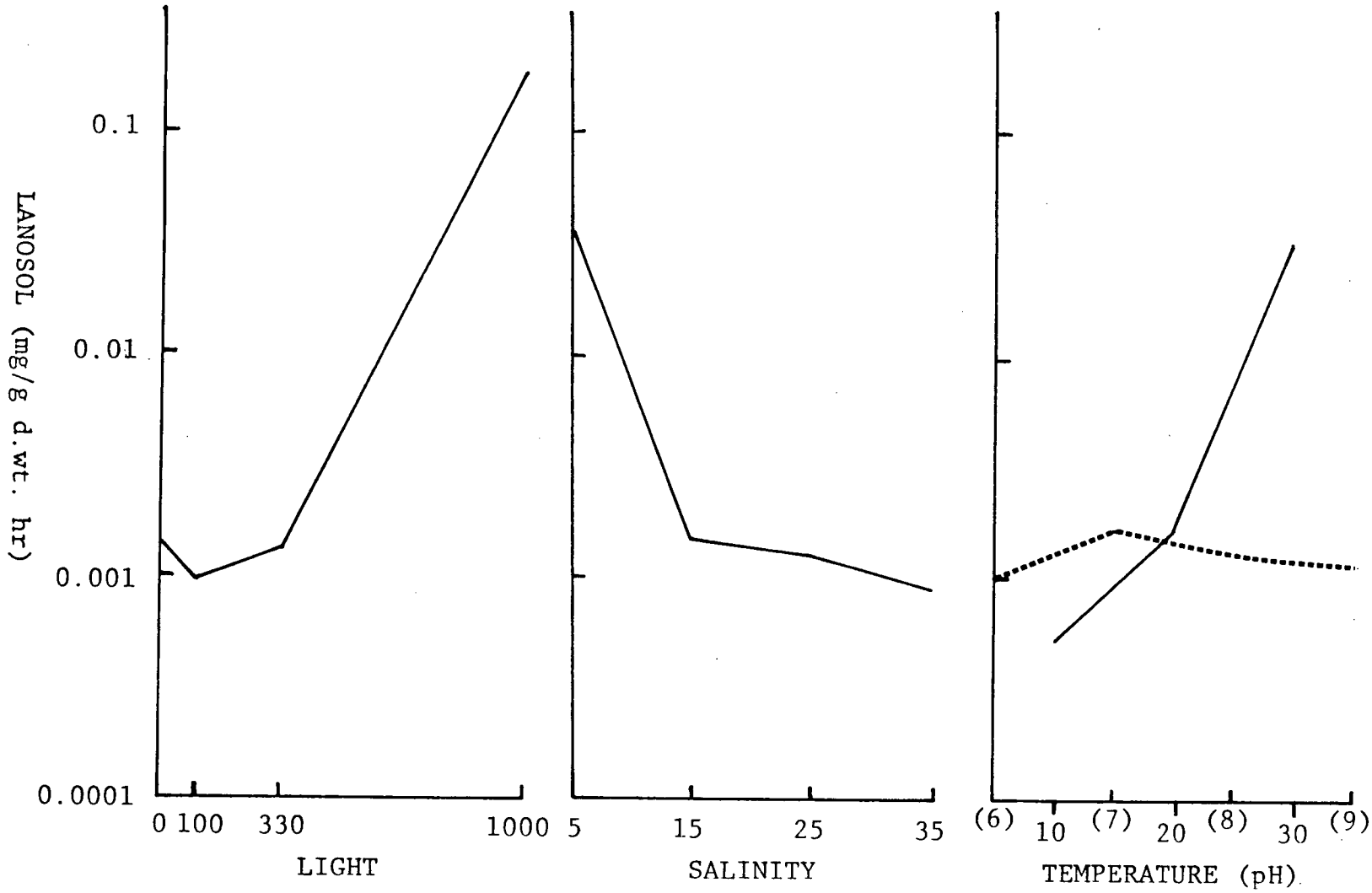

Figure 16. Effect of light, salinity, temperature and pH (·····) on exudation of lanosol by Rhodomela larix.

R. larix contains from 1-4% of lanosol on a dry weight basis (see Chapter IV). At the rates of release found here, this alga is exuding 0.3-1.6% of its total lanosol content per hour, indicating an active turnover and, hence, synthesis of this compound within the plant. The ecological and physiological implications of these findings are thus most intriguing.

As noted in Ragan and Jensen (1979), it has been suggested that increased rates of exudation may be more significant in the spring and summer months. In Chapter IV I examined the levels of lanosol in R. larix over the period of a year and found that concentrations in the plants are lowest during the summer months, precisely when the environmental conditions are most conducive to high exudation rates. The lower levels of lanosol in the plants from May through August may in fact be due to increased exudation and not to slowed biosynthesis of bromophenols. The correspondingly low levels of both polyphenols (Ragan and Jensen 1978) and bromophenols (assuming lanosol to be an indicator of total phenols) during this period of maximum potential epiphytization of the algae (spring to mid summer) may be a reflection of increased exudation as a response to higher levels of attempted epiphytization.

