OCS Study
MMS 2006-054

Advancing Marine Biotechnology: Use of OCS Oil Platforms as Sustainable Sources of Marine Natural Products

Final Technical Summary

Final Study Report

U.S. Department of the Interior
Minerals Management Service
Pacific OCS Region

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Prepared under MMS Cooperative Agreement No. 14-35-0001-31063
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U.S. Department of the Interior
Minerals Management Service
Pacific OCS Region

Camarillo
January 2008

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Availability of Report

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Minerals Management Service
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Suggested Citation

The suggested citation for this report is:

Schmitt, R.J., R.S. Jacobs, H.M. Page, J.E. Dugan, L. Wilson, S.D. Gaines and S.A. Hodges. Advancing marine biotechnology: use of OCS oil platforms as sustainable sources of marine natural products. MMS OCS Study 2006-054. Coastal Research Center, Marine Science Institute, University of California, Santa Barbara, California. MMS Cooperative Agreement Number 14-35-0001-31063. 45 pages.

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FINAL TECHNICAL SUMMARY

STUDY TITLE: Advancing marine biotechnology: use of OCS oil platforms as sustainable sources of marine natural products

REPORT TITLE: Advancing marine biotechnology: use of OCS oil platforms as sustainable sources of marine natural products

CONTRACT NUMBER: 14-35-0001-31063

SPONSORING OCS REGION: Pacific

APPLICABLE PLANNING AREA(S): Southern California

FISCAL YEAR(S) OF PROJECT FUNDING: FY 00/01, FY 01/02, FY 02/03, FY 03/04, FY 04/05

COMPLETION DATE OF REPORT: October 2007

COST(S): FY 00/01: $500,000; FY 01/02: $130,817; FY 02/03: $171,474

CUMULATIVE PROJECT COST: $802,291

PROJECT MANAGER: Russell J. Schmitt

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KEY WORDS: natural products, marine biotechnology, invertebrate assemblage, offshore oil platform, oceanographic gradient, Santa Barbara Channel, mussel growth, exotic species, bryostatin, population genetics, *Bugula neritina*, cryptic species, mtDNA, colonization, dispersal, sea urchin embryo bioassay, mitotic poisons, bryozoa, anthozoa, chlorophyta, sea amemone

BACKGROUND: The discovery and potential commercial use of pharmaceutically important products contained in marine invertebrates has led to numerous studies of these species. Interest in marine natural products for use in medical and industrial applications continues to grow worldwide. However, there is concern over the ecological impacts of the harvest of organisms from natural reefs because large quantities of organisms are typically

needed to extract a small amount of a natural product. The harvest of organisms from man-made structures, including offshore oil and gas platforms, may eliminate impacts to natural reefs. Unfortunately, little information is available on the distribution and abundance or dynamics of invertebrates, including those possessing potentially useful natural products on oil platforms.

The Santa Barbara Channel (SBC) is an ideal location to investigate the possibility of using OCS oil platforms as sustainable sources of biomedically important invertebrates because oceanographic gradients may provide for diversity in biotic assemblages, increasing the liklihood that invertebrates with valuable natural products will occur on one or more platforms. Several offshore oil and gas platforms are arrayed along the length of the SBC from near the southeast entrance, extending to the northwest south of Pt. Conception. The platform structures are covered intertidally and subtidally by an assemblage of sessile and semi-mobile invertebrtes typically found on inshore natural reefs and pier pilings in southern California as well as other species that are relatively rare in the inshore environment. The spatial distribution of platforms along the SBC also presented an opportunity to explore variability in invertebrate assemblages across oceanographic gradients in the absence of the habitat heterogeneity that characterizes natural rocky reefs.

Marine organisms that inhabit the subtidal structures of offshore oil production platforms are a potential source of novel compounds for pharmaceutical use. These organisms provide an unparalleled opportunity to study natural product chemistry from populations of organisms living in ecologically unique habitats. Research has shown that growth rates of certain invertebrate species living in the platform community can be quite high. The platform encrusting invertebrate community also supports many encrusting and soft-bodied organisms, which rely on rapid growth rates, alleopathic effects, and chemical warfare to compete for space in the habitat and avoid predation. Such habitat characteristics (as in the example of coral reefs) have been shown to sustain many organisms that produce compounds with potential pharmaceutical application. This component of the project focused on the study of natural products from sea anemones, marine alga, and bryozoa living on the intertidal and subtidal portions of offshore oil production OCS (outer continental shelf) platforms in the Santa Barbara Channel.

Knowledge of the population genetics of marine organisms will be imperative for advancing marine biotechnology. Genetic markers allow the accurate identification of species, the determination of genetic diversity, both within and between populations, the determination of the degree of gene flow among populations and the identification of processes such as hybridization. Each of these aspects of population genetics could be important for the successful development of marine biotechnology. Advances in marine biotechnology depend not only on the identification of useful compounds that organisms produce, but also on the accurate identification of the organisms that produce them. Without accurate identification, sampling of individuals may result in variable yields of target compounds or worse, no yield at all. It has become increasingly clear that molecular-genetic markers can be extremely useful for the identification of taxa of marine organisms. Using molecular markers, the identification of cryptic marine sibling species (species indistinguishable by morphology) has

increased and the general conclusion has been that the current systematic treatments of many groups is characterized by excessive 'lumping' rather than excessive 'splitting'.

One example of why identifying cryptic species is important for marine biotechnology is the bryozoan, *Bugula neritina*. Though identified by morphological assessment as a single cosmopolitan species, mtDNA has revealed that *B. neritina* is actually at least two distinct species. Furthermore, the anticancer drug candidate Bryostatin 1 is found in only one of these two species.

OBJECTIVES: To examine the possibility of using OCS oil platforms as sustainable sources of, or as culturing sites for, invertebrates containing important marine natural products, we: 1) investigated spatial and temporal patterns in the distribution and abundance of invertebrates on selected offshore oil platforms in the Santa Barbara Channel, 2) explored whether the recruitment and growth of common invertebrates varies among platforms (both spatially and temporally), and 3) examined the relationship between patterns of distribution and abundance and recruitment found at the platforms, and selected environmental factors (e.g., location, water temperature).

The goals of the pharmocology portion of this study were to 1) collect and identify novel species of organisms from the OCS platforms which possess potential marine natural products and 2) isolate and characterize extracts from these organisms to investigate any compounds which possess biological activity.

The objectives of the population genetics component of the study focused on the branching bryozoan *Bugula neritina*. Two cryptic species have previously been identified in the morphological species of the branching bryozoan, *Bugula neritina*, and these species differ in whether they associate with a bacterium, *Endobugula sertula*, that produces bryostatin compounds. We sought to determine if there were further cryptic species within *Bugula neritina* by sequencing larger and more variable regions of mtDNA for samples of this organism from oil platforms and natural reefs in the Santa Barbara Channel.

DESCRIPTION: We conducted this study at 7 oil and gas platforms in the Santa Barbara Channel. The platforms are arrayed geographically from offshore of Oxnard, California in the southeast, approximately northwestward ~65 km towards Point Conception, and encompass a range of water depths and distances from shore. To examine spatial variation in water temperature, we attached one HOBO temperature logger to each of the 7 platforms at a depth of 15 m. Water temperature was recorded hourly and the loggers were retrieved and downloaded at approximately 3 month intervals in summer, fall, and spring. Data were also collected during the winter, but are not included here.

To explore spatial variation in patterns of invertebrate distribution and abundance among our study platforms, photographically sampled the invertebrate assemblage August-November 2001. The distribution and abundance of invertebrate taxa was measured by photographing a single 0.25 m^2 quadrat located on the inside and outside of the 4 corner legs and on 4 randomly selected conductor pipes at depths of 6, 12, 18, and 24 m for a total of 128

photoquadrats per platform. In the laboratory, we identified and estimated the percent cover of major invertebrate taxa within each photoquadrat using point-contact methods.

To investigate spatial variation in barnacle recruitment, unglazed ceramic tiles attached to PVC frames were suspended vertically between adjoining conductor pipes at a depth of 15 m beginning in June 2001. Tiles were retrieved and replaced after three months and transported to the laboratory where barnacles were identified and counted. To explore spatial variation in the growth rate of the mussel, *Mytilus galloprovincialis*, among platforms, we caged in a vexar mesh cage and attached one cage to each of the PVC frames above (n=4 replicates per platform). Mussel shell-length was measured initially and after 3 months of deployment when the cages were retrieved and replaced by cages of new mussels of ~30 mm shell-length.

Species of anthozoans, bryozoans, poriferans and chlorophytes were collected from OCS platforms and other habitats for preparation of crude extracts. Samples were cleaned in the laboratory before preparation of extracts. Crude extracts were then tested using the Sea Urchin Embryo Model assay involving the first cleavage of *S. purpuratus* (sea urchin) embryos and other human cancer cell lines. The sea urchin assay is highly selective for agents that are targeted to the tubulin and microtubules important in mitotic spindle formation. If biological activity was identified in the crude extract, subsequent bioassay guided fractionation of the crude extract was performed to isolate the bioactive compound and perform additional testing.

Samples of *Bugula neritina* were collected using SCUBA from natural populations on reefs in Santa Barbara County and Santa Cruz and Catalina Islands as well as from populations inhabiting two OCS oil platforms, Hogan and Houchin, to determine the types/species of *B. neritina* they harbored. Samples were brought to UCSB where they were sorted, identified and cleaned of any contaminating organisms. DNA was isolated using standard Qiagen DNA prep kits and quantified. A subset of samples were first amplified for the COI gene using the primers and conditions described by Davidson & Haygood (1999). Sequences from an individual were aligned and manually corrected using Sequencer 4.6. All sequences were then aligned using CLUSTAL and manually aligned. Sequences were searched using BLAST to identify genes.

