EPIDERMAL PAPILLOMAS IN THE DOVER SOLE

Microstomus pacificus

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Environmental Health Science

by

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and

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ABSTRACT

EPIDERMAL PAPILLOMAS IN THE DOVER SOLE

Microstomus pacificus

by

Philip A. Sanderson

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David H. Pierce

Master of Science in

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Samplings by otter trawl in the Santa Monica Bay confirmed the presence of epidermal papillomas on Dover sole (Microstomus pacificus) and served to obtain samples of tissue to be used in histological analysis and ultrastructural studies. Water samples taken at the same time were used to obtain a chemical profile of the water column where these fish were found. Numbers of fish collected and the length of these fish, both tumorous and nontumorous, were used to describe the epidemiology of the diseased fish. Chemical parameters from the water column were examined in an attempt to obtain any consistent pattern of association between some
environmental condition and tumor prevalence. Numbers were too small to make valid statistical tests.

Normal and tumorous tissue were prepared for histological and ultrastructural analysis. The results of this analysis indicate a disease that is essentially the same as has been found in other pleuronectid fish along the northwest coast of North America. In particular, the specific cell type reported in the tumors of other species of pleuronectid fishes was found in the tumorous tissue of Dover sole.
Chapter 1

INTRODUCTION

The Teleost family of Pleuronectidae in the Pacific waters off North America have been studied considerably during the last ten years. The stimulus for this study has been the epithelial tumors found on these flatfish. Research has been done primarily in four areas: (1) the sand sole (Psettichthys melanostictus) of British Columbia; (2) the flathead sole (Hippoglossoides elassodon), English sole (Parophrys vetulus), and starry flounder (Platichthys stellatus) of Washington; (3) the English sole in San Francisco Bay; and (4) the Dover sole (Microstomus pacificus) of Southern California.

To date, the thrust of this research has been in two directions. The first has been the sampling of the fish population in selected areas. The occurrence of the disease process on any species was noted, and when numbers were significant, a prevalence value was computed.

Second, there has been considerable histological work done on the diseased tissue itself compared with normal dermal and epidermal tissue. Together with this histological analysis, there have been studies of ultrastructural anatomy along the same lines. A start has
been made to correlate prevalence with environmental parameters of the water column, but no conclusions have, as yet, been drawn.

The purpose of this study was to delineate what seems to be a consistent pattern of the integumental disease among demersal fishes along the northwest coast of North America. This disease process seems to primarily affect the dominant species of flatfish in an area, and to affect the young of the species. This disease has been well documented in areas up and down the coast (8, 27, 35, 40, 44, 54, 64, 66). Yet, although noted in 1956 (67), no serious inquiry has been made concerning the Dover sole in Santa Monica Bay.

It was our working hypothesis that the disease process found among Dover sole in Santa Monica Bay is essentially the same as the process affecting other dominant species of flatfish in areas north along the coast. Our reason for taking this approach is that the gross appearance of the tumors and the prevalence information (54) seem to indicate the same kind of disease process and that other literature (36, 37, 38) indicates that Dover sole is the dominant species of flatfish in the South Bay area. The primary problems encountered in this kind of inquiry are the facts that Dover sole show more seasonal migration and bathymetric tolerance than other
pleuronectids and are the hardest species to catch in an otter trawl (19).
Chapter 2
LITERATURE REVIEW

2.1 Literature on the Dover sole.

The Dover sole (Microstomus pacificus), a pleuronectid fish, is, in fact, a flounder and not a true sole. The work of Hagerman (19) in 1952 lists other common names for Microstomus pacificus as slippery sole, lemon sole, smear dab, rubber sole, short-finned sole, slime sole, and tongue sole. Daniel Miller and Robert Lea (41) describe Dover sole as having a range from Baja California to the Bering Sea, averaging thirty inches in length, and found at depths from 90 to 3,000 feet.

The physical appearance of Dover sole (see Figure 1) is a uniform brown color with black fins on the right-hand, or eyed, side of the body. The left side, or eyeless side, is essentially white. There are two yellowish-brown to black spots along the straight lateral line on the eyed or pigmented side. The mouth extends to the anterior part of the lower eye, and the teeth are developed only on the blind or white side. Probably the most distinctive characteristic of the Dover sole, and the reason for a number of its common names, is the fact that it is uniformly covered by an exceptionally heavy mucous coat, or slime coat.
Figure 1. Macro-photograph of normal juvenile Dover sole, pigmented side.
The reproductive cycle of the Dover sole is reasonably well understood. The onset of spawning is approximately September, reaching a peak the last of December or early January, and essentially ending by April. The larvae of the Dover sole, like most pleuronectids, are buoyant and pelagic. The Dover sole larvae, however, stay in a pelagic form, with the plankton, for several months; and metamorphosis is delayed for a few months longer than that of most pleuronectids. Metamorphosis is not completed until the fish is at least 35 millimeters long; and immature fish as long as 48 millimeters have been found where the left eye is not yet completely in the adult position. The juvenile fish do not complete metamorphosis and take up their demersal lifestyle until the early fall.

2.2 Literature on the Epizootic Nature of Epidermal Tumor Disease.

This review of the areas previously sampled will start at Canada and move down the coast to Southern California. Multiple studies in the same general area will be presented in chronological order.

In Northern Hecate Strait, British Columbia, in 1965 (44), Nigrelli, et al., sampled at eleven stations on the east coast of Graham Island. Of 1,626 sand sole taken, 729 were examined for tumors, and 231 were found to be tumor bearing, yielding an overall prevalence of
31.7 percent, that ranged, by station, from 7.7 percent to 43.4 percent. The article does not explain why only 729 were examined. However, even if the 729 fish were not randomly selected, but pre-selected by some process that was 100 percent accurate so that all fish at risk of being tumor bearing were included in the 729, the overall prevalence out of a total of 1,626 would still yield a prevalence of 14.2 percent. Significant differences in the prevalence found at different stations seem to be accounted for by the also significant difference in the length distribution at each station, those stations yielding more small, young fish gave a higher prevalence figure. In this same study, tumors were also noted on juvenile rock sole (*Lipivopsetta bilineata*), but not seen on English sole or butter sole (*Isopsetta isolepis*).

Off the San Juan Islands in Washington (66), from July, 1963, to July, 1964, 4,364 flathead sole were taken. Of these, 281 were found to be tumor bearing, yielding a prevalence of 6.4 percent. No statistics were presented, but on inspection, the maximum prevalence, approximately 10 percent, was seen in July, and the minimum prevalence, approximately 2 percent, was seen in April. This could be relevant when one considers that of 281 tumor bearing fish only 1 was over three years of age (74 were from zero to one year old, 146 were from
one to two years old, 16 were from two to three years old, and 1 was from three to four years old). Data were not presented on the total number of fish taken by age during each month. Tumors were also noted on sand sole, English sole, and rex sole (Glyptocephalus zachirrus), but in insufficient numbers to compute prevalence data. Tumors were found to occur on all areas of the fish, but a significant tendency was noted for them to be located on the pigmented, or eyed, side of the fish.

In the East and West Sounds of Orcas Islands in Washington (40), from July, 1964, to August, 1964, 5,250 flathead sole were taken, and 336 found to be tumor bearing, yielding a prevalence of 6.4 percent. Tumors were also noted on 3 rex sole, 2 sand sole, and 1 of the 2 English sole. No tumors were found on any fish older than three years of age.

In Port Gardner, Port Susan, and Bellingham Bay, Washington (35), fish were taken from June, 1965, to July, 1966. Tumors were noted on four pleuronectids as follows: 57 of 1,063 starry flounder, prevalence equal to 5.4 percent; 93 of 1,792 English sole, prevalence equal to 5.2 percent; 3 of 90 sand sole, prevalence equal to 3.3 percent; and 3 of 430 butter sole, prevalence equal to 0.7 percent. No tumors were found on 68 flathead sole, 24 slender sole (Lyopsetta exilis), 116 rock sole,
24 Dover sole, 11 C-O sole (*Pleuronichthys coenosus*), and 1 rex sole.