CHAPTER VI

BROAD SPECTRUM ANTIBIOTIC ACTIVITY OF BROMOPHENOLS FROM *RHODOMELA LARIX*

INTRODUCTION

The significance of antibiotic production to the ecology of land plant populations has long been recognized (Whittaker 1970). Only recently has there been an effort to determine a similar significance in the marine environment (Burkholder 1973 and references therein). Marine algae produce a variety of "secondary" metabolites, many of which are known to be biologically active (Silva and Bittner 1979). The diversity of these compounds encompasses virtually every chemical class, and they have been found in all the major divisions of algae (Scheuer 1973, Faulkner and Fenical 1977).

Most of the available information regarding algal antibiosis comes from testing with crude algal extracts. However, the number of compounds isolated and identified has increased substantially and, subsequently, several reviews have appeared on the subject (Lewin 1962, Wolters 1964, Sieburth 1964, Baslow 1969, Bhakuni and Silva 1974). Unfortunately, few of these compounds have been examined for broad spectrum antibiotic activity. Hence, we have little knowledge of the benefit afforded an alga by the compound or group of compounds which it produces.

Antimicrobial tests, although rapid, simple and widely used, often provide little information regarding the range of biological effects a particular compound may possess.

With this in mind, I chose to examine the activity of a bromophenolic substance, lanosol, and its naturally occurring salt (here called "lanosalt," 2,3-dibromo-5-hydroxy-benzyl-1',4-disulfate) on a variety of organisms, many of them marine. Lanosalt is the major phenolic constituent of the marine alga Rhodomela larix (Turner) C. Agardh (Weinstein et al. 1975), which is abundant all along the Pacific coast of North America (Abbott and Hollenburg 1976). Little is known of the ecological or physiological significance of bromophenols to this red alga; however, on numerous occasions mention of the antibiotic activity of bromophenols has been made (Mautner et al. 1953, Saito and Sameshima 1955, Allen and Dawson 1960, Weinstein et al. 1975). The only truly quantitative study of bromophenol antibiotic activity was done by McLachlan and Craigie (1966), in which they examined the effect of lanosol and two other phenolics on a number of planktonic algae. In this study I have made an effort to include the quantitative aspects of bromophenol antibiosis, both from extracts and exudates as well as from purified compounds.

MATERIALS AND METHODS

Algal Extraction and Isolation of Compounds

R. larix (approx. 2.5 kg, 500 g dry weight) was collected in the upper subtidal zone off Bath Island in January, 1980. The alga was lyophilized, ground in a Wiley mill (2 mm mesh) and extracted in a Soxhlet apparatus with a series of solvents (Weinstein *et al.* 1975). Evaporation of the methanol extract produced a large amount of solid (44.7 g) which was collected, washed with and recrystallized from methanol. Physical data for this compound were identical to those reported for the dipotassium sulfate salt of lanosol (Weinstein *et al.* 1975).

Lanosol was prepared by the method of Hodgkin *et al.* (1966). One g of lanosalt upon hydrolysis produced 123 mg of the free alcohol. This compound was identical to the authentic standard. The purity of the isolated compounds was checked by HPLC (see Chapter III for details).

Other extracts were prepared by grinding 1 g of *R. larix* in hot 80% methanol. The methanol was removed *in vacuo* and the water acidified to pH 2 with dilute

HCl and heated for 15 min at 60°C. Extraction with ethyl acetate and subsequent removal of the solvent left a dark green residue which was taken up in 1.0 ml of ethanol (50%) and used for the antibiotic experiments. The quantity of lanosol in the extracts was ascertained by HPLC and comparison to a calibration curve prepared using the standard.

Preparation of Algal Exudates

Ten g of the algae were placed in 100 ml of 0.22 um filtered sea water and kept at 20°C while being exposed to bright light (1000 uE $m^{-2}sec^{-1}$) for 8 hrs. The algae were removed and the water extracted with ethyl acetate and worked up as in the 1 g extract already described. The quantitative determination of lanosol in this exudate was again achieved by HPLC.

Screening for Activity with Standard Test Organisms

Initial screening using Escherichia coli, Staphylococcus aureus, Saccharomyces cerevisiae and Candida albicans as test organisms was done on either nutrient agar (Difco) for bacteria or Sabouraud dextrose agar (Difco) for fungi. Agar plates were seeded with the microorganism to be tested and small filter paper discs (diameter, 6mm) previously loaded with a known concentration of lanosol or lanosalt were placed on the surface of the plates. The diameter of the zone of growth

inhibition was determined after 24 hrs at 35°C.

Subsequent Experiments with E. coli

Liquid media for bacterial growth rate experiments were prepared by the addition of 1.0 g glucose and 5.0 g bactopeptone (Difco) to one liter distilled water. In one set of experiments media were made to pH 6-10 with either dilute HCl or NaOH. To 2.0 ml of each medium in small test tubes was added 0.5 ml of a bacterial suspension grown up to stationary phase to give an optical density of 0.1. Fifty ul of 50% ethanol containing 0.5 mg of lanosol or 5 mg of lanosalt were added to each tube and the growth rate (change in optical density) monitored over a 10 hr period (incubation at 35°C). Optical density was read on a Pye Unicam SP 6-500 spectrophotometer set at 520 nm.

In other experiments media were prepared at pH 8 and bacteria added as before. Varying concentrations of lanosol, algal extracts or exudates in small amounts of 50% ethanol were added and growth rates monitored over a 24 hr period.