STUDY RESULTS: Mean water temperature decreased from platforms in the southeast (Gail) to platforms in the northwest (Holly) in summer 2001 with a less pronounced gradient in fall 2001 and spring 2002. Temperatures at Gina were quite variable during this period and lower overall than the other three southern platforms (Gail, Gilda, Grace). The major macroinvertebrate taxa (e.g., sea anemones, mussels, barnacles, tubiculous amphipods, hydroids and sponges) were common to all platforms studied. Across all platforms, the most widely distributed and abundant higher taxa, together accounting for 83% of the total cover in our photoquadrats, were anemones, tubiculous amphipods, hydroids, and sponges. Other widespread taxa included mussels, barnacles, and tunicates. Filamentous red algae were the most widely distributed algal taxon. However, in general the cover of algae was low (~5%). However, Discriminant Function Analysis (DFA) revealed that the assemblages of two platforms (Gilda, Gail) were clearly different from the other platforms, a pattern attributable, in part, to the presence of conspicuous exotic species (the anemone, *Diadumene* sp. and

encrusting bryozoan, *Watersipora subtorquata*) on these platforms. If the exotic species were excluded from the analysis, platforms in close proximity to one another generally tended to have invertebrate assemblages more similar to each other than to platforms located further away. Spatial variation in barnacle recruitment onto ceramic plates and mussel growth rate reflected prevailing oceanographic gradients created through the advection of warmer waters into the channel from the south.

The structure of invertebrate assemblages varied among platforms. Anemones occurred in higher cover overall (up to 50 to 60%) than most other invertebrates, but the dominant species varied with location. *Corynactis californicnus* was the dominant anemone on platforms at the southeast end of the channel. In contrast, mean cover of *Metridium* sp. was generally highest at the most northwest platforms. Tubiculous amphipods, hydroids, and mussels also generally occurred in higher cover on platforms with increasing distance along the channel from the southeast to the northwest.

Three species of barnacles recruited to the plates deployed at the platforms. Recruitment of *Balanus trigonus*, which occurred during the summer and fall, was highest on plates at the two most southeasterly platforms (Gina, Gail). Recruitment of *Megabalanus californicus* occurred during the fall and spring. During the fall, there was no apparent gradient in recruitment along the SBC, whereas during the spring, recruitment of this species and *Balanus regalis* occurred primarily at the most southeasterly platform (Gina) with markedly lower recruitment onto plates deployed at the other platforms.

Growth rate of *Mytilus galloprovincialis* was most rapid during the summer and at the southeasterly platforms. There was a significant correlation between mussel growth and location in the channel during the summer, but not during any of the other seasons. During the summer only, mussel growth was also correlated with degree-days. However, there was no correlation between mussel growth and distance from shore or water depth during any time of the year.

Extracts of the sea anemone, *Diadumene* sp., collected from OCS Platform Gail were prepared and tested with the Sea Urchin Embryo Model assay. The crude organic extract was active in inhibiting the first cleavage of sea urchin embryos division in a concentration dependant manner with 50% inhibition occurring at approximately 118 μg g/ml. The organic extract was also examined for the ability to inhibit proliferation in the human lung cancer cell line, A549, and found to be highly active. The extract inhibited cell proliferation in a concentration dependant manner with 50% inhibition occurring at 23 μg g/ml.

In studies on the anti-mitotic actions of coumarin compounds from species such as the chlorophytes, we initially discovered that dicoumarol (a coumarin anticoagulant chemically designated as 3,3'-methylenebis[4-hydroxycoumarin]) inhibits the first cleavage of *S. purpuratus* (sea urchin) embryos in a concentration dependent manner, with 50% inhibition occurring at approximately 10 μM drug. Because the sea urchin assay is highly selective for agents that are targeted to tubulin and microtubules, we reasoned that the active compounds might inhibit cell division by interfering with the polymerization or dynamics of the mitotic spindles. Dicoumarol reduced the rate and extent of shortening, it increased the percentage of

time the microtubules spent in an attenuated (paused) state, and it reduced the overall dynamicity of the microtubules. Using fluorescent spectroscopy, we determined that dicoumarol binds directly to tubulin dimers in vitro with a moderately high affinity (Kd, 23 μM). In addition, we demonstrated that taxol inhibition of sea urchin embryo division is potentiated by low concentrations of dicoumarol.

For the bryozoan, *Watersipora subtorquata*, the bioactivity assay of purified compound associated with the red pigment (WC01-A) using the Sea Urchin Embryo Model found that WC01-A inhibited the first cleavage of *S. purpuratus* (sea urchin) embryos in a concentration dependent manner, with 50% inhibition occurring at approximately 8.3 μM drug. Initial organic extraction with traditional organic solvents yielded less than 1% yield of the red organic soluble pigment. The red pigment was shown to have specific binding to an individual protein. This association of the pigment to a single protein is very unique in that most molecules with the ability to attach to a protein are generally ubiquitous in most systems (ie. they are generally attached to all proteins present; nonspecific). This binding presumably also allows for the red compound to be water soluble in its native marine environment. The largest difficulty faced in the bryozoa study was the instability of the natural product. This difficulty made it very challenging to obtain pure quantities of the natural product for NMR analysis. It is also noteworthy that the red compound is highly reactive which may also contribute to possible breakdown on silica or other chromatographic substrates.

For the branching bryozoan, *Bugula neritina,* direct sequencing of COI PCR products and sequence comparisons revealed members of both the 'shallow' and 'deep' clades in samples from platforms and reefs in the Santa Barbara Channel. Only members of the "deep" bryostatin-producing clade and no members of the 'shallow' clade were found on either OCS platform (Hogan, Houchin) sampled.

After identifying samples that belonged to the 'shallow' and 'deep' clades of *B. neritina*, we designed PCR primers at the 5' and 3'ends of the COI and 16s genes. The COI-reverse and 16s-forward primers produced a large PCR product from both 'shallow' and 'deep' clades. We used the COI-reverse and 16s-forward primers to amplify the large mtDNA fragment from a wide range of samples including those from natural reefs as well as those from OCS oil platforms Hogan and Houchin. We sequenced these PCR products using the amplification primers and two internal primers designed from our initial sequencing of both 'shallow' and 'deep' individuals of *Bugula neritina*. Phylogenetic analysis of these data revealed that there were two distinct clades within the 'deep' lineage, which we call 'Deep-1' and 'Deep-2'. A large majority of the 'deep' samples were members of the Deep-1 clade, only four individuals of the Deep-2 clade were found. All members of the Deep-2 clade found were from Santa Cruz Island.

Within the Deep-1 clade there appeared to be some suggestion of structure. Notably, a clade with 62% support was found that included all samples from OCS oil platforms Hogan and Houchin. All of the samples from platforms Hogan and Houchin clustered within a single subclade of Deep-1 suggesting dispersal is limited and that dispersal to OCS oil platforms may be a relatively rare event.

SIGNIFICANT CONCLUSIONS: Although the major macroinvertebrate taxa were common to all platforms, the relative abundance of these taxa (as percent cover) varied along the SBC such that platforms in close proximity to one another tended to have invertebrate assemblages more similar to each other than to platforms located further away. However, there were exceptions to this general gradient pattern. For example, the cover of sponge was highest at Platform Gail, one of the southern platforms and cover of *Metridium* was low (3%) at Hogan, one of the northern platforms. Further, conspicuous exotic species were present in high cover on two of the platforms, the anemone, *Diadumene* sp. on Gail and the encrusting byrozoan, *Watersipora subtorquata* on Gilda.

We propose that along-channel variation in platform invertebrate assemblages result, in part, from regional oceanographic gradients created through the advection of waters into the channel from the south via the Inshore Counter Current and Southern California Eddy during the spring and summer. Along-channel variation in water temperature measured at the platforms during this study was consistent with the temperature patterns reported by Otero and Siegel (2004) from the compilation of monthly satellite SST data covering the period 1997 to 2001. The anemone, *Metridium* sp. (probably *M. senile*) appears to prefer cooler waters with a distribution range extending from southern California to Alaska, which may explain the higher density of this species on the northern platforms. Current flow from the south also provides a mechanism for the transport of invertebrate larvae into the channel. The higher recruitment densities for three species of barnacles at the southern compared to the northern platforms was consistent with predictions of oceanographic conditions bringing warm water masses and the longer-lived planktonic larvae of southern taxa, such as *Balanus trigonus* into the channel.

Platform assemblages are probably also strongly influenced by founder effects. This supposition is supported by the presence of exotic species, *Diadumene* sp. and *Watersipora subtorquata*, each on different platforms. These species were probably introduced via barges or crewboats, have limited dispersal ability, but occur in high cover and appear to be superior competitors for space. In addition to founder effects, biological interactions, including predation and competition undoubtedly influence the structure of platform invertebrate assemblages.

Growth rates of caged *Mytilus galloprovincialis* also varied along the SBC during the summer, with highest growth rates at the southeastern platforms. This pattern coincided with the temperature gradient that typically develops from the east to the west end of channel during the summer. However, gradients in water temperature *per se* do not satisfactorially explain spatial and temporal variation of mussel growth in the channel. Alternatively, spatial and temporal variation in mussel growth rate may reflect food availability, or the interaction of food availability and water temperature.

The existence of along-channel patterns in the composition of platform assemblages, and in invertebrate recruitment and growth, suggests that assemblages attached to platforms may be useful as barometers of short and long-term change in oceanographic climate. Over the short-term, changes in oceanographic conditions may influence invertebrate recruitment and

growth. Over the longer-term, changes in climate that alter ocean currents (e.g. ENSO events, global warming) may shift the composition of platform invertebrate assemblages.

Our results indicate that invertebrates, such as sponges, tunicates, and bryozoans, that may contain potentially useful marine natural products can be abundant on offshore oil platforms. However, the significant variation found in the distribution, recruitment and growth of these invertebrates among platforms in the SBC suggests that factors such as location and temperature could affect the potential harvest of these organisms for use in the development of marine natural products.