In San Francisco Bay, California (8), from September, 1965, to September, 1966, 15,739 English sole were taken. Of those collected, 12 percent were tumorous. Overall geographic and seasonal fluctuations varied from 3 percent to 28 percent. The Bay was divided into north and south areas. The north yielded 7,200 fish, prevalence equal to 15.5 percent tumor bearing. The south yielded 8,454 fish, 8.9 percent tumor bearing.

San Francisco Bay (27) was re-examined in January, 1970, to July, 1970, with interesting results. Of 4,651 English sole taken, 347 were found to be tumorous, yielding an overall prevalence of 7.5 percent. However, the pattern from north to south had reversed, so that 1,230 fish taken from the north bay yielded only 21 tumor bearing fish, with prevalence equal to 1.7 percent, while the south bay yielded 4,651 fish, of which 328 were tumorous, yielding a prevalence of 9.6 percent.

Off the Southern California Coast (67), in 1956, Young noticed tumors on Dover sole in the Santa Monica Bay. No data are given on number of fish taken. However, Young did note that fish over 10 inches long were not found with the disease and hypothesized that this was a disease process affecting only the younger fish.
From 1969 to the present, the Southern California Coastal Waters Research Project (54) has been carrying out an ongoing trawling program. Between 1969 and 1973, 303 trawls were made, and the samples obtained by 267 of these were examined for anomalous fish. Of the 8,774 Dover sole taken, 118 were tumor bearing, yielding a prevalence of 1.3 percent, varying by area from 0.4 percent to 2.4 percent. Tumors were also noted on 1 of 2,515 slender sole, 1 of 533 turbot (Pleuronichthys verticalis), and 1 of 2,656 tongue fish (Symphurus atricayba).

2.3 Theories on the Epidemiology of Tumors Among Pleuronectids

The disease process affects juvenile fish almost exclusively. By the age of three years, the fish population demonstrates almost no disease. By four years of age, tumors are not found. Of all affected fish, those whose ages range to one year have the highest average number of tumors per affected individual. Prevalence and mean number of tumors per affected fish drops off very rapidly to a low at three years of age.

It has been suggested by Wellings, et al., (64, 66) that there is a significant tendency for tumors to occur on the pigment side. Cooper, et al., (8) and Kelly (27), however, demonstrated that this was not the case on the English sole of San Francisco Bay. Whether or not
there is an eyed-side bias is of importance with regard to the timing of the initial tumorigenic event. If there is a bias to either side of a flatfish, then the critical event must have occurred after metamorphosis, yet before the fish were taken as juveniles. If one accepts that the findings concerning San Francisco English Sole can be generalized to include other species and areas, then the timing of the critical event is pushed back to a time before metamorphosis. This is troublesome in that the fry before metamorphosis inhabit a different ecological niche than the fish caught in the otter trawl and examined for tumors. Therefore, the environmental parameters of the habitat from which these fish were taken may bear no resemblance to the environment in which the initial tumorigenesis occurred.

Aquarium studies have used fish already possessing tumors and control fish of the same approximate weight, length and age (27). Never has a control fish developed tumors de novo even when kept in the same tank with tumorous fish.

Wellings, et al., (66) found that the AEN (angioepithelial nodule, a small, rounded protuberance 1 to 2 mm in diameter) is a primary lesion that develops into a PAP (epidermal papilloma, a larger, cauliflower-like proliferation of tissue 1 to 6 cm in diameter). As it develops, the overlying epidermal layers experience
extensive growth, both thickening and spreading outward. This process is always progressive. Regression of a tumor has not been observed. This also accounts for the decrease in the number of tumors per affected individual in that the PAPs of adjoining AENs tend to grow together and become indistinguishable as individual tumors. Given the exclusively progressive nature of the disease, the fact that one does not find tumors on fish older than four years old, and the fact that tumors occur over eyes, mouths, gills and other vital structures, it has been generally accepted that the infected fish die before age four from the disease. This effectively removes them from the population.

Kelly (27) confirmed the finding that AENs are the primary lesion and do develop into papillomas. However, his aquarium study dealing with the fate of the AENs had a strikingly different finding in terms of the survival of infected fish. In Kelly's aquarium study, he found that 83 percent of the nodules were sloughed. This was inferred by the fact that most of the fish which had possessed AENs at the beginning of the experiment possessed a normal epidermis at the end of the two month duration of the experiment. Only 5.6 percent of the nodules developed into PAPs, the remainder were still at the AEN stage. Further work demonstrated that PAPs undergo the same developmental fate, with 57 percent being
sloughed and 42 percent persisting. Although this does not rule out death as the ultimate fate of the infected fish, it would seem to indicate that sloughing is the primary result rather than death or disability.

The etiology of the disease could be one or a combination of the following four components: genetic, parasitic, viral or environmental. The genetic aspect can only be ruled out with aquarium breeding and long-term study.

Parasitic involvement has primarily two possibilities. First, it has been hypothesized (4) that the X-cell, which has been found in all tumorous tissue is a unicellular parasite of unknown type rather than a transformed fish cell. This view has some support (28, 39, 47, 61). The most direct test of this hypothesis would be to grow the X-cells in pure culture and transmit the disease via this culture to unaffected fish. This has not as yet been done. Second, the parasite may be a vehicle which induces an inflammatory response to the skin which may be necessary for viral entry, parasitic entry or stimulating expression of a genetic mutation.

Possible viral involvement has been suggested (27, 35, 44, 63). A number of virus-like particles have been seen in electron micrographs of tumorous tissue and never seen in normal tissue. These particles have been classified as unknown virus types by all but McArn, et al.,
(35), who feels that they are identical to a human enterovirus, the coxsackie virus (an RNA-type virus). The X-cell, however, gives the appearance one would expect to see in tumorigenesis induced by a DNA-type virus (17).

The alteration of the environment by man's activities has been suggested as a main factor (67) in studies of the effects of sewer effluent on the fauna of Long Beach in terms of weight gain, flesh texture and skin tumors. The possibilities of environmental effect are four. First, some pollutant may be ingested in biologically magnified amounts sufficient to cause tumors. Second, some pollutant may act directly on the integument causing the tumor. Third, some pollutant may directly cause an inflammatory skin response allowing the entry of an infectious agent or stimulating the expression of mutant genes. Fourth, pollution in vast amounts usually causes tremendous nonspecific changes in the entire community and tumor incidence could well be linked directly or indirectly to these massive changes in food supply, species dominance or to population changes (7, 53, 67). In any event, it is a certainty that the environment plays its role in this disease process. The size and complexity of this role has not yet been delineated. In fact, it has not as yet been properly approached. It should be noted that a greater understanding of the life history of the affected species and the timing of the
tumorigenetic event must be known before the particular environment which must be studied can be determined.

2.4 Literature on Environmental Factors.

The Southern California Coastal Waters Research Project (SCCWRP) has defined the Southern California bight as "the open embayment of the Pacific Ocean bounded on the east by the reach of the North American coastline extending from Point Conception, California, to Cabo Colnett, Baja California, Mexico, and on the west by the California current." (54) The California current is the convergence of the eastern branch of the North Pacific gyre. It flows undisturbed 1,500 kilometers south to Point Conception. At Point Conception, the land mass makes a sharp easterly break. The indentation continues to Cabo Colnett on the coast of Baja California. The resultant climatology in this vicinity influences the entire floral and faunal productivity. The stalling of the California current creates a countercurrent, which resembles a return eddy as described by Gross (18). The consequence of this circular pattern of water mass movement is four fold: (1) a warming of the California bight waters; (2) an overall movement of food materials into the bight from areas farther north along the coast; (3) a high net productivity, partially due to the fact that many organisms maintain a breeding stock in these waters; and (4) a cumulative buildup of chemicals and compounds
expelled into the waters anywhere along the northwestern coastline. Concentration is due in part to the prolonged retention time of the waters within this embayment and has been implicated as a possible reason for environmental contamination and ecological destruction (15).