Experiments with Marine Fungi

Three marine fungal isolates were kindly provided by T. Thompson, Department of Botany, University of British Columbia, Vancouver, B. C. These were Sigmoidea sp. Crane, Dendryphiella salina (Suth.) Pugh et Nicot and

Zalerion maritimum (Linder) Anastasiou. All experiments with these fungi were performed on Difco corn meal agar using sea water (pH 7.0). Stock cultures of each species were grown up on the above agar and small agar blocks (0.5 cm^2) were excised and placed in the center of test plates. Around the inoculum at a distance of about 2 cm was placed a series of filter paper discs containing specific lanosol concentrations (3.0-0.03 mg/cm^2). A control disc contained no lanosol. Growth was slow for two of the three species, and the zone of inhibition was examined after 1-2 weeks at room temperature. For Dendryphiella an alternate method was used. Stock culture plates of sporulating mycelia were inverted and tapped over clean test plates to seed the entire agar surface with conidia. Lanosol-containing filter paper discs were placed on the surface as before. Germination of the conidia and mycelial growth could be observed within two days and the zone of inhibition measured.

Tests with Tidepool Snails

Littorina scutulata Gould. was collected on Bath Island and maintained in sea water at $10^{o}C$. Antibiotic testing with this organism was done by a modification of the method of Ohta (1979). Nine cm filter paper discs (Whatman No. 1) were placed in the bottom of slightly larger glass petri dishes, and 3 ml of sea water were

added to each. A series of test solutions (100 ul each) containing lanosol (0.001-0.1 mg/cm^2) in ethanol or lanosalt (0.1-10.0 mg/cm^2) in water were evenly applied over the surface of filter paper rings (outer diameter, 5.5 cm; inner diameter, 4.0 cm; area, 11.2 cm^2) and the rings hung up and allowed to air dry. Only solvents were applied to control rings. Once dried, the rings were positioned in the center of the petri dishes and five snails (diameter, 4-6 mm) were placed inside each ring. The dispersal of the snails was noted after 10 min. This experiment was repeated on three separate occasions with two replicates per concentration in each case. The results were very consistent.

RESULTS AND DISCUSSION

Of the organisms used in the initial screening of lanosol and lanosalt activity, E. coli proved the most susceptible (Figure 17). As a result, this bacterium was used in further antibiosis experiments. Lanosalt showed no effect at concentrations as high as 1 mg/cm^2, while the lanosol effects on E. coli in particular ranged from very effective at 1 mg/cm^2 to barely detectable at 0.01 mg/cm^2.

For liquid culture experiments a lanosol

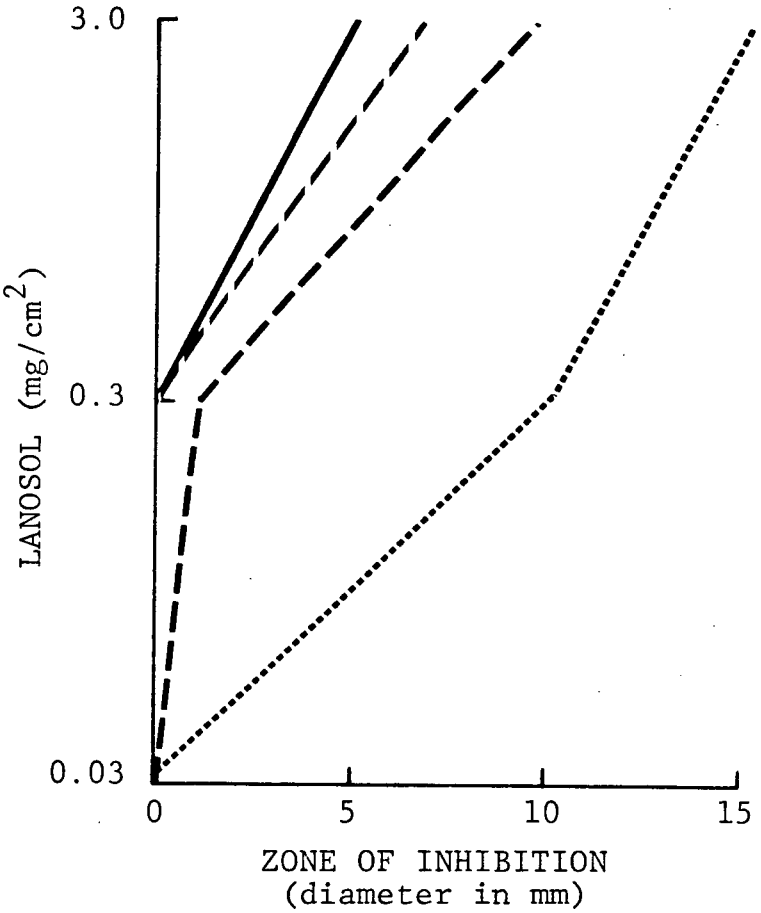

Figure 17. Antibiotic activity of lanosol against Candida albicans (———), Escherichia coli (·····), Saccharomyces cerevisiae (— — —) and Staphylococcus aureus (- — - —).

concentration of 0.2 ppt was found to be effective but not to stop completely the growth of E. coli. This concentration corresponds to the midrange dose response of the initial plating experiments (e.g. 0.1 mg/cm^2). Lanosalt was again ineffective, even at concentrations ten times those of lanosol (see Figure 18).