Significant biological activity in the crude extracts of a number of species of platform-dwelling organisms were found in this study, including species of anthozoa, bryozoa, and chlorophytes. Crude extracts of the exotic anemone, Diadumene sp. from Platform Gail showed strong biological activity in the sea urchin bioassay and a human cancer cell line. Our results on specific action and activity suggest that coumarins, such as dicoumarol, possess a unique anti-proliferative mechanism of action and possibly related pharmacophores are mediated by tubulin binding and the kinetic stabilization of spindle microtubule dynamics. The coumarin pharmacophore thus may represent an attractive compound for development of new drugs for cancer treatment.

The red pigment (WC01-A) found in the exotic species of foliose bryozoan, *Watersipora subtorquata,* from Platform Gilda appears to be a compound which has not been previously described before in the literature. The compound shows a high degree of bioactivity in the sea urchin embryo model assay and in other human cancer cell lines. These results suggest it is a viable candidate for further cell division studies. High instability of the compound is also indicated by our results. However, further analytical study of derivatives could provide key information for the characterization of the complete compound.

For the branching bryozoan, *Bugula neritina*, no members of the 'shallow' clade were found on either OCS platform sampled. This may have been due to sampling only at moderate to deep depths though Davidson and Haygood (1999) found members of this clade at depths up to 10 meters. The very high frequency of the bryostatin-producing Deep-1 clade members on the platforms suggests that collections of *B. neritina* from these artificial habitats would contain high yields of bryostatins, undiluted by members of the Shallow clade that does not contain them. Very little population structure was identified though samples from the two oil platforms were members of a single subclade suggesting limited dispersal of this species.

Sequencing larger and more variable regions of mtDNA between the 16s and COI genes, allowed us to identify a new cryptic species of *B. neritina* that is very closely related to the cryptic species that associates with the bacterium *E. sertula* and produces bryostatins. The new cryptic species of *Bugula neritina* that we identified was rare in our samples and was only obtained from reefs on Santa Cruz Island. The bryostatin-producing capabilities of this new cryptic species of *Bugula neritina* were not evaluated.

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Published work:

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FINAL STUDY REPORT

CHAPTER 1. Oceanographic gradients and patterns in invertebrate assemblages on offshore oil platforms

H. Mark Page, Jenifer E. Dugan, Carolynn S. Culver and Brent Mardian (authors only)

Introduction

The discovery and potential commercial use of pharmaceutically important products contained in marine invertebrates such as sponges, tunicates and bryozoans has led to numerous studies of these species (e.g. Proksch et al 2002). Interest in marine natural products for use in medical and industrial applications continues to grow worldwide. However, there is concern over the ecological impacts of the harvest of organisms from natural reefs because large quantities of organisms are typically needed to extract a small amount of a natural product. These means that that even drug discovery and testing could be limited by ecological concerns. The harvest of organisms from man-made structures, including offshore oil and gas platforms, may eliminate ecological impacts to natural reefs. Unfortunately, little information is available on the distribution and abundance or population dynamics of invertebrates, including those taxa possessing potentially useful natural products on offshore oil platforms.

The Santa Barbara Channel (SBC) is an ideal location to investigate the possibility of using OCS oil platforms as sustainable sources of biomedically important invertebrates because oceanographic gradients may provide for diversity in biotic assemblages, increasing the liklihood that invertebrates with valuable natural products will occur on one or more platforms (Culver et al. 2005). The channel, ~100 km long and 50 km wide, is bordered on the north by the California mainland and on the south by the Northern Channel Islands. Circulation in the SBC is variable, but beginning in late spring, warmer waters from the Southern California Bight are typically advected into the channel through its eastern entrance. These waters move westward where they meet the cooler waters of the California Current, which enter the SBC through its western entrance at Point Conception (Hendershott and Winant 1996, Harms and Winant 1998, Otero and Siegel 2004). As a result of this circulation pattern, a gradient of water temperature occurs beginning in late spring, peaking in early summer extending the length of the channel (Fig. 1). The mixing of warmer waters from the south with cooler waters from the north creates a biogeographical transitional zone in the channel between the Oregonian faunal province, located to the north of Pt. Conception, and the Californian faunal province to the south (Horn and Allen 1978).

Several offshore oil and gas platforms are arrayed along the length of the SBC from near the southeast entrance, extending to the northwest south of Pt. Conception. The platform structures are covered intertidally and subtidally by an assemblage of sessile and semi-mobile invertebrtes typically found on inshore natural reefs and pier pilings in southern California (Wolfson et al., 1979, Page et al. 1999, Bram et al., 2005) as well as other species that are relatively rare in the inshore environment (e.g., *Metridium sp.*). The spatial distribution of platforms along the SBC presented an opportunity to explore variability in invertebrate

assemblages across oceanographic gradients in the absence of the habitat heterogeneity that characterizes natural rocky reefs; the vertical structure of platforms also permits sampling and experiments at known depths uncomplicated by spatial variation in tides and swell. In addition, platforms provide hard substrate habitat along portions of the channel where other hard substrate habitat (rocky shore) is sparse.

To examine the possibility of using OCS oil platforms as sustainable sources of, or as culturing sites for, invertebrates with important marine natural products, we have: 1) investigated spatial and temporal patterns in the distribution and abundance of invertebrates on selected offshore oil platforms in the Santa Barbara Channel, 2) explored whether the recruitment and growth of common invertebrates varies among platforms (both spatially and temporally), and 3) examined the relationship between patterns of distribution and abundance and recruitment found at the platforms, and selected environmental factors (e.g., location, water temperature).

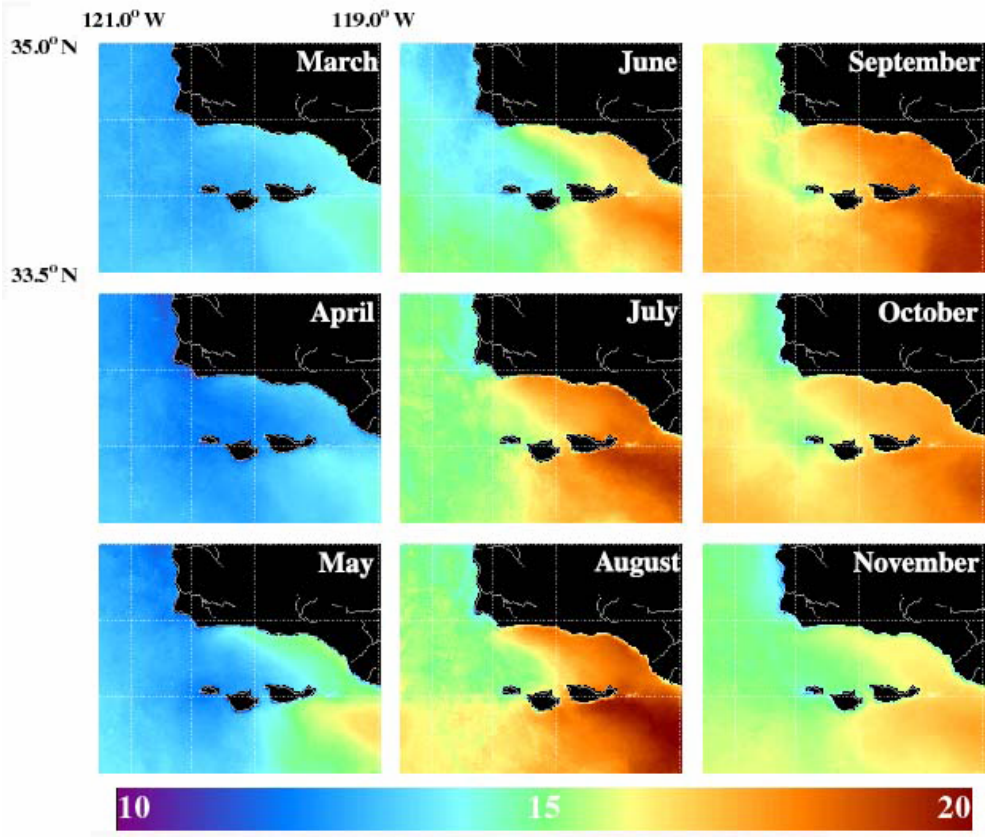


Figure 1. Monthly mean sea surface temperature (SST) composited from satellite images made from October 1997 to June 2001 showing the intrusion of warm water from the Southern California Bight into the Santa Barbara Channel beginning in Spring. Data from Otero and Siegel (2004).

Materials and Methods

Study sites

We conducted this study at 7 oil and gas platforms in the Santa Barbara Channel. The platforms are arrayed geographically from offshore of Oxnard, California in the southeast, approximately northwestward ~65 km towards Point Conception (Fig. 2), and encompass a range of water depths (29 to 225 m) and distances from shore (2.9 to 14.4 km: Table 1). The platforms differ in size (Table 1), but their general configuration is similar with the subtidal portion consisting of steel vertical, oblique, and horizontal steel cross members together with conductor pipes through which the wells are drilled. Subtidal reef habitat at the water depths of our study (≤ 24 m) is rare along this length of the Santa Barbara Channel.

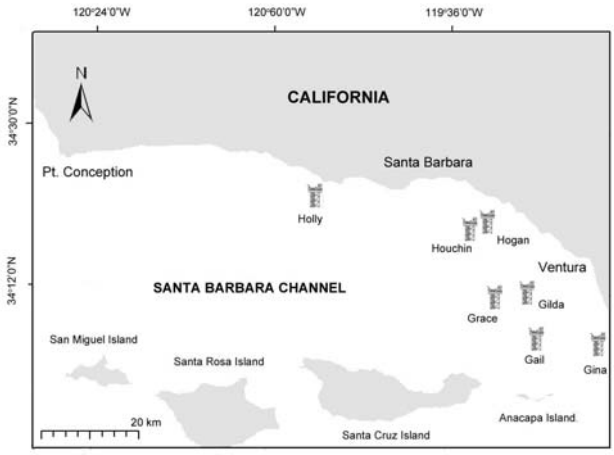


Figure 2. Locations of 7 offshore oil and gas platform in the Santa Barbara Channel sampled in this study.