Another phenomenon which radically affects the balance of this area is upwelling. Upwelling is an oceanographic response to a climatologic stress. Winds along the coast of California and a component of the Earth's rotation, known as coreolis, initiate the process. When intensified northerly or northwesterly winds combined with the coreolis effect create a net transport of surface waters offshore, upwelling results. The waters driven offshore are replaced from below by waters which are colder, lower in dissolved oxygen content, and rich in organic nutrients. Since the causative factor for upwelling is the intensity and direction of the winds, different areas along the coast are susceptible to upwelling at various times of the year. Prime conditions for upwelling exist in the central and southern California coastal areas during May and June. This corresponds to the time that Dover sole larvae are in their free-floating pelagic state. Upwelling has profound effects upon marine productivity and population distributions. As waters from approximately 150 meters below the surface move inshore and upward, demersal fish populations also move inshore,
while pelagic organisms migrate to the surface and out to the open ocean. Photosynthetic phytoplankton also increase due to an overabundance of organic nutrient, primarily phosphorous and nitrogen compounds.

The ecological continuance and distribution of biological populations is also dependent upon the actual quality of the water (7, 15, 56, 60). Because they produce a general profile of the health of the marine community, water temperature, salinity, pH, and dissolved oxygen have been utilized as major water quality indices (14). Fluctuations of these parameters determine the quality of life in the marine environment.

The primary influence is temperature. All of the other environmental parameters are dependent upon it and, thus, the entire balance and distribution of all marine organisms. In general, an increase in the temperature reduces the solubility of gases in the water column (e.g., increased temperature reduces the solubility of oxygen in the water). The decrease in oxygen solubility reduces the amount of oxygen available for animal respiration. The affect of temperature on the marine organism is magnified due to the very nature of most species. Unlike the homeothermic animal, the poikilothermic organism has no mechanism for maintaining a relatively constant body temperature. The individual is directly influenced by temperature variation, because its
body temperature is nearly that of the surrounding environment. There is a narrow range of temperatures to which the organism can acclimate. The effect of increased temperature within this tolerable range is a consequent increase in the metabolic rate of the organism (46, 49, 51). Keeton (25) noted in his discussion of comparative metabolisms that, in general, for every ten degree centigrade increase within the tolerable range, the metabolic rate of the poikilotherm doubles. Increase in temperature has also been found to have a synergistic effect with the toxicity of heavy metals in a variety of vertebrate species (52, 62). A decrease in temperature produces lethargy in the animal. Within the scope of temperature variations, it is the aperiodic fluctuations with which the organism's physiology is not able to cope.

The actual fluid environment of the sea is a complex mixture of atmospheric gases, metallic salts, and organic materials. Classically, the amount of solid material in the water column has been quantified by measuring the salinity. Zottoli (68) has defined salinity as ". . . the total amount of solid material in grams contained in one kilogram of sea water when all the carbonate is transformed to oxide, bromide and iodine ions are replaced by chloride ions, and all organic materials are oxidized." Salinity is dependent
upon the temperature, the stratified density of the water column and, at the surface, on evaporation. As temperature increases, evaporation occurs, and the salinity will increase. The effect of temperature is reversed below the photic zone. Here, as the temperature of the water column decreases, the water becomes more dense and, consequently, more saline. In areas of high seasonal runoff, increased evaporation and upwelling, the salinity is most variable in approximately the first meter of the water column. For this reason, there is less need for deep oceanic organisms to acclimate to change in ionic concentrations in the water.

Most organisms seek out an area of optimum salinity suited to their own particular physiology. Zottoli (68) has described four major control mechanisms utilized by marine organisms to insure the appropriate salinity. An organism may (1) reduce its body contact with the surrounding water; (2) control the rate of movement of water in and out of its body; (3) crawl or swim to a new location; or (4) burrow into the substrait. Specifically, Dover sole (*Microstomus pacificus*) secretes a heavy mucous coat, high in lipid content, which reduces the degree of body contact directly made with the environment.

Control of the relative alkalinity or acidity of sea water is also necessary to maintain biological
life. Due to the mixing of carbon dioxide gas and sea water at the atmosphere-water interface, an equilibrium is created between atmospheric CO₂ in water, H₂CO₃, HCO₃⁻, and CO₃²⁻. Because of this carbonate system equilibria, sea water maintains an average pH of 8.3. The solution is slightly alkaline due to the removal of hydroxyl ions from solution to form the carbonates. Since the removal of hydroxyl ions from any solution raises the pH, the carbonate system in sea water provides a chemical mechanism for a neutralization of excess acids by the formation of bicarbonate.

The ability of the carbonate system to buffer the environment is influenced by the two major metabolic processes. During photosynthesis, CO₂ is utilized by phytoplankton. A shift in the equilibrium back to CO₂ in water effectively removes hydrogen ions from solution and raises the pH of the system. In respiration, the antithesis of this mechanism is observed. CO₂ is produced as a byproduct of metabolism. The equilibria shifts to replenish the depleted carbonates causing an increase of hydrogen ions in solution and a lowered pH. Maximum variation of pH is obtained at the surface due to extensive mixing, photosynthesis, respiration and decomposition. Below the photic zone, the pH is less variable and more acidic due to the predominance of respiration activity.
Little is known concerning the direct effects of pH fluctuations on the marine biota. Its indirect effects are, however, extremely significant. Even a slight change in the pH indicates that the buffering system has been radically altered and signals either a potential or actual carbon dioxide imbalance which could be deleterious to marine life (14).

The dissolved oxygen in ocean waters is an essential component necessary to initiate metabolic activity of all organisms which function by means of respiration. The solubility of oxygen in the water column is dependent upon a number of factors (30). The major natural influences are temperature and salinity. An increase in either temperature or salinity will result in a decrease in the solubility of oxygen as was previously noted. Biological activity is at a maximum in the photic zone, and the oxygen requirements are greatest. Because of gaseous diffusion across the air-water interface and photosynthetic activity, the dissolved oxygen concentration is very near saturation levels. At greater depths, the oxygen demand decreases due to a reduction in metabolic rates as a result of lower temperatures, and a large reduction in food production, causing the populations to remain small. In specific localities, distribution of the amount of dissolved oxygen is also dependent upon upwelling, which seasonally brings a
high salinity, low temperature, oxygen depleted water mass to the surface.

Dissolved oxygen minimums are considered to be in the range of two to three mg/l, optimum values of most species being five to eight mg/l (14). It should be kept in mind, however, that oxygen levels in deeper water are as a rule considerably less.

In conjunction with natural fluctuations of environmental indices, conditions in the California bight have been found to be altered by waste water discharge (7, 15, 21, 36, 37, 54, 56, 59). These changes have been manifested by alterations in population distribution, influences upon normal food chains, accumulations of biological toxins and increased prevalence of abnormalities and disease in fish populations.

In an investigation of the inputs to the California bight, the SCCWRP (54) delineated two major categories of sources. The diffuse, or nonpoint, sources are four in number: (1) bottom antifouling paints and spent fuel residues from the densely populated areas which harbor a large number of recreational, commercial and naval vessels, which release trace amounts of lead, mercury, copper, cadmium and polychlorinated biphenyls into the marine environment; (2) ocean dumping of refinery, chemical and radioactive waste, along with refuse; (3) aerial and rain-induced fallout of metals and
hydrocarbons; and (4) the most important, is the influx of 14,000,000 million gallons per day (mgd) of water from external sources to the California bight via the California current system. The pollutants being avected into the bight are low in concentration. However, considering this massive water influx daily and an average retention time of three months for waters entering the bight, it is not unreasonable to assume that a buildup of these trace pollutants could occur.

The second category, that of discrete sources, is more quantifiable and is subdivided into three major contaminating inputs: (1) on the order of 1,000 mgd of treated municipal wastewater is discharged into the California bight (94 percent of this total is contributed by five major dischargers, the largest being the Los Angeles County Sanitation District discharging off White's Point); (2) 180 mgd of discrete industrial wastewater is discharged (mostly from petroleum-related industry); and (3) an average flow of 410 mgd of surface runoff which contains silt and a wide range of biological, as well as environmental, contaminants provides additional input.