Conover and Sieburth (1966) have suggested that the alkaline pH of sea water promotes the activity of algal tannins (e.g. reducing substances, polyphenols, etc.) as toxic substances to planktonic animals. This increased toxicity probably includes effects on other plants and herbivorous animals as well as on microorganisms. As a means of examining this effect using bromophenols, the toxicity levels of lanosol and lanosalt against E. coli were tested at different pHs (Figure 18). The results not only illustrate the differences in the effects of the two compounds, but they also give some indication of the effect of pH on the chemistry of lanosol antibiosis. Comparing the lanosol curve to the control at pH 10, one notes that the maximal levels of bacterial growth are roughly equivalent. The higher pH not only slows the growth rate of E. coli (control), but it seems in some way to detoxify lanosol. The mechanism of this detoxification procedure is unknown. A possible explanation is the production of phenolate anions from the

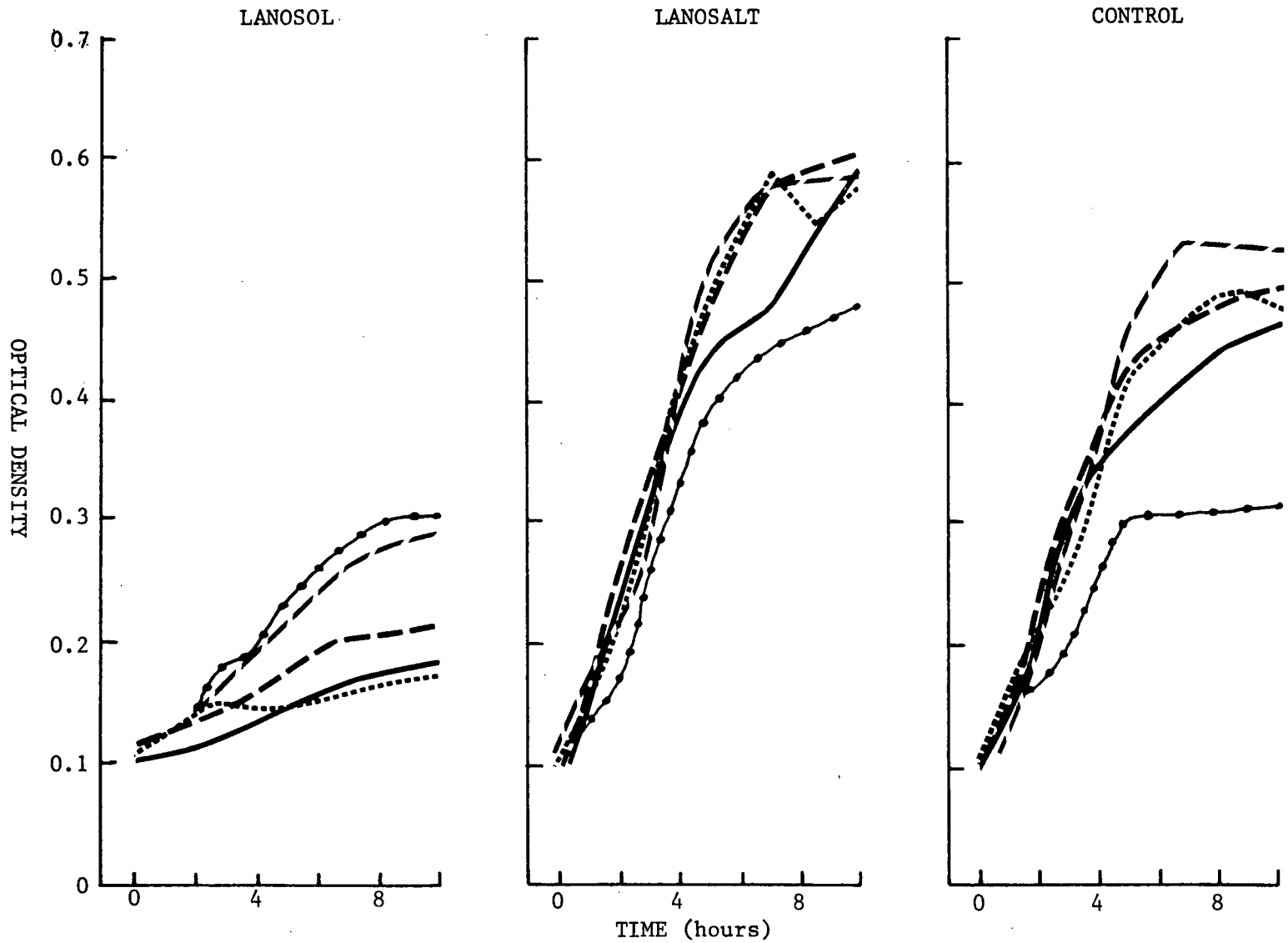

Figure 18. The effect of pH on the antibiotic activity of lanosol and lanosalt against Escherichia coli (pH 6——, 7– – –, 8·····, 9–·–·–, 10 —•—•—).

bromophenols. These as salts would approximate the inactivity of the lanosalt as shown in Figure 18.

In Chapter V I have examined the exudation of bromophenols from tidepool R. larix and found that during periods of most copious phenol release there is a definite and rapid change in pH of the water surrounding the algae. As a result the surface of the plant may be quite acidic, which in contrast to the above argument promotes antibiotic activity against, for example, E. coli (Figure 18, pH 6).