Table 1. Characteristics of the study platforms. Key to abbreviations: Gi-Gina, Ga-Gail, Gil-Gilda, Gr-Grace, Hog-Hogan, Hou-Houchin, Hol-Holly.

Variable	Gi	Ga	Gil	Gr	Hog	Hou	Hol
Distance from shore (km)	5.0	13.2	11.9	14.4	5.1	7.0	2.9
Water depth (m)	29	225	64	97	46	49	64
Year of installation	1980	1987	1981	1979	1967	1968	1966
Distance along channel (km)	0	12	15	19	33	36	65
Platform size (m^2 on bottom)	560	5600	2340	3120	1444	1444	1728

Water temperature

To examine spatial variation in water temperature, we attached one HOBO temperature logger to each of the 7 platforms at a depth of 15 m. Water temperature was recorded hourly and the

loggers were retrieved and downloaded at approximately 3 month intervals in Summer (June-August 2001), Fall (September-November 2001), and Spring (March-May 2002). Data were also collected in winter 2002, but are not included here. To compare the temperature regime among sites, we calculated degree-days, the average daily water temperature mulitplied by the number of days spent at that water temperature, for each 3 month period.

Spatial variation in invertebrate assemblages

To explore spatial variation in patterns of invertebrate distribution and abundance among oil platforms along the Santa Barbara Channel, we used a Nikonos V 35 mm camera fitted with a 15 mm lens to photographically sample the invertebrate assemblage following methods similar to those suggested in Coyer et al. (1999). The camera and two strobes were mounted on a PVC frame designed to photograph 0.25 m2 quadrats. Quadrats measured 41 x 62 cm internal diameter (0.25 m2) to accommodate the dimensions of the platform legs and conductor pipes. The distribution and abundance of invertebrate taxa was measured by photographing a single 0.25 m2 quadrat located on the inside and outside of the 4 corner legs and on 4 randomly selected conductor pipes at depths of 6, 12, 18, and 24 m for a total of 128 photoquadrats per platform. Photographs were taken August-November 2001.

In the laboratory, we identified and estimated the percent cover of major invertebrate taxa within each photoquadrat using point-contact methods. Percent cover of taxa was estimated by projecting the photographic slide images onto 100 randomly located points and recording contacts to the lowest possible taxonomic level (Table 2). Because the platform invertebrate assemblage may be several centimeters thick, only organisms occupying the surface layer were counted. These organisms were often attached to secondary substratum (e.g., mussels, encrusting bivalves). Mussels and encrusting bivalves were thus under sampled in the photoplots because they were often covered by other species. Categories of nonliving substrata if present (e.g., bare pipe) were also included.

Barnacle recruitment

To investigate spatial variation in barnacle recruitment, we used unglazed ceramic tiles (15 x 15 cm). Two tiles were attached to a PVC frame and 4 frames were suspended vertically between adjoining conductor pipes at a depth of 15 m beginning in June 2001. Each tile possessed a smooth and grooved side; we used the grooved side of each tile as the sampling surface. Tiles were retrieved after three months and transported to the laboratory where barnacles were identified and counted. Data from the two tiles of each frame were composited to form a single replicate from each frame.

Mussel growth

To explore spatial variation in the growth rate of the mussel, *Mytilus galloprovincialis*, among platforms, we enclosed 10 mussels of ~30 mm shell-length in a vexar mesh cage and attached

one cage to each of the PVC frames above (n=4 replicates per platform). Mussel shell-length was measured initially and after 3 months of deployment when the cages were retrieved and replaced by cages of new mussels of ~30 mm shell-length. Growth rates were calculated as the difference in shell length between the beginning and end of the experiment standardized to 30 days of deployment.

Statistical analysis

The percent cover data were arcsin transformed ($p'=\arcsin(\sqrt{p})$) prior to statistical analysis (Zar, 1999). We tested for significant differences in invertebrate assemblage composition and cover across platforms using multivariate analysis of variance (MANOVA). We tested for significant differences in the cover of selected taxa between platforms using Tukey post hoc tests. We also examined assemblage patterns using Canonical Discriminate Function Analysis and explored relationships between these patterns and physical variables using multiple regression analysis. Mobile taxa such as crabs and starfish were excluded from statistical analysis, as were algae (~5% cover). Individual invertebrate species with low overall cover or that were difficult to identify from photographs were grouped into higher taxa (e.g., tubicolous amphipods, sponges) for statistical analysis.

Table 2. Taxa identified and quantified in terms of percent cover in photoplots and taxa identified, but quantified under higher taxa.

Higher taxon	Quantified (percent cover)	Identified
Algae	Green (filamentous)	
	Red (filamentous)	
	Red (bladey)	
	Red (branching)	
Amphipoda	Unknown tubiculous	
Annelid	Sabellidae	
Anthozoa	*Anthopleura* sp.	
	Corynactis californica	
	Diadumene sp.	
	Metridium sp.	
	Tealia	
	Unknown	
Barnacle	Barnacle	*Megabalanus californicus*, *Balanus* sp.
Bivalve	*Mytilus* sp.	*Mytilus galloprovincialis*, *M. californianus*
	Crassadoma gigantea	
	Unknown	
Bryozoa	Unknown encrusting	
	Watersipora subtorquata	
	Erect	*Crisia complex, Bugula neritina*
Decapod	*Cancer antennarius*	
	Loxorhynchus sp.	
Echinoderm	*Pisaster* sp.	*Pisaster ochraceus*, *P. giganteus*
	Cucamaria sp.	
	Ophioroid	
	Strongylocentrotus purpuratus	
Porifera	Sponge	*Halichondria panicea*, *Spheciospongia confoederata*, *Haliclona* sp.
Tunicate	*Styela* sp. (solitary)	
	Unknown colonial	

Results

Water temperature

There was a pronounced gradient in water temperature with degree-days decreasing from Platform Gail in the southeast to Platform Holly in the northwest in summer 2001 (Fig. 3). However, water temperatures at Gina were quite variable during this period and lower overall than at the other three southern platforms (Gail, Gilda, Grace). The along-channel gradient in temperature as degree-days was also evident, though less pronounced in fall 2001 and spring 2002.

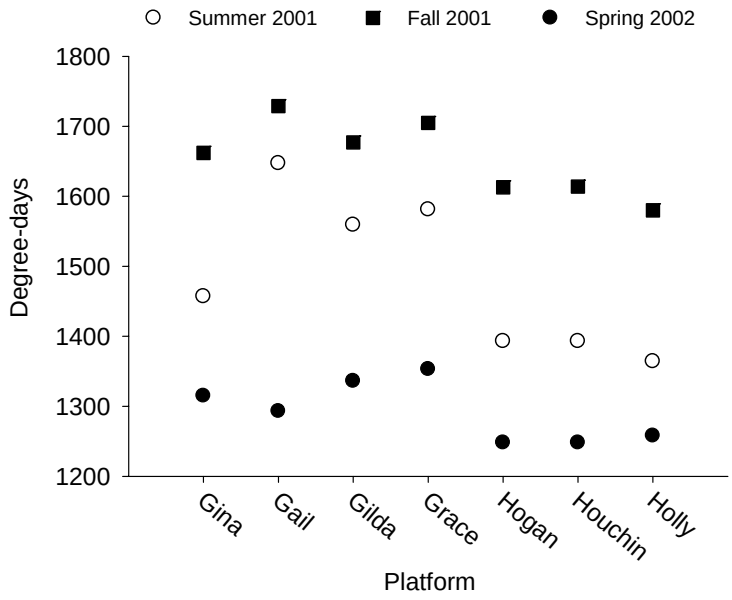


Figure 3. Water temperature, as degree-days, at the study platforms during the Summer and Fall 2001 and Spring 2002. Platforms arranged along the x-axis from the most southeast (Gina) to the most northwest (Holly) in the SBC.

Distribution and abundance of selected taxa

Across all platforms, the most widely distributed and abundant higher taxa, together accounting for 83% of the total cover in our photoquadrats, were anemones (e.g., *Corynactis californicus*, *Metridium* sp.), tubiculous amphipods, hydroids (Plumaria, Agalophenia), and sponges (e.g., *Halichondria panicea*, *Spheciospongia confoederata*) (Fig. 4). Other widespread taxa included mussels, (*Mytilus californianus, M.galloprovincialis*), barnacles (*Megabalanus californicus, Balanus* spp.), and tunicates (e.g., *Styela montereyensis*). Exotic species were conspicuous on two platforms; the encrusting bryozoan, *Watersipora subtorquata*, was observed only on Platform Gilda and the anemone, Diadumene sp. was recorded only on Platform Gail (see also Page et al. 2006). Filamentous red algae were the most widely distributed algal taxon. However, in general the cover of algae was low (~5%).