There are a multitude of chemical accumulations that have been investigated. This research, however, is specifically concerned with only those constituents having major biological impact. Three important
components of the discharged effluent have been implicated in affecting the general biological health of the marine community. The three constituents are trace metals, bacteria and viruses, and organic chlorinated hydrocarbons, notably dichloro-diphenyl-trichloroethane (DDT) and polychlorinated biphenyls (PCBs).

Except for iron, manganese and cobalt, a number of investigations have found that concentrations of heavy metals are highest in municipal wastewater discharge (14, 54). Again, the major discharge of trace metals was from the White's Point station. Extensive profiling of the area also revealed that concentrations of trace metals in the bottom sediments were highest in the immediate vicinity of the outfall.

Recently, viral or pathogenic bacterial organisms have been considered in research endeavors to delineate the etiology of a number of anomalies which have been discovered in various marine populations off the coast of Washington, Oregon, and California (8, 27, 28, 39, 47, 61, 66). Isolation and identification of viruses in the waste water is difficult, since the concentrations are relatively low. It has also been found that the concentration of enteric bacteria in seawater exponentially decreases with time and distance from the outfall.
In the 1960's, it was discovered that a certain portion of municipal wastewater discharge was of a chemical toxicant nature. The chemicals were chlorinated hydrocarbons, and the two groups found to be most common were DDTs and PCBs. Further research discovered that both of these groups of compounds were ubiquitous in both the terrestrial and marine environments (12, 16, 45, 54). DDT and its derivatives, along with PCBs, have been used as plasticizers and in insecticides and paints. In a survey of municipal wastewater dischargers and an analysis of the effluent, the SCCWRP found that during the course of 1971, 19,000,000 tons of DDT and 10,000,000 tons of PCBs were released into the nearshore bight waters (54). Levels of concentration were highest around the White's Point outfall, partially attributable to the previous discharge, up until 1970, of a large amount of DDT from the Montrose Chemical Company. Assays have determined that organic chlorinated hydrocarbons have an average biological half-life of five years. The structural nature of these compounds grants their persistence. Both DDTs and PCBs are halogenated dual-ring hydrocarbons. In either case, the stability of the compound in the environment is a function of the percent halogenation (in the case of DDT and PCB, it is chlorination) of the compound (58). At levels of toxicity, both PCBs and DDT compounds are
extremely threatening to the marine ecosystem. The danger exists because organic chlorinated hydrocarbons are insoluble in water, soluble in lipid tissue, and have an affinity for sediment. Most important is the fact that they are, in many cases, not available for degradation or metabolism by the organism.

Effects of pollutant loads on marine individuals vary depending upon the nature of the pollutant, the concentration, the organism's response, and the general quality of the environment to which the pollutant is being introduced. The major concern over heavy metals is with biomagnification through the food chain. It is generally recognized that most metals in acute doses are toxic to marine organisms. It is, however, less certain what the effects are of continual trace levels of heavy metals in the sediments. Studies conducted in the California bight reveal two important facts. There is no specific positive relationship between high levels of heavy metals in the intertidal mussel (Mytilus californianus) and distance from a point source of pollution (54). Secondly, tissues of Dover sole collected in areas of waste effluent discharge were found to have accumulated no greater concentration of heavy metals than control individuals collected in the vicinity of Catalina (36, 37, 54). It has also been discovered that organisms feeding on invertebrate benthic filter feeders in areas
of high metal concentrations do not exhibit any excessive accumulation of the metal constituents in their tissues. The inference necessarily drawn from this research is that there is a mechanism which binds the metal components of sewage effluent to particulate matter in the sediment, such that they are generally nonbiologically available. Two other theories have been proposed which may still implicate trace metals either directly or indirectly in the disruption of biological systems. Contact with the heavy metals in the sediments may initiate an external inflammatory response. This may be a possible etiology for fin erosion, which is seen primarily in demersal fish. The other thought is that a metal's toxicity varies dependent upon temperature and/or salinity fluctuations. This type of synergistic effect was observed by Rehwoldt (52), who reported that with increased temperatures fish survival rates were radically decreased in the presence of sublethal mercury concentrations. Vernberg, et al. (62), noted a similar complimentary effect of environmental parameters and mercury concentrations in the adult fiddler crab (Uca pugilator). Abnormal stressing of salinity and temperature was found to cause organisms to be more susceptible to environmental hazards.

As was previously discussed, direct effects of bacterial or viral agents on marine organisms are difficult
to quantify. A number of pathogenic epidemics have been reported along the Pacific northwest coast (28, 47, 61). The bacterial agent isolated was a marine vibrio (Vibrio anguillarum). Diseases caused by such agents are difficult to identify and categorize because the epidemiology of the disease process is largely unknown, the natural reservoir is uncertain and, due to a large number of strains, the immunological responses are inconsistent. Recently, researchers have been pointing out the necessity to investigate the possible role played by marine vibrios in the prevalent fin erosion disease of demersal species such as the Dover sole (54). The SCCWRP has also proposed that there may be a positive correlation between maximum bacterial concentrations in the Southern California bight waters during the summer months and the concurrent appearance of the nodular stage of the epidermal disease found most frequently in the predominant demersal fish species (8, 27, 66).

Virus infections have been cited as etiological agents of lymphocystis, fishpox and some papillomatous growths (43, 44, 48). Nigrelli (43) further proposes a criteria for evaluating a disease and its agent to determine the presence or lack of a virus. One, examine for the development of lesions in different individuals of the same species to determine host specificity. Two, observe seasonal appearance and disappearance of the
disease. Three, utilize electron microscopy and examine for the presence of inclusion bodies in the hyperplastic cells. The findings of these examinations allow one to draw certain inferences. "Spontaneous development of tumors, simultaneously, progressively and repeatedly in individuals of the same species of a single defined population living under the same environmental conditions would lead one to believe that an infectious agent was a possible agent."(43) Nigrelli also states that when no parasite can be isolated a virus causing transformation of normal cells must be suspected. In the case of the hyperplastic papillomatous growth in flathead sole and English sole studied by various authors (8, 27, 66), Nigrelli's criteria are satisfied. In each case, the affected fish have been the dominant species in the demersal population. The prevalence of the disease is seasonally cyclic, unlike fin erosion which appears to be chronic and virtually noncyclic. In all cases, a comparatively large number of individuals of one species were found to be diseased, while only a few of all other species were affected. The final criteria of inclusion body presence was met in every study of tumorigenesis in the aforementioned species. Nigrelli and Ketchen (44) reviewed the history of virus-type diagnoses and concluded that in fish diseases which are of viral etiology (e.g., lymphocystis found in European
flounders (48) and several of the fish pox diseases) generally single out the juvenile population in which to initiate tumorigenesis. The association to the papillomatosus disease being examined here is obvious. In each species predominately affected in a given area, results suggest juvenile population susceptibility.

The effects of chlorinated hydrocarbons to the marine biota are diverse. The organism can concentrate the toxic chemicals in its tissues (9, 12, 16, 26, 45, 58). Conservation of fats permits this concentration to be passed up the food chain to consecutively higher trophic levels, with the consequence being that the organisms at higher trophic levels are obtaining larger and sometimes lethal doses of the toxin.

Off the Southern California coast, analysis of Dover sole muscle tissue found PCB and DDT levels which were highest in the vicinity of the major sewage outfalls. Concentration of both pollutants were greatest in Dover sole taken from the White's Point outfall area. Here the DDT concentration was 22ppm and the PCB concentration was 4.1ppm as opposed to the respective concentrations of 0.1ppm and 0.04ppm found to be present in samples taken from a controlled area off Santa Catalina Island (54).

Growth decreases and RNA synthesis reductions were observed in marine diatoms (Clindorothea)
closterium) exposed to 0.1ppm PCB (26). Reproductive inhibition was observed in California sea lions (Zalophus californianus) having high tissue concentration of chlorinated hydrocarbons (12). Anatomical anomalies have been induced in guinea pigs, rats, rabbits and chickens using small doses of chlorinated hydrocarbons (16).