When dealing with an antibiotic substance in pure form, one is never sure if this is the only source or at least the major source of activity seen when whole plants or crude extracts are used. I attempted to examine this question by making extracts and producing exudates of R. larix, quantifying the lanosol in these, duplicating these quantities with pure compound (controls) and testing all three in another series of bacterial growth experiments. This type of experiment was thought to be an "action spectrum" of biological activity for R. larix. Technical problems, especially in tests with extracts, minimized the potential of the method. For example, the high chlorophyll concentration of the extracts and its progressive degradation masked the growth of the bacteria as monitored by visible spectrophotometry

(Figure 19). Therefore, one can say nothing about the quantitative aspects of lanosol activity in extracts except to refer the reader to the literature, which gives numerous examples of activity of suspected bromophenols in extracts of red algae. The experiments with exudates, however, showed a close correlation with the lanosol controls (Figure 19). This information and the results of the bacterial experiments given here lead to the conclusion that lanosol is produced as an antibiotic and that it is the primary substance of this kind found in R. larix.

The results of the assays with marine fungi are given in Table IX. As far as I am aware, no other work has been done utilizing antibiotics derived from marine algae and testing their effects on marine fungi. The procedure in which D. salina conidia were peppered onto the agar surface proved the most rapid and informative in that no long experimental periods were necessary in which lanosol degradation could occur. Using this procedure lanosol was shown to inhibit spore germination and not just mycelial growth alone.

The snail L. scutulata is very common in local tidepools and essentially throughout the intertidal zone. It is often found on and around R. larix, but there is no evidence that it ever consumes the alga. The reason for this may be the high concentrations of bromophenols

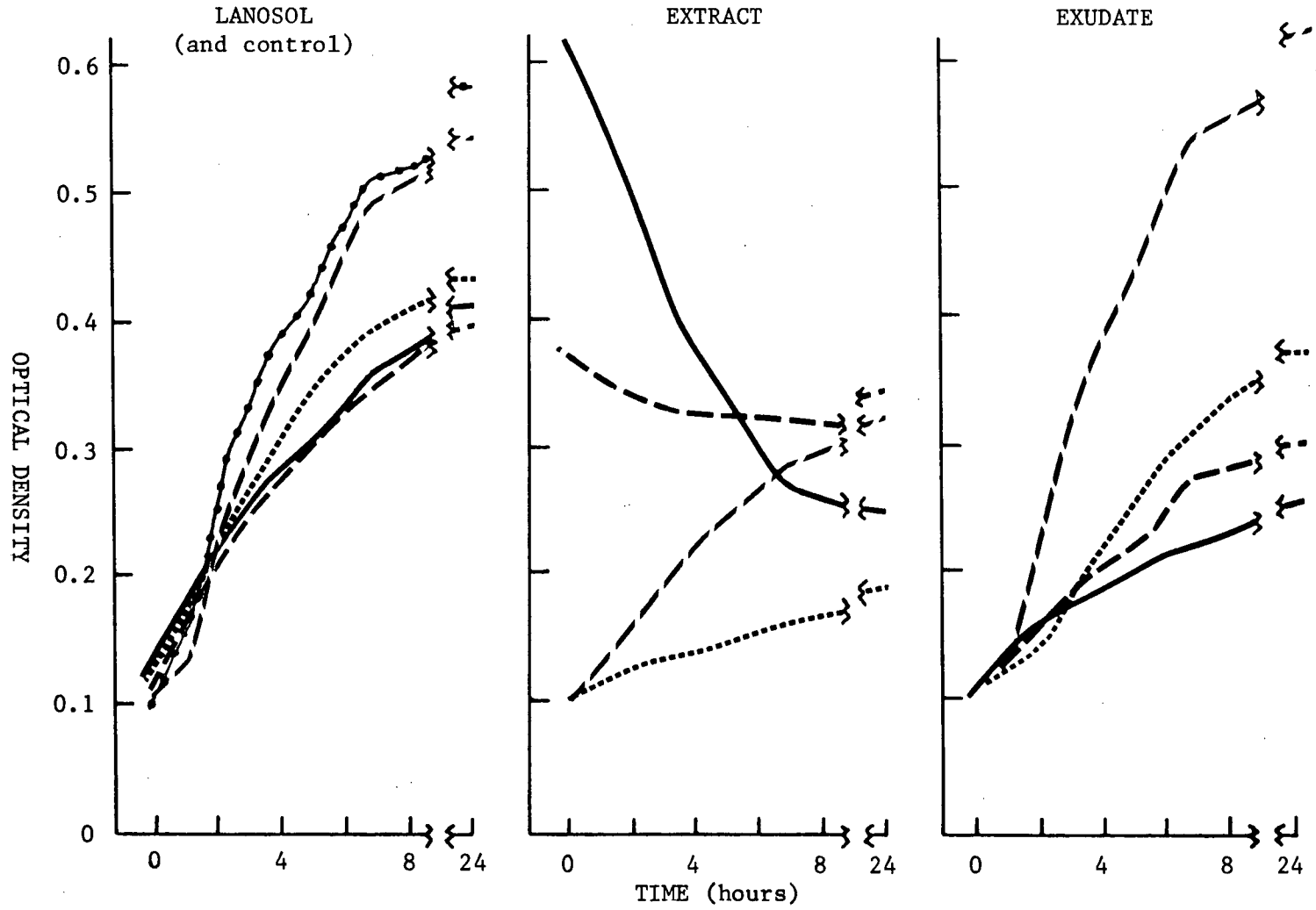

Figure 19. Effect of varying concentrations of lanosol in pure form, in algal extracts and in exudates on the growth rate of Escherichia coli. The initial concentration was 0.25 ppt (———) and the dilutions are 0.13 (— — —), 0.06 (·····), 0.03 (— — —) and 0 (—•—•—) ppt.