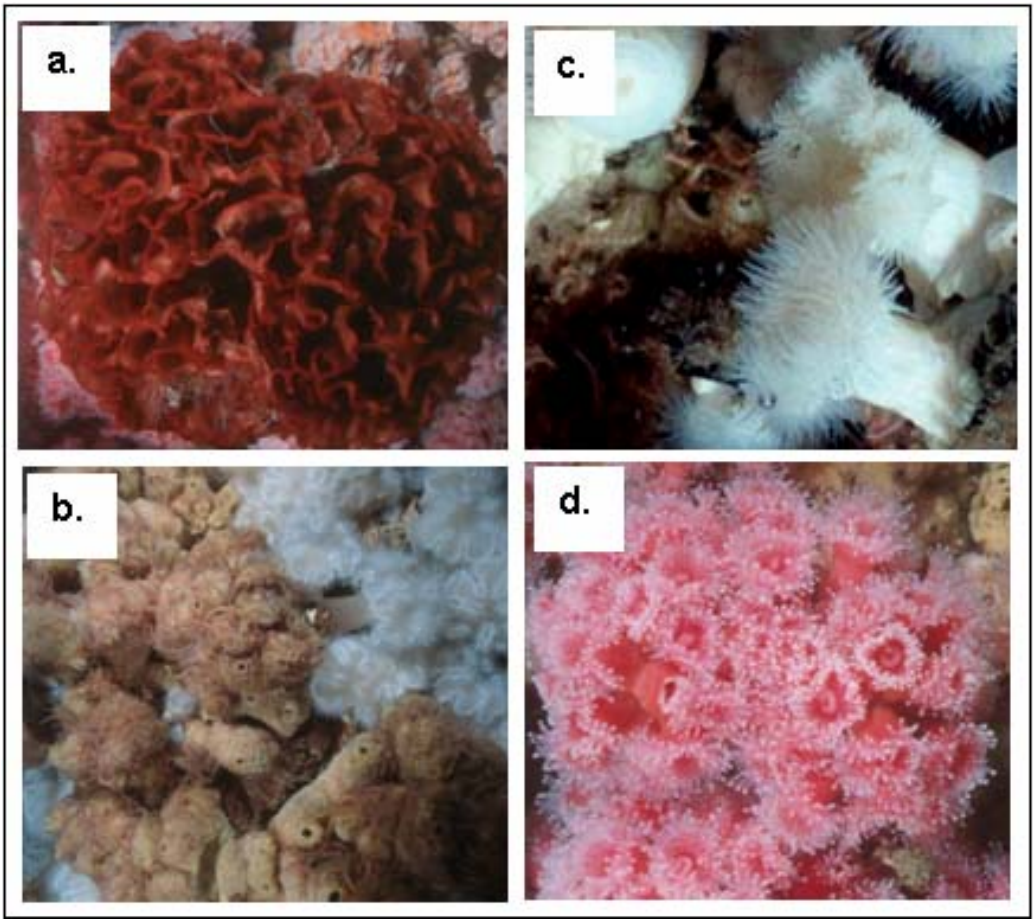


Figure 4. Examples of a) the encrusting bryozoan, *Watersipora subtorquata*, b) sponge, *Halichondria panacea*, and the anemones, c) *Metridium sp.* and d) *Corynactis californicus*.

The structure of invertebrate assemblages varied significantly among platforms ($P<0.001$, $F=13.729$, df=120, 1082.43, MANOVA). Anemones occurred in higher cover overall (up to 50 to 60%) than most other invertebrates, but the dominant species varied with location (Fig. 5). *Corynactis californicnus* was the dominant anemone on platforms at the southeast end of the channel (e.g., Gina, 59 ± 18%, mean ±1SD); cover of this anemone tended to be lower on platforms to the northwest (e.g., 5 ± 2% at Holly). An exception to this pattern occurred at Gail where mean cover of C. californicus was only 7 ± 5% and the most abundant anemone was the exotic species, *Diadumene* sp. (26±7%). In contrast, mean cover of *Metridium* sp. was generally highest at the most northwest platforms (Holly, 51 ± 13) and lower on platforms to the southeast (Gina, 3 ± 2%) (Fig. 5). An exception to this pattern was evident at Hogan where cover of *Metridium* was only 2 ± 1%.

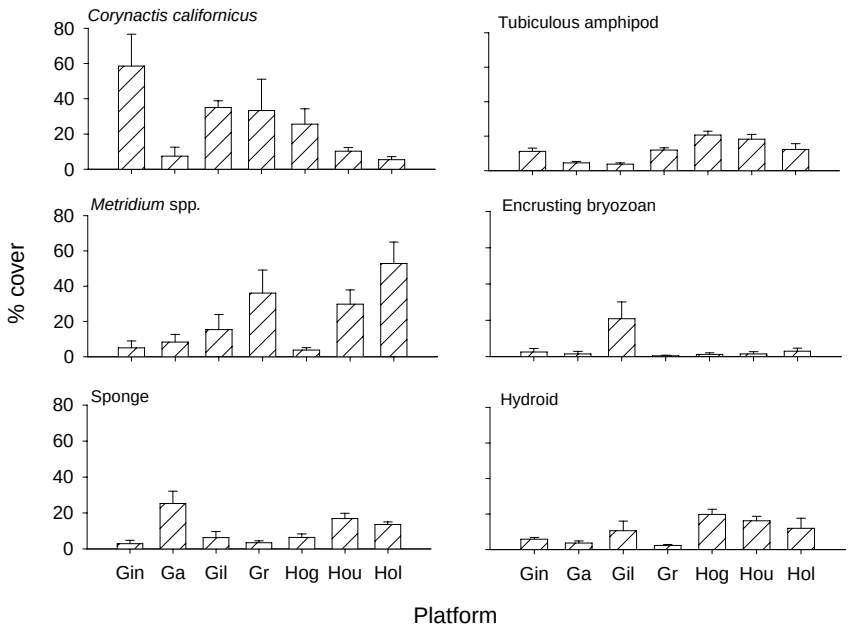


Figure 5. Distribution and abundance of the anemones, Corynactis californicus and Metridium sp., sponges, tubiculous amphipods, mussels (Mytilus spp.), and hydroids among study platforms. Data composited for each leg or conductor pipe from photoplots taken inside and outside and across depths of 6, 12, 18, and 24 m.

Tubiculous amphipods, hydroids, and mussels also generally occurred in higher cover on platforms with increasing distance along the channel from the southeast to the northwest (Fig. 5). For example, tubiculous amphipods occurred at 15 to 20% cover on Hogan and Houchin, but <5% on Gail and Gilda. In contrast, the cover of sponges was more variable, with highest cover at Gail (up to 35%) and the two most northerly platforms (Houchin, Holly). The bryozoan, *Watersipora subtorquata*, occurred only on Gilda with mean cover of 21%.

Assemblage patterns

Discriminant Function Analysis (DFA) revealed that the assemblages of Gail and Gilda were clearly different from the other platforms, a pattern that can be attributed, in part, to the presence of conspicuous exotic species on these platforms (Fig. 6a). Canonical Discriminant Functions (CDF) 1 and 2 explained 80% of the variation in the data. Cover of the anemone, Diadumene sp. was positively correlated ($P<0.05$, 0.482) with CDF1, and an important source of the separation of Gail from the other platforms along the CDF1 axis. The significant negative correlation of cover of the bryozoan, *Watersipora subtorquata*, with CDF2 (-0.379) for Gilda contributed to the separation of this platform from the others (Fig. 6a).

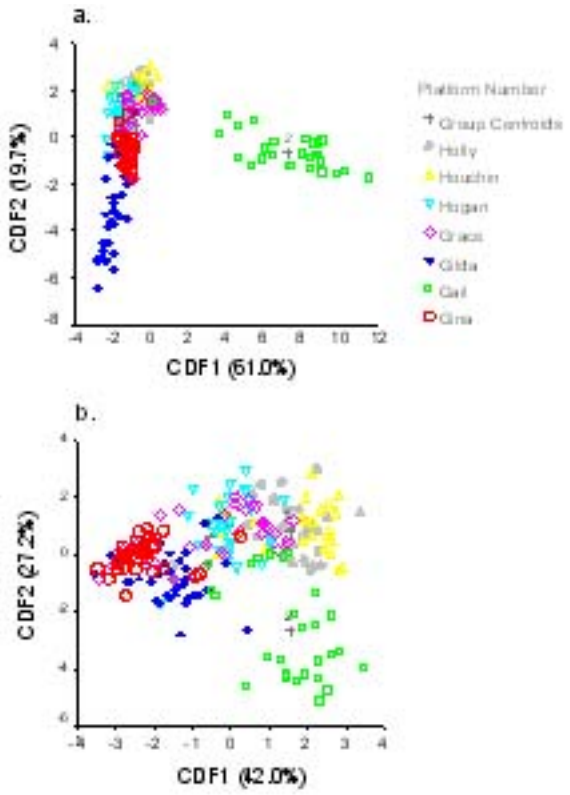


Figure 6. Results of Canonical Discriminant Function Analysis of invertebrate communities on the seven study platforms: a) all species, b) exotics species excluded. Each point represents one leg or conductor pipe.

To explore the effect that inclusion of the exotic species might have on the patterns observed with DFA, we repeated the DFA, but excluded *Diadumene sp.* and *Watersipora subtorquata* from the analysis (Fig. 6b). Removal of *W. subtorquata* from the analysis reduced variability in the Gilda data and assemblage patterns at this platform tended to become more similar to that of Gina and Grace. In contrast, the structure of the invertebrate assemblage at Gail remained distinct from the other platforms (Fig. 6b). The significant positive correlation of sponges (0.584) and negative correlation of *Corynactis californicus* (-0.614) with CDF1 contributed to the separation of all platforms except Gail along the CFD1 axis. In contrast, the positive correlation of cover of *Metridium sp* (0.605) and negative correlation of hydroids (-0.428) with CDF2 contributed to the separation of Gail from the other platforms along the CDF2 axis.

Assemblage patterns and environmental variables

To explore relationships between assemblage patterns and environmental variables, we used the calculated values of CDF1 for each platform (excluding *Diadumene sp.* and *Watersipora subtorquata*), and the independent variables of location along the channel, water depth, proximity to shore, and platform size (Table 1) in backward stepwise multiple regression analysis. Prior to this analysis, we tested for co-linearity among the independent variables. There was a significant correlation between platform size and both water depth ($P<0.001$, $r =$

0.974) and proximity to shore (P = 0.049, r=0.758). However, depth and proximity to shore were not significantly correlated (P>0.10). Therefore, we excluded platform size from the analysis, but included water depth and distance from shore. There was no relationship (P>0.10) between variation in CDF1 and any of the independent variables if the data from Gail were included in the analysis. With data from Gail excluded from the analysis, variation in CDF1 was significantly related only to location along the channel (P=0.014, t=4.203) (Fig. 7).