Recently it has been proposed that organic chlorinated hydrocarbons do not behave as other aromatic hydrocarbons. A study of metabolic pathways revealed that aromatic hydrocarbons, such as benzopyrene (a byproduct of oil degradation) are metabolized and excreted in the waste products of the organism. With DDT, however, there is no sign of hydroxylation or metabolization, but rather an accumulation of the toxin in lipid tissue (31). The halogenation of the aromatic ring appears to block normal metabolism. A mechanism of blockage has been proposed independently by Kutcompf and Kinter (10, 29). Their experimental evidence substantiates a rising belief that PCBs and related polychlorinated hydrocarbons inhibit the normal osmo-regulatory mechanism of many marine species by blocking the necessary ATPase energy driving enzymatic functions. The specific mechanism hypothesized is that chlorinated hydrocarbons are lipophilic compounds. The ATPase enzyme is a lipoprotein enzyme. Since PCBs and DDTs are insoluble in water, they enter the organism and
are conserved. They likely bind with such lipid molecules as the enzyme. The immediate effect is an inhibition of the enzyme's normal catalytic properties. The long-term effect is the blockage of critical metabolic processes necessary for cell and organism maintenance.

2.5 Theories and Observations on the Environmental Parameters.

Based on the fact that the California bight has been shown to be a long-term retention zone to which large quantities of known pollutants are admitted daily, it is hypothesized that there is an environmental factor or factors which must be considered as a component in the etiology of the tumorigenesis in the Dover sole population. The specific cause may be body contact with trace metals, introduction of an oncogenic virus, an accumulation of toxic chlorinated hydrocarbons and a subsequent metabolic inhibition and/or cellular degeneration, or some combination of these factors. What the conflicting etiological research seems to indicate is that the probable cause of the anomalous growths in the Dover sole is a combination of factors. To completely describe the onset of the disease, the epidemiology of the population must be understood. Quantification of the effects of such variables as nutrient levels, habitat and population crowding under specific environmental conditions are necessary. Specifically, quantification of the
environmental parameters, which have been shown to be indices of the health of the biological community, should allow one to specify the rank order of sources of deleterious effects and to seek to curtail those which are of major biological importance.

2.6 Literature on the Histology and Ultrastructure of Normal Integument and Epidermal Tumors.

The normal integument of flatfish consists of an overlying epidermal area with an underlying dermal area. The epidermis consists of a layer of stratified squamous epidermal cells from five to nine cells thick (1, 3, 6). Interspersed among these squamous cells are a number of mucous cells, which progress from less mature cells toward the interior of the fish to most mature cells out at the periphery.

These mucous cells are thought to be modified squamous cells that follow a typical holocrine secretory pattern, such that when they discharge their cell contents at the outer surface of the fish, the cell itself is dead and sloughed. The squamous cells are characterized by many desmosomes with their associated cytoplasmic filaments. Mucous cells are also seen with desmosomosomal attachments to the squamous cells (6). The surface-most cells are also characterized by proliferations of microvilli, and the whole of the epidermal layer is characterized by complex finger-like interdigation
between the squamous cells. This interdigitating complex of fibers is shown as a matting between the cells, and as one moves distally to the exterior of the fish, they actually seem to form a fibrous web over the outermost cells at the periphery of the skin below the microvilli. The immature mucous cells most proximally located are characterized by many mitochondria and a few mucus-containing vacuoles. As these cells mature, the interior central portion of the cell fills with these mucous droplets, while the nucleus and cell organelles are crowded out to the periphery of the cell.

The epidermis is proximally underlain by a basement membrane. Immediately underneath this basement membrane, in the superficial dermal area, one finds an interlocking network of collagen fibers, fibroblasts and pigment cells. This loose fibrous network immediately overlies the layer of dermal scales seen as tooth-like projections found in the outer area of the dermis (5).

The squamous cells display the complex finger-like interdigitations between the adjacent cells and also a characteristic division within the cytoplasm of the cells themselves. The cells can be divided into a perinuclear zone which contains the Golgi complex, mitochondria and most of the cell organelles and a peripheral cytoplasmic zone characterized by excessive filamentation (6, 23).
The tumors, on histological analysis, are essentially classified as one of two types or an intermediate form. The first type of lesion is the smallest and is found with the greatest frequency on younger fish. It has been named an angio-epithelial nodule or an AEN. It is well defined by Wellings, et al., (66) as "... small, one to two millimeter, hemispherical, smooth surfaced, pink or red, sessile nodules ... Microscopically these were angiomatous proliferations of vascular tissue capped by a layer of mildly hyperplastic and pleiomorphic epidermis ... Mucous gland cells were generally absent from the hyperplastic epidermis in contrast to their relative abundance in normal skin."

Transitional stages were noted between AENs and the second type, epidermal papillomas or PAPs. PAPs are found primarily in fish from one to three years of age and are again best described by Wellings (66) as "... much larger, one to six centimeter cauliflower-like proliferations of epidermal cells with relatively inconspicuous stroma ... Centers of tumors were often raised and warty; edges were often flat and plaque-like. The flat edges graded imperceptibly into the surrounding epidermis of normal gross and microscopic appearance. Some tumors were ovoid and cauliflower-like without a spreading plaque-like edge. The dermal angiomatous component of the epidermal papillomas was usually
inconspicuous microscopically, but the epidermal portion extended as thick, closely spaced papillary folds supported on thin strands of connective tissue."

The ultrastructural anatomy of the PAPs has been well researched (4, 27, 63, 64, 65). The dominant significant finding of ultrastructural observation on PAPs has been the discovery of an unidentified cell type that has been called the X-cell. The X-cell is found in the epithelial layer. It does not have desmosomal connections with normal epithelial cells and has been delineated by Brooks, et al., (4) as "... characterized by membrane bound, pleiomorphic, cytoplasmic granules; large mitochondria with small, round cristae, and prominent nucleoli." The X-cell is essentially recognized by its uniformly ovoid nucleus containing a large, densely stained nucleolus and a very vesicular appearance to the cytoplasm. The cytoplasm also contains a number of membrane bound dense particles, possibly viral in origin. These particles are usually round in appearance, very electron dense, usually appearing singularly, but also in couplets or triplets and as demonstrated by Kelly (27) and Wellings, et al., (64) as an astral body which demonstrates a stellate appearance.
The theoretical basis for a viral etiology of pleuronectid epidermal tumors has been well established (11, 17, 34, 42, 43, 50, 55, 57). The current working hypothesis is that the epithelial tumors found on flatfish have a viral etiology. The next step in the process is to try to isolate the virus or isolate viral antibodies. Complicating the problem is the lack of understanding of the epidemiology of the disease. It is entirely possible that there are multiple causation effects at work here and without an environmental stimulus one would not be able to induce an oncogenic viral change. The evidence would seem to support an etiology related to a DNA-type virus due to the vesicular nature of the cytoplasm, the highly altered cells, the lack of obtainable free virus, the presence of inclusion bodies in the cytoplasm and the massive changes in the tissue structure such that the X-cell, if it is indeed an altered fish cell, is no longer in harmony with the squamous cells of the epidermis; and the order and integrated nature of the epidermis and dermis have been completely disrupted.
Chapter 3

MATERIALS AND METHODS

3.1 General.

Dover sole (Microstomus pacificus) were collected from fish samples obtained in an otter trawl approximately 8 feet wide and 30 feet long with a 3/4 inch mesh net and a 1/8 inch mesh shrimp liner. The trawling was carried out aboard the "R. V. Nautilus," a converted purse seiner. The trawling was carried out during the summer and fall of 1973. Individual runs were made at the speed of 2 to 3 knots along isobathymetric lines and along sandy bottoms. The location of the survey was less than one mile from either White's Point or the Orange County Sewage Outfall (see Figure 2). Each trawl was approximately 15 minutes in duration at a relatively constant depth recorded by the fathometer on board. All invertebrates collected were discarded. Every vertebrate was counted and all flatfish were examined for species determination (41). Only the Dover sole were singled out for close scrutiny and separate counting and measuring. Any anomaly other than one produced by the organism being damaged in the net was photographed with an Olympus OM-1 35 mm camera with a 49 mm lens, equipped with close-up lenses. The tumor was then excised and
Figure 2. Map of sampling area.
placed in 4 percent gluteraldehyde for fixation of tissue. A normal juvenile Dover sole taken from the same location by the SCCWRP was later obtained for histological and ultrastructural comparisons.