Table IX. Antibiotic activity of lanosol against three species of marine fungi (given as the diameter of the zone of growth inhibition in mm). * Activity against D. salina conidial germination, not the adult mycelium as in the other three examples. **

LANOSOL (mg/cm^2)	SPECIES			
	Dendryphiella salina	Dendryphiella salina*	Sigmoidea sp.	Zalerion maritimum
3.0	10	18	9	20
0.3	7	12	7	16
0.03	0	7	0	10
0	0	0	0	0

** - Three replicates were used for each species tested and the above data are averages obtained from each set of experiments.

in the alga. Results of a typical test with both lanosol and the salt are pictured in Figure 20 and the combined results of all experiments are shown in Figure 21. Lanosol produced repulsion of the snails at levels of 0.001 to 0.1 mg/cm^2 while lanosalt was only effective at concentrations 100 times those of the free phenol. This method of testing (Ohta 1979) was modified so that a better control of the quantities applied to the sample area could be obtained. Also, problems of snail repulsion by residual solvent were overcome by thoroughly drying the filter paper rings before surrounding the snails with them.

Evidence has been presented here for the large scale production or at least utilization of lanosol as an antibiotic substance by R. larix. The assignment of this compound to a particular role in the ecology of red algae would at this stage be merely conjecture. Demonstration of the broad spectrum antibiotic activity of lanosol is enough to encourage further investigation into the implications of such phenomena in the marine environment. More detailed microecological work coupled with quantitative chemical analysis will invariably help to determine the reasons for the production of bromophenols by red algae.

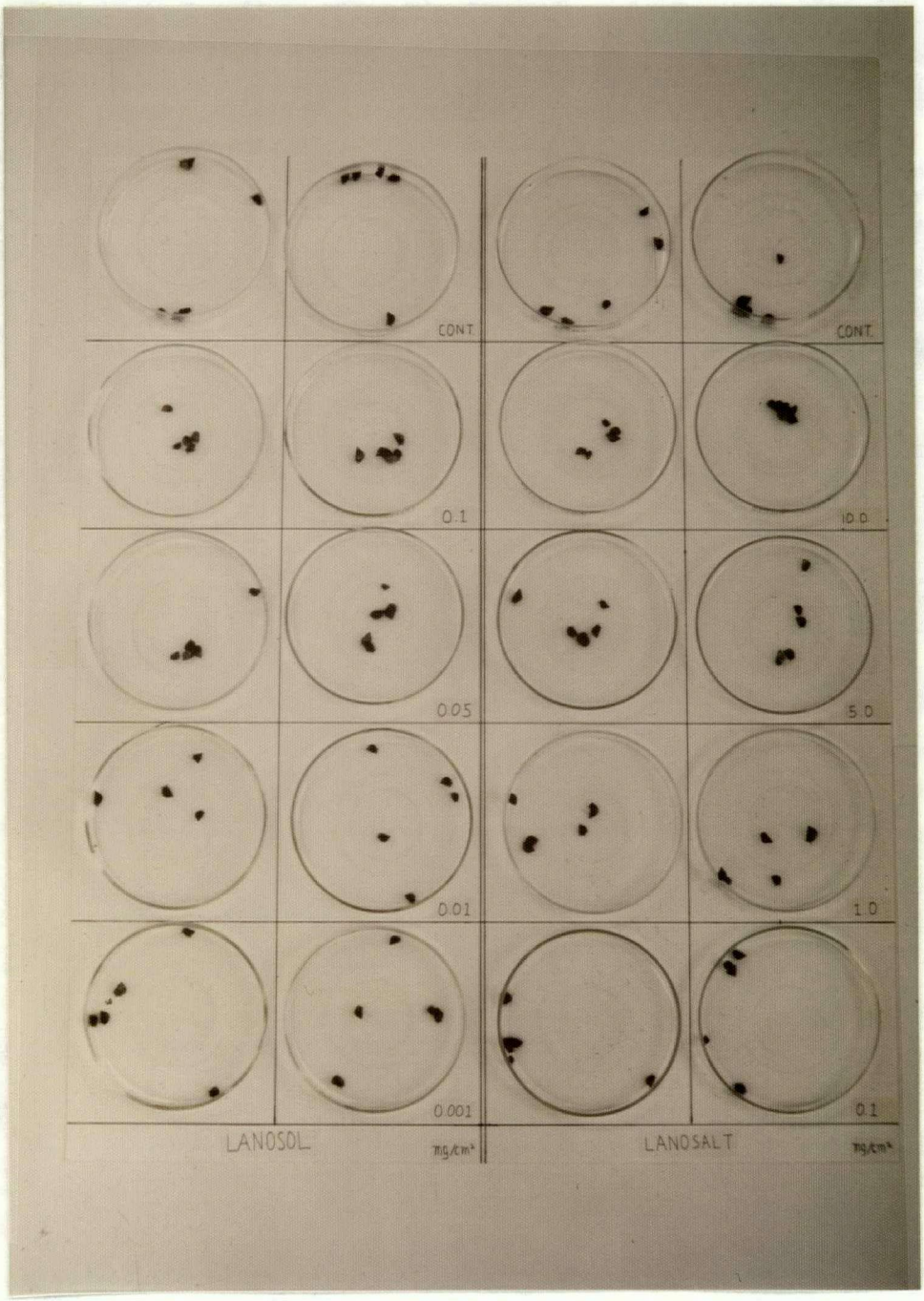

Figure 20. A typical snail repulsion experiment showing the effects of lanosol and lanosalt on the behavior of Littorina scutulata. This photograph was taken 10 min after five snails had been placed in the center of each dish.