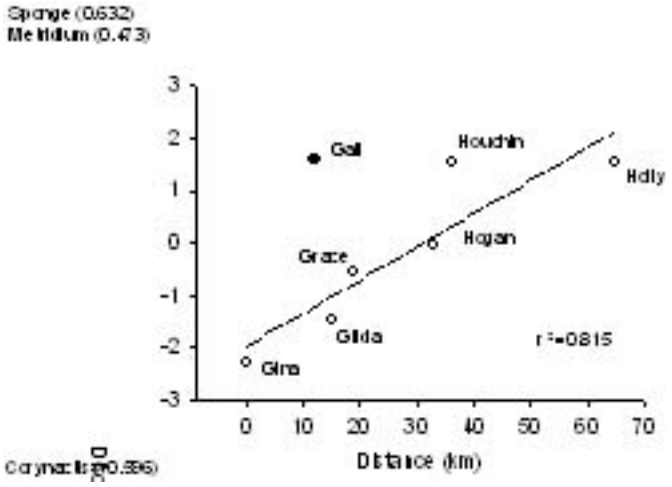


Figure 7. Relationship between canonical discriminant function 1 and location of platforms along the Santa Barbara Channel. The r2 value was calculated excluding the data from Platform Gail. Taxa most positively or negatively correlated with CDF1 are also shown on the y-axis.

Barnacle recruitment

Three species of barnacles recruited to the plates deployed at the platforms: *Balanus trigonus*, *Megabalanus californicus* and *Balanus regalis* (Fig. 8). Recruitment of *Balanus trigonus*, which occurred during the summer and fall, was highest on plates at the two most southeasterly platforms (Gina, Gail), declining with distance towards the more northwesterly platforms. Recruitment of *M. californicus* occurred during the fall and spring. During the fall, there was no apparent gradient in recruitment along the SBC, whereas during the spring, recruitment of this species and *B. regalis* occurred primarily at the most southeasterly platform (Gina) with markedly lower recruitment onto plates deployed at the other platforms (Fig. 8).

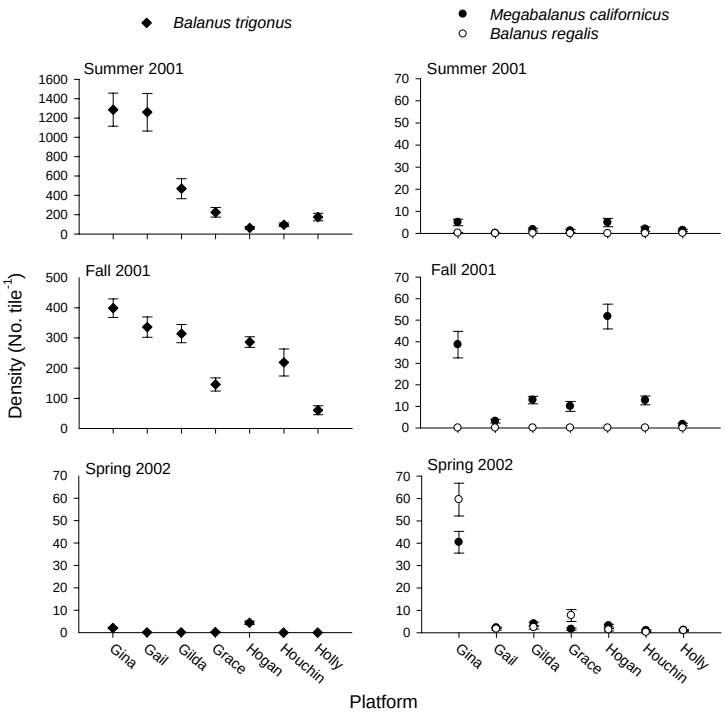


Figure 8. Recruitment of barnacles, *Balanus trigonus*, *Megabalanus californicus*, and *B. regalis* onto ceramic tiles deployed at a depth of 15 m for approximately 3 months at each platform. n=4 per platform. Mean values ±1SE.

Mussel growth

Growth rate of *Mytilus galloprovincialis* was most rapid during the summer and at the southeasterly platforms, declining with distance towards the more northwesterly platforms (Fig. 9). In general, mussels grew more slowly in the fall and spring and without the pronounced spatial pattern along the channel present in the Summer. There was a significant correlation between mussel growth and location in the channel during the summer ($r2 = 0.60$, $p < 0.05$), but not during any of the other seasons. During the summer, mussel growth was also correlated with degree-days ($p<0.01$, $r2=0.81$, $df=5$). However, there was no correlation between mussel growth and distance from shore or water depth during any time of the year.

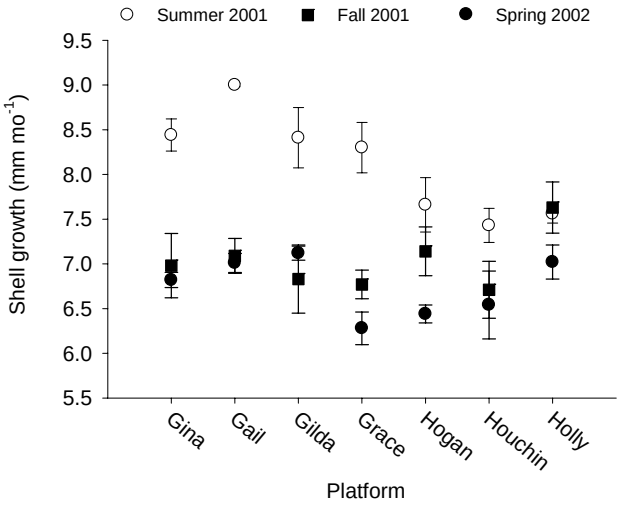


Figure 9. Growth rate of mussels, *Mytilus galloprovincialis*, in cages deployed at a depth of 15 m for approximately 3 months at each platform. Mean initial shell-length of mussels was ~30 mm. n= 4 cages of 10 mussels at each platform. Mean values ±1SE.

Discussion

Although the major macroinvertebrate taxa (e.g., sea anemones, mussels, barnacles, tubiculous amphipods, hydroids and sponges) were common to all platforms, the relative abundance of these taxa (as percent cover) varied along the SBC such that platforms in close proximity to one another tended to have invertebrate assemblages more similar to each other than to platforms located further away. Notably, there was a general decrease in the cover of the anemone, *Corynactis californicus*, and increase in cover of *Metridium sp.*, tubiculous amphipods, hydroids and sponges (at least to a depth of 24 m) on platforms from the southeast to the northwest along the channel. However, there were exceptions to this general gradient pattern. For example, the cover of sponge was highest (23%) at Platform Gail, one of the southern platforms and cover of *Metridium* was low (3%) at Hogan, one of the northern platforms. Further, conspicuous exotic species were present in high cover on two of the platforms, the anemone, *Diadumene* sp. on Gail and the encrusting byrozoan, *Watersipora subtorquata* on Gilda.

We propose that along-channel variation in platform invertebrate assemblages result, in part, from regional oceanographic gradients created through the advection of waters into the channel from the south via the Inshore Counter Current and Southern California Eddy during the spring and summer. Along-channel variation in water temperature measured at the platforms during this study was consistent with the temperature patterns reported by Otero and Siegel (2004) from the compilation of monthly satellite SST data covering the period 1997 to 2001. The anemone, *Metridium* sp. (probably *M. senile*) appears to prefer cooler waters with a distribution range extending from southern California to Alaska (Morris et al. 1983), which may explain the higher density of this species on the northern platforms.

Current flow from the south also provides a mechanism for the transport of invertebrate larvae

into the channel. The higher recruitment densities for three species of barnacles at the southern compared to the northern platforms was consistent with predictions of oceanographic conditions bringing warm water masses and the longer-lived planktonic larvae of southern taxa, such as *Balanus trigonus* (Werner 1967) into the channel. In general, regional patterns of current flow would interact with the location of source populations to influence the recruitment of species with longer-lived planktonic larvae to platforms in the SBC.

Local physical and biological factors also play an important role in structuring platform assemblages. For example, disturbance from wave action or platform cleaning operations, which occurs mainly in the upper 6 m (Page et al. 1999), removes large masses of attached mussels and other invertebrates, opening primary space for colonization by other species (Wolfson et al. 1979, Page et al. 1999). At present, the variation in physical disturbance or cleaning regime among our study platforms is not known. The invertebrate assemblage may develop to a greater thickness on platforms closer to shore in shallower water compared with platforms farther from shore in deeper water (MBC 1987). However, proximity to shore/water depth, were not satisfactory predictors of encrusting invertebrate assemblage patterns at our study platforms.

Platform assemblages are probably strongly influenced by founder effects, whereby initial colonists that arrive by chance through recruitment from the plankton (or human introduction), and are superior competitors for space, persist in the assemblage over time. This supposition is supported by the presence and high cover of the exotic species, *Diadumene sp*. and *Watersipora subtorquata*, each on a different platform. These species were probably introduced via barges or crewboats, have limited dispersal ability, but occur in high cover and appear to be superior competitors for space (Page et al. 2006). Once established, species that reproduce asexually (anemones, sponges), or have short larval lifespans (bryozoans, hydroids), or crawl away juveniles (amphipods) would be "self-seeding" and less dependent on the vagaries of current flow for recruitment. In addition to founder effects, biological interactions, including predation and competition undoubtedly influence the structure of platform invertebrate assemblages. However, we have no information on whether the strength of these biological interactions varies among platforms.

The taxa sampled photographically comprised a mixture of those species attached to primary space (directly to platform support member or conductor pipe) and those attached to secondary space (other encrusting invertebrates). The subtidal portions of the platform structure are almost entirely covered by invertebrates. Those species that can recruit to secondary substratum, such as the shells of mussels or encrusting bivalves (hydroids, barnacles, anemones), would be favored over those species that recruit poorly to these surfaces (some encrusting bivalves, pers. obs.).