At the end of the trawl, a Watanabe Keiki Nansen bottle No. 206TSK was lowered to the depth of the trawl and a discrete 2,000 ml water sample was collected for analysis. Reversing thermometers secured to the Nansen bottle provided pressure independent, pressure dependent and ambient temperatures. All temperatures were recorded, but only the pressure independent temperature was tabulated.

3.2 Sampling Techniques.

A number of test trips were made in each of the finally sampled areas, including a totally unproductive trip to the seaward side of Santa Catalina Island (November 16, 1973). It was originally thought that this area could be utilized as a control site, but no fish of any species were taken in four trawls, so the area was never resampled. During the test runs, emphasis was placed on locating areas of juvenile Dover sole and perfecting water sampling and analysis techniques.

Being unable to conduct water analysis on board the vessel, a method was sought to preserve the dissolved oxygen in the water sample. A trial test was conducted with water samples taken from the Santa Monica
Bay off the Santa Monica pier. The results of the tests showed that water samples immediately placed on ice would maintain the original dissolved oxygen concentration for as much as twenty-four hours. Based on this finding, all water samples were immediately placed on ice and transported to the Environmental Health Laboratory at California State University, Northridge, in an ice chest.

3.3 Environmental Analysis.

Water samples were taken to determine water quality by chemical analysis. Three hundred milliliters of the Nansen bottle sample was collected for dissolved oxygen assay in a 300 ml glass stopper bottle. A six inch length of 3/8 inch diameter Tygon tubing was affixed to the outlet of the Nansen bottle so that the dissolved oxygen (DO) bottle was filled from the bottom and allowed to overflow for approximately five minutes. The bottle was then stoppered, letting no air inside the bottle. This was to insure that the sample would not be contaminated by atmospheric mixing. A 1,000 ml sample was then collected in a glass jar to be used in a determination of pH and salinity. Both samples were placed on ice. The survey originally included an examination of suspended solids, but the accessible equipment was unable to register fine enough precision and reliability for the test so it was deleted from the experimental design.
Laboratory analysis was carried out to determine the salinity, pH and DO content of the water at each trawling site. Salinity was measured directly with a salinometer of specific gravity type which is calibrated at 20° Centigrade. The pH of the sample was determined utilizing a Corning digital No. 110 expanded scale pH meter. Dissolved oxygen analysis was carried out by a standard iodometric titration method (33), utilizing the Hach water and wastewater analysis Model No. DR-EL direct reading engineering lab.

3.4 Histological Procedures.

Dover sole, both normal and tumorous, were sacrificed for histological and ultrastructural purposes. Excised tissues for histology were fixed in 4 percent gluteraldehyde buffered with .05M phosphate buffer. Dehydration and paraffin impregnation was carried out utilizing an Auto-technicon tissue processor, Model No. 2A. A serial increase in ethyl alcohol concentrations from 70 percent to 100 percent followed by 100 percent xylene was used for dehydrating the tissue (22). All tissues were imbedded in Paroplast and sectioned on either the AO Spencer Model No. 820, or the AO Spencer Model No. 815 Rotary Microtome. Both Microtomes were used throughout the course of the work. Tissue ribbons were placed on a Boekel water bath at 42° C and allowed to set in order to produce increased flatness of the tissue on
the glass slide. Selected sections 5 to 7 microns thick were mounted and stained with Hemotoxylin and Eosin (32). Micrographs were then taken of the better slides using a Nikon microscope model L-Ke with a Micro-flex model AFM Automatic Photo-micrographic attachment and a Nikon M35-S camera. Histological comparative analyses were made between the normal and tumorous sections.

3.5 **Electron Microscopy Procedures.**

Tissue sections both normal and tumorous which were excised for ultrastructural use were diced into one millimeter cubes, fixed in 4 percent gluteraldehyde buffered with .05M phosphate buffer and secondarily fixed in 1 percent osmium tetroxide (22). Normal tissue samples were taken from both the unpigmented and pigmented surfaces. Tissues were dehydrated in increasing ethyl alcohol concentrations and imbedded in Araldite epoxy. Araldite blocks were cut into pyramids and one micron sections were taken and stained with methylene blue and a determination of the specific area desired was made (20). The pyramids were then trimmed to smaller dimensions and thin sections were cut on a Sorvall MT2B ultra-microtome and mounted on a Formvar coated, copper grid and stained with uranyl acetate and lead citrate. Thin sections were examined and photographed with a Philips 300 electron microscope. The Ilford rapid print processing Model No. 1501 printed the
pictures and a Durst enlarger 45s produced micrographs 2.5 times the size of the negatives. Again a comparative analysis was made of the two tissues.
Chapter 4

RESULTS

4.1 General.

After four shake-down trawling runs, made primarily to find fishing grounds containing juvenile Dover sole and secondarily to refine the sampling technique, three testing days were obtained. Two of these were off the White's Point Sewage Outfall and one off the Orange County Outfall. Each day produced three or four test samples. No Dover sole were taken at Orange County. Of 42 total Dover sole taken at White's Point, 3 were found to be tumor bearing, yielding a prevalence of 7.14 percent. The difference, if significant, between this result and the result of other workers (54), yielding 1.3 percent prevalence, is probably accounted for by the fact that our sampling was confined to areas yielding juvenile fish (mean length of tumorous fish was 10.8 cm, mean length of normal fish was 18.4 cm).

Of the three affected fish, two possessed a single PAP (one was round, approximately 1.5 cm in diameter and medially sited approximately 5 cm behind the snout on the blind side; the other was irregularly round and approximately 2 cm in diameter centered approximately between the two eyes and spreading around the snout). The
third possessed two PAPs and one intermediate form (one PAP was round, approximately 1.3 cm in diameter, medially sited approximately 3 cm behind the snout on the eyed side; the other PAP was round and approximately 1 cm in diameter and medially sited approximately 3 cm behind the first. The intermediate form was ovoid and approximately 3 mm by 7 mm at the base of the fin web immediately dorsal to the second PAP).

The small sample size obtained denies this study the opportunity to work with larger numbers, compute prevalence values by one year age increments, compare tumor location on eyed versus blind side or look at other species known to be effected in other areas whose range extends into the Santa Monica Bay. Even given these problems, it can still be inferred on the basis of this and other work (54) that the epithelial disease of Dover sole in Santa Monica Bay is essentially the same as that effecting other dominant species of pleuronectids north along the coast. Dover sole shows the same epizootic pattern effecting the juvenile population, the same lack of disease in more mature fish, the same apparently random siting of tumors and the same lesion in gross, histological and ultrastructural appearance.

4.2 Results from the Sampling Procedures and Discussion.

The results of samplings in which Dover sole were collected are summarized in Table 1. Only 42 Dover sole
Table 1. Summary of population sampled at White's Point by date and by individual trawl, with prevalence, total vertebrates taken, and percent population Dover sole.