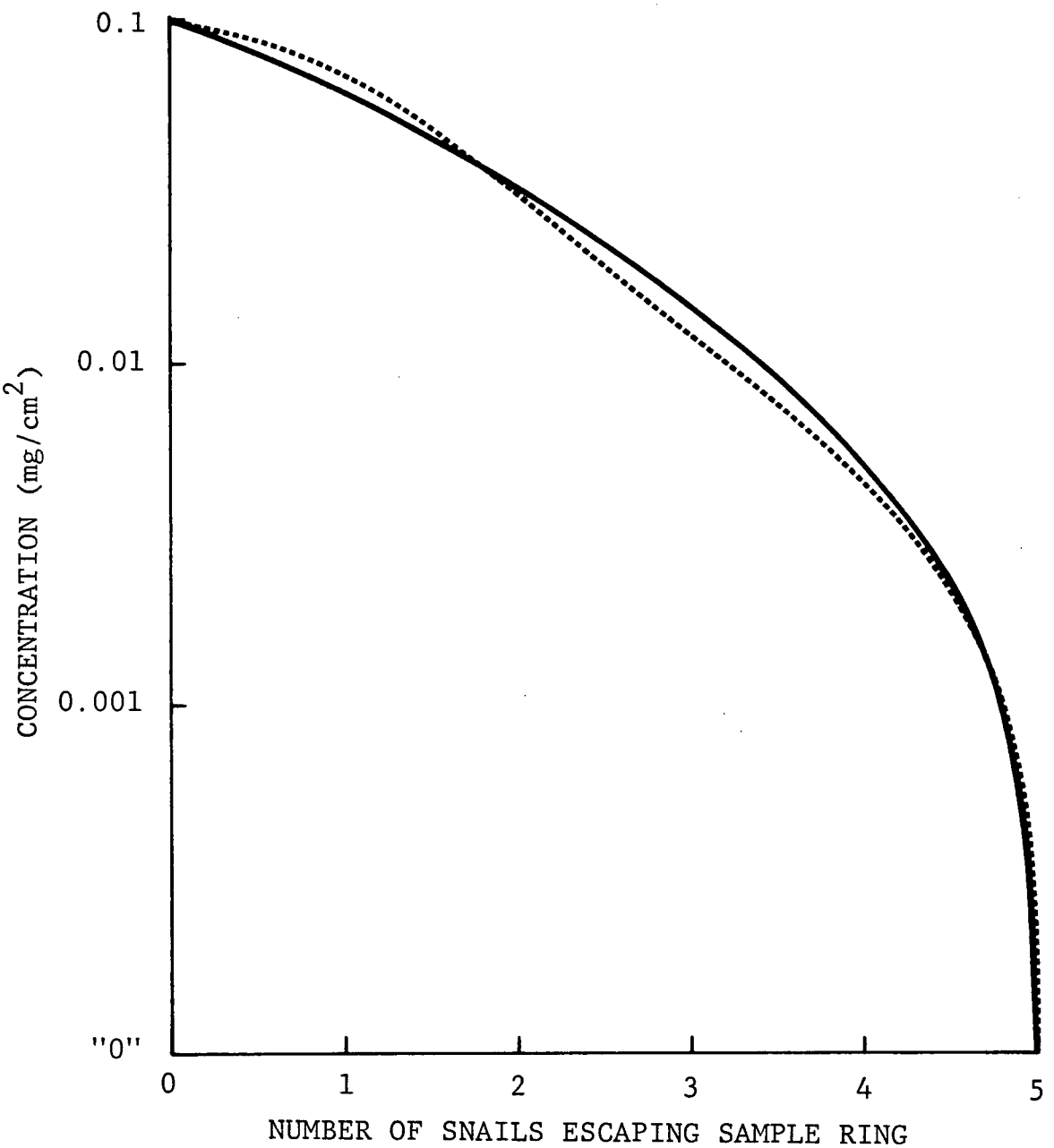

Figure 21. Degree of repulsion of Littorina scutulata by varying concentrations of lanosol (——) and lanosalt (·····). Lanosalt concentrations are 100 times those listed on the vertical axis.

PERSPECTIVES

This dissertation has been concerned with assigning a role to the production of bromophenols by red algae. Each chapter represents a step taken towards the formulation of a conclusion regarding this role. Because of the diversity in the subject matter of the chapters, the contribution of each to the dissertation as a whole may need minor clarification. I will therefore attempt to explain the impetus for and the significance of each separate step.