Growth rates of caged *Mytilus galloprovincialis* also varied along the SBC during the summer, with highest growth rates at the southeastern platforms. This pattern coincided with the temperature gradient that typically develops from the east to the west end of channel during the summer. However, mussel growth was not associated with water temperature during the spring (cool temperatures) or fall (warm temperatures) or for data grouped across the three seasons. In addition, median water temperature (12.6 - 15.8ºC) and degree days

(1364 - 1580) varied considerably among seasons at the one location (Holly) where mussel growth varied little. Thus, gradients in water temperature per se did not satisfactorially explain spatial and temporal variation of mussel growth in the channel.

Alternatively, spatial and temporal variation in mussel growth rate may reflect food availability, or the interaction of food availability and water temperature. Temporal varation in the growth rate of *Mytilus galloprovincialis* correlated with chlorophyll a and particulate organic carbon (POC) concentrations at Platform Holly, after incorporation of a 3-week time lag (Page and Hubbard, 1987). In the present study, mussel growth rates at Holly varied less than at the other platforms. This platform is closest of the study platforms to Point Conception where upwelled waters first enter the channel and thus could be in a location that experiences episodic pulses of food throughout the year without sustained periods of low food availability (Page and Hubbard, 1987).

The existence of along-channel patterns in the composition of platform assemblages, and in invertebrate recruitment and growth, suggests that assemblages attached to platforms may be useful as barometers of short and long-term change in oceanographic climate. Over the short-term, changes in oceanographic conditions (e.g., El Nino or La Nina conditions) may influence invertebrate recruitment and growth; we observed lower invertebrate recruitment on settlement plates attached to Platform Houchin associated with the La Nina of 1999 compared with the following year (Bram et al. 2005). Over the longer-term, changes in climate that alter ocean currents (e.g. ENSO events, global warming) may shift the composition of platform invertebrate assemblages. Longer-term monitoring of platform invertebrates would permit an evalution of the concept that biogeographic transition zones, such as the Santa Barbara Channel, are particularly susceptible to shifts in the composition of marine species driven by climate fluctuations.

Our results indicate that invertebrates, such as sponges, tunicates, and bryozoans, that may contain potentially useful marine natural products can be abundant on offshore oil platforms. However, the significant variation found in the distribution, recruitment and growth of these invertebrates among platforms in the SBC suggests that factors such as location and temperature could affect the potential harvest of these organisms for use in the development of marine natural products.

CHAPTER 2. Pharmacology of selected organisms of offshore oil platforms

Robert J. Jacobs, Leslie Wilson, Claudia Moya, Dainel Day (authors only).

Background

Marine organisms that inhabit the subtidal structures of offshore oil production platforms are a potential source of novel compounds for pharmaceutical use. These organisms provide an unparalleled opportunity to study natural product chemistry from populations of organisms living in ecologically unique habitats. Research has shown that growth rates of certain invertebrate species living in the platform community can be quite high (Page, 1986; Page and Hubbard, 1987). As well, the platform community supports many encrusting and soft-bodied organisms, which rely on rapid growth rates, alleopathic effects, and chemical warfare to compete for space in the habitat and avoid predation. Such habitat characteristics (as in the example of coral reefs) have been shown to sustain many organisms that produce compounds with potential pharmaceutical application (Look et al, 1986; Jacobson and Jacobs, 1992, 1992). This project focused on the study of natural products from sea anemones, marine alga, and bryozoa living on the intertidal and subtidal portions of offshore oil production platforms in the Santa Barbara Channel.

Objectives

The goals of this project are to: 1) collect and identify all genus and species of organisms from the OCS platforms, 2) test crude extracts for biological activity and 3) isolate and characterize extracts from these organisms for any compounds which possess biological activity.

Descriptions of Taxa Investigated

Anthozoans

Sea anemones are sessile, soft-bodied members of the phylum Cnideria and class Anthozoa. In coastal Santa Barbara, they inhabit shallow intertidal zone areas, as well as the intertidal and subtidal zones of oil rigs and pier pilings. Sea anemones have been a source of interesting natural toxins including neurotoxic and cardiotoxic peptides (Bruhn et al. 2000), and cytolysins (Pederzolli et al. 2001). Most sea anemones contain symbiotic dinoflagellates in their gastroepidermal tissue. These symbionts aid in nourishing the anemone by translocating lipids to the host. Recent discoveries in our laboratory have shown that dinoflagellate symbionts have biosynthetic capabilities to produce bioactive natural products.
Several genera of anemones inhabit the OCS oil platforms. Anthropleura, a symbiont-containing anemone, is found on several of the platforms. The asymbiotic anemones such as *Epiactus irregularis*, and *Corynactis californica* are also found on several platforms while *Diadumene* sp. is found solely on platform Gail.

Chlorophytes

One group of natural products we focused on were the coumarins. The coumarins, members of a large family of structurally related compounds that occur widely in terrestrial plants, have only recently been observed in marine algae. Menzel et al (1983) first discovered the presence of 3,6,7-trihydroxycoumarin in the green algae, *Dasycladus vermicularis*. The genus *Dasycladus* belongs to the order Dasycladales, a group of primitive marine unicellular green algae that are members of the kingdom Protoctista (Bonotto, 1988; Floyd and O'Kelly, 1990). We have successfully isolated semi-pure extracts containing the 3,6,7-trihydroxycoumarin compound in *Batophora oerstedii*, a species of the same family indigenous to the gulf coast. *Batophora oerstedii* has also been found to grow abundantly on several oilrigs in the Gulf of Mexico. In view of the relative simplicity of the coumarin molecules thus far reported in green algae and our recent discovery that these compounds inhibit mitosis, suppress microtubule dynamics, and potentiate taxol. We invested a significant portion of our effort to define the mode of action of dicoumarol, a model pharmacophore, at the molecular level as a prelude screening for new species of courmarins in marine algae.

Bryozoa

The bryozoan, *Watersipora subtorquata,* is an exotic species of fouling invertebrate that occurred on only one of the study platforms. This species was collected from Platform Gilda located approximately 9 miles off of the Ventura coastline. Divers reported an overwhelming dominance over other nearby sessile organisms suggesting the ability to somehow outcompete other organisms for the substrate. Also interesting was the colonial size which was achieved presumably by the high currents produced in the channel. Upon disturbance the organism was known to release a dark red pigment into its surroundings. This pigment would readily stain dive suits, clothing, and other equipment which made it unique to the collection team. Subsequent bioassay guided fractionation geared towards identifying potential mitotic spindle poisons revealed a potent compound with very interesting chemical properties. Included in these unique properties were the rare chromophore of the compound (red) and the proposed mode of release in nature. Further evidence suggests that this animal utilizes the compound as an allelopathic agent to inhibit growth of invasive species. It should also be noted that organic soluble red pigments are quite uncommon among marine species.

Study Results

Anthozoa

Extracts of the sea anemone *Diadumene* sp. were prepared and tested in the sea urchin embryo assay. The crude organic extract was active in inhibiting the first cleavage of sea urchin embryos division in a concentration dependant manner with 50% inhibition occurring at approximately 118 μg/ml (Fig. 1). The organic extract was also examined for the ability to inhibit proliferation in the human lung cancer cell line, A549, and found to be highly active. The extract inhibited cell proliferation in a concentration dependant manner with 50% inhibition occurring at 23 μg/ml (Fig. 2).

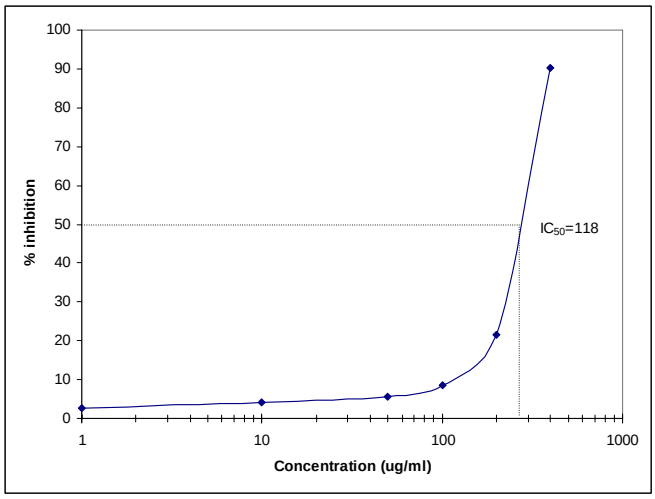


Figure 1. Concentration dependent inhibition of sea urchin embryo division by *Diadumene* sp.extract.

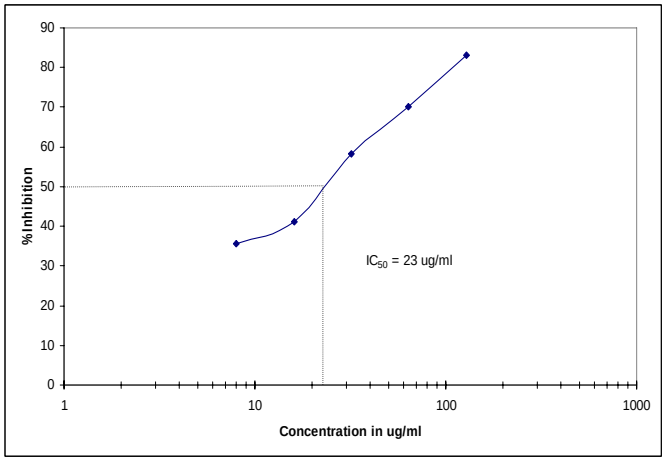


Figure 2. Concentration dependent inhibition of A549 lung cancer cell proliferation by *Diadumene* sp extract.

Chlorophytes

In studies on the anti-mitotic actions of coumarin compounds, we initially discovered that dicoumarol (a coumarin anticoagulant chemically designated as 3,3'-methylenebis[4-hydroxycoumarin]) inhibits the first cleavage of *S. purpuratus* (sea urchin) embryos in a concentration dependent manner, with 50% inhibition occurring at approximately 10 μM drug (Fig. 3).