<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>Total Fish</th>
<th>Dover Sole</th>
<th>Tumorous D. Sole</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-07-73</td>
<td>93</td>
<td>10</td>
<td>0</td>
<td>0</td>
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<td>10-07-73</td>
<td>11</td>
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<td>10-07-73</td>
<td>162</td>
<td>16</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>11-02-73</td>
<td>342</td>
<td>11</td>
<td>1</td>
<td>9.09</td>
</tr>
<tr>
<td>11-02-73</td>
<td>261</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>869</strong></td>
<td><strong>42</strong></td>
<td><strong>3</strong></td>
<td><strong>7.14</strong></td>
</tr>
</tbody>
</table>
were taken, and, of these, 3 were tumor bearing. These numbers are too small to allow any meaningful statistical analysis. Had larger numbers of Dover sole been obtained, it is conceivable that multiple regression analysis could have elucidated an association between population dynamics and prevalence of tumor bearing Dover sole. In an area such as the one sampled off White's Point where there is a tremendous influx of nutrients and pollutants from upwelling and sewage effluent, increases in fish population density are thought to result. Crowding, although a difficult factor to evaluate, may alter the nature of the relationship of a species to its habitat such that it becomes a susceptible candidate for the tumorigenic event. However, this is only a hypothesis, and a true test of its validity would necessitate a number of controlled aquarium studies which would vary both the population dynamics as well as the concentrations of nutrients and pollutants.

4.3 Results from Chemical Analysis and Discussion.

The results of the chemical analysis of the water column are summarized in Table 2. The number of stations is too small to permit determination of patterns of water quality. Concomitant with this limitation is the fact that due to constant dynamic mixing and continuous inputs the California bight is highly unstable. For these reasons, fluctuations in any given index such
<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>Depth (Fathoms)</th>
<th>Temp. (°C)</th>
<th>Salinity (o/oo)</th>
<th>pH</th>
<th>DO (mg/1)</th>
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<tr>
<td>10-07-73</td>
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<td>31.5</td>
<td>8.31</td>
<td>7.50</td>
</tr>
<tr>
<td>10-07-73</td>
<td>13</td>
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<td>31.0</td>
<td>8.74</td>
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</tr>
<tr>
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<td>23</td>
<td>12.19</td>
<td>30.5</td>
<td>8.29</td>
<td>7.49</td>
</tr>
<tr>
<td>11-02-73</td>
<td>20</td>
<td>15.75</td>
<td>31.7</td>
<td>8.19</td>
<td>7.06</td>
</tr>
<tr>
<td>11-02-73</td>
<td>20</td>
<td>15.90</td>
<td>32.5</td>
<td>7.87</td>
<td>7.46</td>
</tr>
<tr>
<td>Mean</td>
<td>18.4</td>
<td>14.35</td>
<td>31.4</td>
<td>8.28</td>
<td>7.49</td>
</tr>
<tr>
<td>11-30-73</td>
<td>16</td>
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<td>30.0</td>
<td>7.93</td>
<td>7.20</td>
</tr>
<tr>
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<td>30.5</td>
<td>7.97</td>
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<td>12.19</td>
<td>30.5</td>
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</tr>
<tr>
<td>Mean</td>
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<td>13.00</td>
<td>30.3</td>
<td>7.94</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Table 2. Summary of environmental indices by area sampled and individual trawl, with depth, temperature, salinity, pH, and dissolved oxygen.
as salinity could occur daily or even more frequently without affecting other indicies. The open ocean and quiet embayments tend to conform to stable, integrated patterns such that measurement of the temperature and salinity allow prediction of many others with good reliability. In the inshore waters of the California bight, there is this tendency for water quality indicies to vary independently of one another. Thus, an environmental agent associated with disease which also caused a change in water quality indicies would be uniquely recognized in this inshore habitat. The problem encountered, however, was the fact that highly dilute solutions were being analyzed, and it is entirely possible that significant variations were obscured by insufficient sensitivity of the equipment.

4.4 Results from Histological Analysis.

Histological results confirm (1) Dover sole integument is that of a typical teleost and (2) PAPs of the Dover sole are typical of other PAPs found on other pleuronectid fish.

Normal Tissue

Normal integument (see Figure 3) shows the lack of keratinization at the surface, an orderly array of epidermis 5 to 8 cells thick, a basement membrane, an underlying fibrous net and a basement layer of dermal cells.
Figure 3. Light micrograph of epidermis and underlying collagen web. Note the orderly layering of the epidermis, underlying melanophores and orderly structure of collagen web. 175X.
The outer epidermis (see Figure 4) is composed primarily of squamous cells. These cells tend to be flattened with their long axis parallel to the outer surface of the fish. This flattening becomes more pronounced toward the outer surface. There are complex and ubiquitous fiber connections between all cells. The other cell type seen in the epidermis is the mucous cell. This cell type is seen throughout the epidermal layer, but becomes more distinct toward the outer surface of the fish.

The basement membrane is only visible under light microscopy as the interface between the epidermal layer and the underlying open fibrous web at the dermis and appears as a dark strip. This is due to the presence of melanophores in the uppermost area of dermis immediately below the basement membrane. This fibrous web fills the area between the epidermis and a stratified dermal layer, 2 to 4 cells thick.

Tumorous Tissue

In tumorous tissue, the basement dermal layer is virtually unaffected. The start of distortion is found in the fibrous web which varies from compressed to distended, depending on the lobate structure of the overlying epidermis (see Figure 5).

The epidermis exhibits the maximum changes. The stratified thin appearance is completely lost and
Figure 4. Detail of epidermis. Note the orderly array of cells, close packing, non-vesicular nature, and cellular flattening along the long axis of the fish. 1750X.
Figure 5. Light micrograph of cross section at tumor margin. Note the distention of the collagen web on the left portion of the picture and compression of web beneath the lobe on the right of the picture. Note the swollen appearance of cells, vesicular appearance of some cells, the lobate tumor structure, and complete loss of the tissue's normal array. 90X.
replaced by an invaginating, lobate structure, tens of cells thick (see Figure 6). Normal squamous and mucous cells are still seen, however, the mucous cells are much less prominent and the squamous cells show a swollen and vesicular appearance. The swelling is so pronounced that normal tissue at 175X, 900X and 1750X is comparable to tumorous tissue viewed at 90X, 175X, and 900X, respectively, in terms of apparent size.

A third cell type has appeared and far outnumbers the mucous cells. This is the X-cell (see Figure 7). The X-cells are characterized by their size, highly vesicular appearance, a densely staining body in the nucleus (electron micrograph shows this to be the nucleolus) and an overall eosinophilic staining (as opposed to the primarily hemotoxylinophilic staining of the squamous and mucous cells). Although they do occur as single cells or pairs, X-cells usually appear in clusters.

4.5 Results from Electron Microscopy Analysis.

Ultrastructural analysis confirms (1) Dover sole integument is that of a typical teleost; (2) PAPs of the Dover sole are typical of other PAPs found on other pleuronectids; and (3) the X-cell is most probably a modified epidermal cell.

Normal Tissue

Ultrastructural study of normal tissue shows the same orderly arrangement of tissue and fills in a
Figure 6. Light micrograph of outer lobe of epidermal tumor. Note the extensive clusters of X-cells in the right lower area. 175X.
Figure 7. Detail of Figure 6. Cluster of X-cells. Note the highly vesicular appearance. Also, note the apparent free space between adjacent cells in contrast to the tight coupling of normal epidermal cells. 900X.
number of details. The outermost layer of cells is characterized by proliferations of microvilli that seem to be extensions of and based on a dense fibrous mat that is continuous over the outermost integument and seems to arise from the extensive fibrous interweaving between adjacent cells.

The squamous cells (see Figure 8) are characterized by a finely granular nucleus with a darker staining, but still finely granular border. The cytoplasm is roughly divided into two concentric areas. The inner area that surrounds the nucleus, the perinuclear area, is characterized by a concentration of mitochondria, endoplasmic reticulum and other cell organelles. The outer or peripheral area is characterized by fibrous structures, desmosomes and complex finger-like projections of plasma membrane locking adjacent cells together.