The importance of the first chapter lay in the discovery that the level of total phenols noticeably increases in the tidepools over the course of a day. I chose to examine the tidepool habitat for two reasons: first, the tidepool is a "closed" system--essentially a large culture flask in nature--and secondly, the rapid increase in yellow brown coloration (phenolic substances) produced by tidepool algae might very well have some effect on other organisms within the pools. Measurements of the range of environmental factors occurring in the pools later enabled me to set up exudation experiments which, because the conditions used fell within

environmental ranges, were assumed to place no unnatural physiological stresses on the algae being tested.

For some red algae there is still confusion as to their assignment at the species level. Populations occurring in different habitats, being exposed to different degrees of natural stress, often change in gross morphology, conceivably as an adaptive response to their environment. The tidepool form of R. larix has a quite different appearance from that growing in the upper subtidal zone. The chemical comparison of these two forms gave me some basis, at least, for calling the tidepool form R. larix.

The chemistry of this species was further examined by HPLC. The method which I developed proved to be rapid and informative for the screening of algal extracts and exudates for bromophenols. Quantification of lanosol was made easy and accurate. The efficiency of the method was further demonstrated in the analysis for bromophenols of two additional species, P. lyallii and C. washingtoniense.

Demonstration of the variation in bromophenol content, both temporal and intrathallial, provided the first real clue into the ecological significance of these compounds. Sieburth et al. (1974) have shown that Polysiphonia lanosa, which also contains lanosol in

concentrations of up to 5% of its dry weight (Table V), supports a diverse and seasonally changing epiflora. In summer the P. lanosa epiflora is composed mainly of yeasts and in winter, of diatoms and filamentous fungi. If the levels of lanosol in this species vary temporally as do those of R. larix, an intriguing correlation arises. Although the epiflora of P. lanosa was shown to be abundant, the alga was in no way threatened (fouled) by the number of organisms present. Variations in the abundance of the epiflora could be due either to seasonal changes in the numbers of a particular species present or to changing levels of tolerance to algal antibiotics (e.g. bromophenols) by that same species. Interestingly, the levels of "contamination" of the P. lanosa thallus appeared to be minimal toward the growing tips.

The second phase of this study led to the discovery that large quantities of bromophenols (lanosol in particular) were present in the exudates of R. larix. Brown algae have long been known to control surface fouling by the production of polyphenolic substances (Conover and Sieburth 1964, Sieburth and Conover 1965, Ryland 1974, also see Sieburth 1968). The presence of bromophenols--particularly lanosol in the free form--in red algal exudates furthers the notion that these compounds have a definite ecological function. The rates

of exudation of lanosol were as high as 0.15 ppt/g d.wt. hr. The effective antibiotic dose of this compound against E. coli was less than 0.3 mg/cm^2, which is approximately 0.1-0.2 ppt. Thus the rate of exudation of bromophenols seems to be on the order of that necessary to control surface fouling, assuming that other microorganisms show the same response to lanosol as did E. coli.

Testing of the antibiotic activity of lanosol on a variety of organisms proved that they did in fact show such a response: all were affected at some concentration between 0.03 and 0.3 mg/cm^2. The effect on E. coli was greatest at lower pHs, and the activity of R. larix exudates closely resembled that seen for pure lanosol. Results of the experiments with tidepool snails opened the door to another array of possible functions for bromophenols in that they may also be involved in the repulsion of potential herbivores. The level at which lanosol was effective as a snail repellent was much lower than that necessary for the compound to be antibiotic. Further testing with a variety of marine animals could demonstrate the full spectrum of this type of effect.

A final and possibly most convincing argument for the ecological significance of bromophenols comes from the work of Roos (1957). He examined temporal variation in the antibiotic activity of extracts of R. subfusca

on several species of bacteria. Kurata and Amiya (1975) have shown this alga to contain 9.2% of lanosalt on a dry weight basis (or as lanosol, 5.1%). Roos observed that the antibiotic activity of R. subfusca was lowest in the summer months and essentially twice as great in the winter. Rhodomela larix contains lanosol in approximately the same concentrations, and the seasonal maxima and minima for this compound correspond to the seasonal maxima and minima of antibiotic activity for R. subfusca. Hence there exists an indirect correlation of lanosol content with temporal antibiotic activity found for extracts of red algae.

Most certainly, I have made but the barest beginning in applying the concepts of chemical ecology, as we understand them in the sense of their terrestrial origins, to the vast and complex marine environment. The potential for further analyses of the interactions among marine organisms is steadily increasing and I hope that there occurs, not too far into the future, much more and detailed research in the chemical ecology of such complex ecosystems.

FOOTNOTES

(1) This light meter and probe combination effectively measures wavelengths between 400 and 800 nm, the region of the spectrum utilized for photosynthesis.

(2) This figure represents a summary of the data presented in Figure 3 and includes some of the factors discussed in the text which might effect changes in environmental conditions occurring in each of the pools.

(3) This figure is based on the maximum level of total phenols reached in the pool over the period of a single day (about 10 hrs). Hence, on an hourly basis, 0.05 mg of total phenols would be produced for each liter of tidepool water (or as amount produced per gram dry weight of alga, 0.14 mg/hr).

(4) No correlation between medium discoloration and lanosol content is intended. The two may be mutually exclusive or some relationship may exist. In addition, use of the word "correlation" implies no mathematical similarity in any of its many uses in this thesis; rather it simply implies that a mutual relationship exists between two events, the basis of which has not necessarily been established.

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