The mucous cells (see Figure 9) are seen developing from bottom to top along a typical holocrine pattern. The mucous cells found closest to the basement membrane show proliferation of mitochondria essentially centrally located around the nucleus. There are a few mucus-containing vacuoles in the cytoplasm. There are also extensive connections with adjacent squamous cells similar to the interlocking pattern found between adjacent squamous cells. The mucous cells follow a progression of development toward the surface-most cellular appearance.
Figure 8. Electron micrograph of classic epidermal cell. Note the finely granular nuclear appearance, darker staining, but still finely granular nuclear margins, mitochondria and ribosomes in the perinuclear area and extensive cellular connections in the peripheral area. The left side of the Figure shows a dense concentration of desmosomes with their associated fibrous processes. The upper area shows proliferations of typical plasma membrane interdigitations. 25000X.
Figure 9. Electron micrograph of a mature mucous cell at surface of fish. Note the underlying epidermal cell, reduction in the amount of plasma membrane interdigitating connections between the mucous cell and adjacent epidermal cells, the lack of desmosomes and the concentration of the nucleus and cell organelles at the lower portion of the cell while the central area of the cell is filled with mucus-containing vacuoles. 9750X.
Here the cytoplasm is filled with mucus-containing vacuoles. The nucleus, with a few mitochondria, Golgi apparatus and endoplasmic reticulum, is compressed against the plasma membrane. The intercellular connections have regressed. Some mucous cells have been seen at the surface of the epidermis contracted and expelling their contents. At this stage, the intercellular connections are completely gone.

The basement membrane is slightly overlain by a fine fibrous mat, similar to that on the surface. It is immediately underlain by melanophores (see Figure 10) and guanocytes (see Figure 11). The melanophores are the dominant pigment cell type on the eyed side. The guanocytes are more characteristic of the blind side. The melanophores display numerous discrete melanin-containing vacuoles. The dendritic cell type seen in some amphibians and reptiles was not present. Rather the melanophore, as well as the guanocyte, assumes an elongated shape with the long axis parallel to the basement membrane and the length to width ratio varying from about 2 to 5. The guanocytes are characterized by plate-like voids left when the guanine crystals are dissolved during processing. These plates tend to be aligned perpendicular to the long axis of the cell.

The open fibrous web contains a number of fibroblasts with lengthy extended processes. This web
Figure 10. Electron micrograph of classic melanophore. Note the basement membrane in the right portion of the picture immediately above the melanophore. Note the elongated appearance of the cell and proliferation of melanin filled vacuoles. Also, note fibroblast processes surrounding the melanophore. 17000X.
Figure 11. Electron micrograph of classic guanocyte. Note the basement membrane with underlying collagen fibers, the elongated appearance of the guanocyte and the plate-shaped voids left with the dissolution of the guanine crystals during processing. Also, note appearance of possible granular cells in the right upper portion of the picture, immediately above the basement membrane. Finally, note fine fibrous mat overlying the coarse collagen layer distal to the guanocyte and proximal to the basement membrane. 11250X.
is underlain by the basement dermal layer whose surface contains the dermal scales and is essentially a dense collagen fiber and fibroblast band between 2 and 4 cells thick.

**Tumorous Tissue**

*Ultrastructural study of PAPs yields evidence of massive changes.* Typical mucous cells are fairly rare and occur exclusively at the outer lobe margin. Epidermal cells are seen with normal appearance throughout the tumor, but in greatest concentration around the outer surface of the lobes.

Mucous cells vary from normal to more densely staining and slightly vesicular with some cytoplasmic inclusions and aberrant mitochondria.

Epidermal cells vary from normal to X-cells. In this progression, the first step is the presence of some inclusion bodies. The progression is then through more encapsulated inclusions, vesiculation of the cytoplasm, degeneration of the cristae of the mitochondria, appearance of heavy grains in the nucleus and degeneration of the intercellular connections. During this progression, the normal perinuclear-peripheral cytoplasmic division also breaks down. This progression is to be considered general and may not be the true order of progression for a cell.

The X-cell is characterized by a large, densely staining nucleolus (see Figure 12), highly vesicular
Figure 12. Electron micrograph of classic X-cell. Note roughly ovoid nucleus large densely staining, but finely granular nucleolus, highly vesicular cytoplasm, loss of orderly array of cytoplasm, complete loss of perinuclear and peripheral cytoplasmic organization, presence of many encapsulated inclusion bodies and the lack of any connection between adjacent cells. 17000X.
cytoplasm (see Figure 13), a number of cytoplasmic inclusion bodies, degeneration of the cristae of the mitochondria (see Figure 14) and a complete lack of intercellular coupling. This lack of coupling is demonstrated by the complete lack of desmosomes and their fibrous components. And, when plasma membrane interdigitations are seen, they are relict and turned inward with no extra-cellular involvement. The cytoplasm has lost all subdivision and is essentially homogeneous from nucleus to plasma membrane. The cell is also quite large, 2 to 3 times the size of a normal epidermal cell.

The inclusion bodies within the X-cells are of essentially two types (see Figure 15). The first is a membrane bound capsule with a small, uniformly round darkly staining sphere. The spheres occur most often as singles, but are also seen as couplets or triplets, not all of which are necessarily of the same radius. Some appear not to stain uniformly, but will stain lighter in the center or around the outer margin.

The second type, a homogeneous body, appears as a round to irregular shaped, nonmembrane bound, lightly staining, finely granular uniform inclusion. The astral body of Kelly (27) and Wellings, et al., (64) was not found.

In the dermis, melanophores, guanocytes and fibroblasts of tumorous tissue appear to be normal.
Figure 13. Electron Micrograph of an X-cell seen without the nucleolus. Note the abundance and variety of cytoplasmic inclusions (homogeneous bodies and encapsulated singles, doublets, and triplets). The minimal and exclusively in-turning plasma membrane invaginations are quite pronounced. 17000X.
Figure 14. Electron micrograph of X-cell cytoplasm. Note degenerate mitochondria above the septum of the large void 25000X.
Figure 15. Electron micrograph of X-cell cytoplasm. Note the encapsulated inclusion bodies, homogeneous bodies and degenerate mitochondria. 50000X.
Some X-cells are found in the dermis, but are confined to the most superficial areas. The basement membrane separating the epidermis and the dermis is distorted, but still intact.
Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions.

1) There is a disease process resulting in the production of epidermal papillomas operating among the dominant species of pleuronectids, *Microstomus pacificus*, in the Santa Monica Bay.

2) The disease process resembles that found north all along the Pacific coast of North America and reported in the literature. Specifically, the disease process is confined to juveniles of the dominant flatfish species; the observable lesion is characteristic with respect to siting; and in gross, histological and ultrastructural appearance, the lesion is identifiable with that found in research efforts with similar populations.

3) Histological and ultrastructural examination of tissues confirms that normal integument of the Dover sole is typical of teleost integument.

4) Comparison of the literature with the findings of this research revealed the classic X-cell as described. The authors postulate that the X-cell is a modified squamous cell, rather than a unicellular parasite. This is inferred by the presence of completely
normal squamous cells and essentially normal but modified squamous cells in tumorous tissue. Modifications were in varying degrees, but all were directed toward an X-cell appearance. Also normal squamous cells possessing one-half of a desmosomal connection against an unreceptive adjacent X-cell extended their peripheral cytoplasm around it, rather than making interdigitating attachments with it.

5) The findings presented here contribute to the investigative research of a specific tumorigenic disease process. Further, this work extended the epizootiology of the disease 300 miles farther south than had been previously described.

6) The difficulty encountered in attempting to sort out valid hypotheses for the etiology has not been lessened by the research. The results seem to indicate that multiple causation must be examined. Areas of sewage discharge, containing material deleterious to overall marine ecology, appear to also be areas of relatively higher tumor prevalence. However, no predictability of disease, based on environmental analysis, has been obtained.

5.2 Recommendations.

1) Delineation of the life history of the Dover sole larvae should be undertaken. Particular emphasis
should be placed on an investigation of the organism's habitat prior to metamorphosis.

2) A long-term aquarium study with larvae rather than fry is needed to more clearly describe the tumorigenic cycle.

3) A more extensive sampling program coupled with a more sophisticated, sensitive laboratory analysis should be conducted for at least one year through a spawning and metamorphosis cycle in order to delineate the dynamics of the marine environment in the Southern California bight and the resultant biological effects.

4) A rigorous virological study should be conducted to determine if a virus is the etiological agent of disease. If such is the case, then increased efforts must be directed toward isolation and identification of the specific viral agent.

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