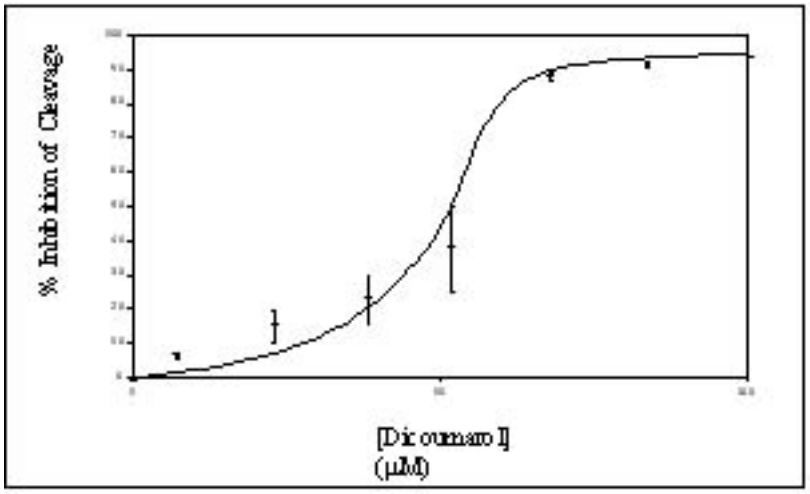


Figure 3. Concentration dependent inhibition curve of sea urchin embryo division by Dicoumarol.

Because the sea urchin embryo model assay is highly selective for agents that are targeted to tubulin and microtubules, we reasoned that the active compounds might inhibit cell division by interfering with the polymerization or dynamics of the mitotic spindles. By video microscopy, we did indeed find that dicoumarol (1 μM) suppresses the growing and shortening dynamics of bovine brain microtubules at plus ends in vitro at concentrations substantially below those required to perturb microtubule polymer mass, an action typical of a number of important antimitotic anticancer drugs including taxol and vincristine. Dicoumarol reduced the rate and extent of shortening, it increased the percentage of time the microtubules spent in an attenuated (paused) state, and it reduced the overall dynamicity of the microtubules. Using fluorescent spectroscopy, we determined that dicoumarol binds directly to tubulin dimers in vitro with a moderately high affinity (Kd, 23 μM). These results suggest that coumarins, such as dicoumarol, possess a unique anti-proliferative mechanism of action and possibly related pharmacophores are mediated by tubulin binding and the kinetic stabilization of spindle microtubule dynamics. We believe the coumarin pharmacophore may represent an attractive one for development of new drugs for cancer treatment. We recently demonstrated that taxol inhibition of sea urchin embryo division is potentiated by low concentrations of dicoumarol (Fig. 4).

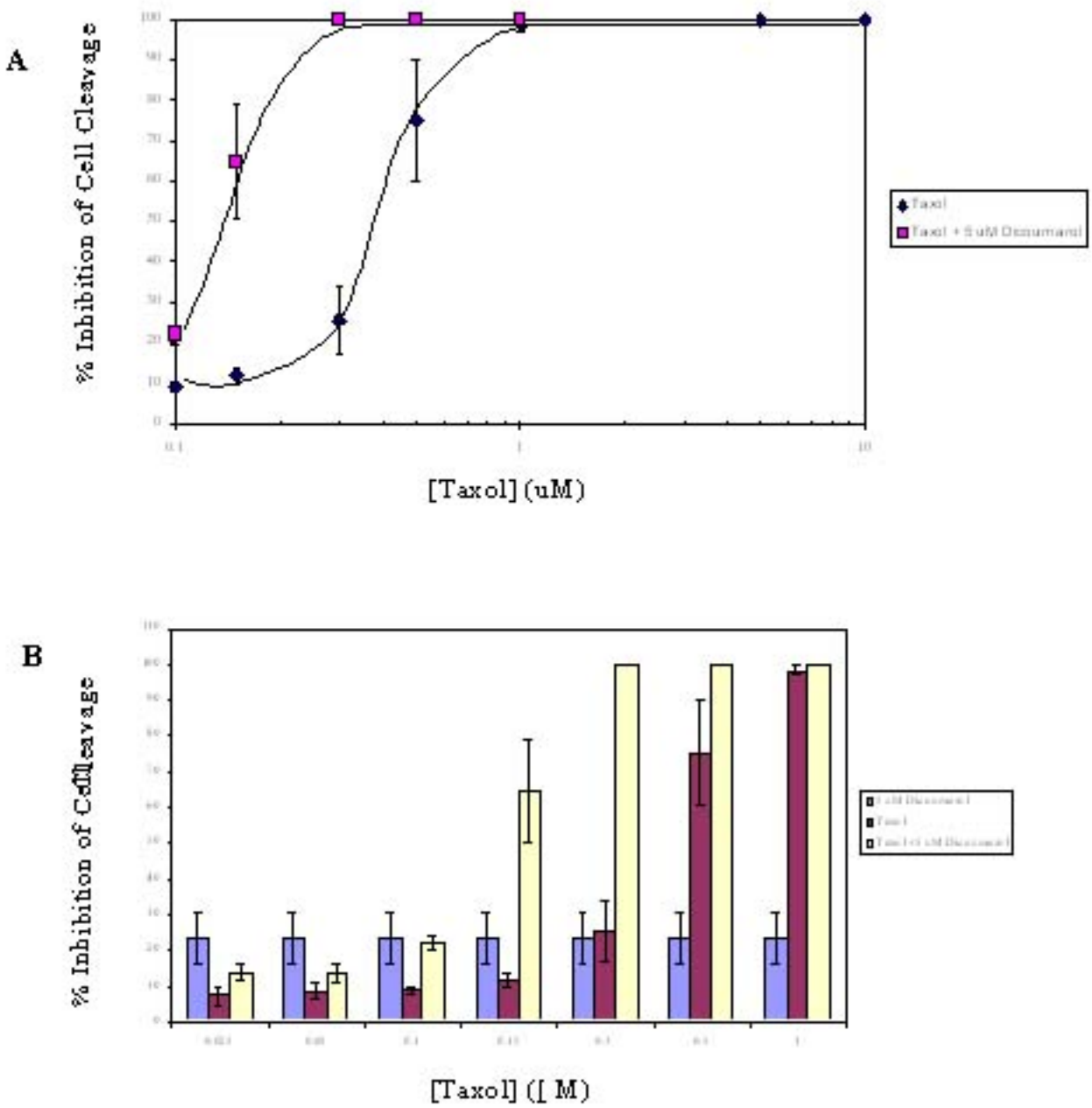


Figure 4. Synergy between Taxol and dicoumarol. The combination of Taxol with dicoumarol decreases the concentration of Taxol required to induce 50% inhibition of cell proliferation in the sea urchin cell division assay. The results are expressed as the percentage of inhibition compared with vehicle; bars, SE.

Bryozoa- *Watersipora subtorquata* (Compound WC01-A)

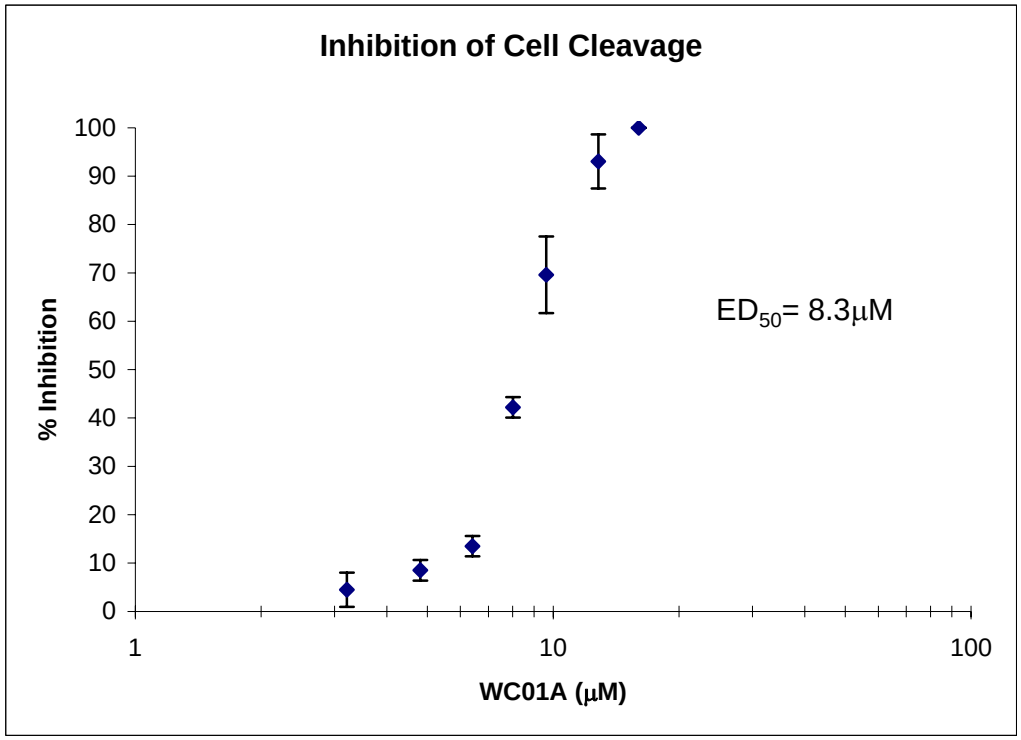


Figure 5. Inhibition of 1st Cell Cleavage in *S. purpuratus* by WC01-A extract from *Watersipora subtorquata.*

The bioactivity assay of purified compound using Sea Urchin Embryo Model indicated that WC01-A inhibits the first cleavage of *S. purpuratus* (sea urchin) embryos in a concentration dependent manner, with 50% inhibition occurring at approximately 8.3 μM drug (Fig. 5).

Initial organic extraction with traditional organic solvents yielded less than 1% yield of the red organic soluble pigment. It seemed that much of the pigment was remaining insolublized in the non organic soluble material. Subsequently an SDS-PAGE was performed to determine if pigment remained with proteins (Fig. 6).

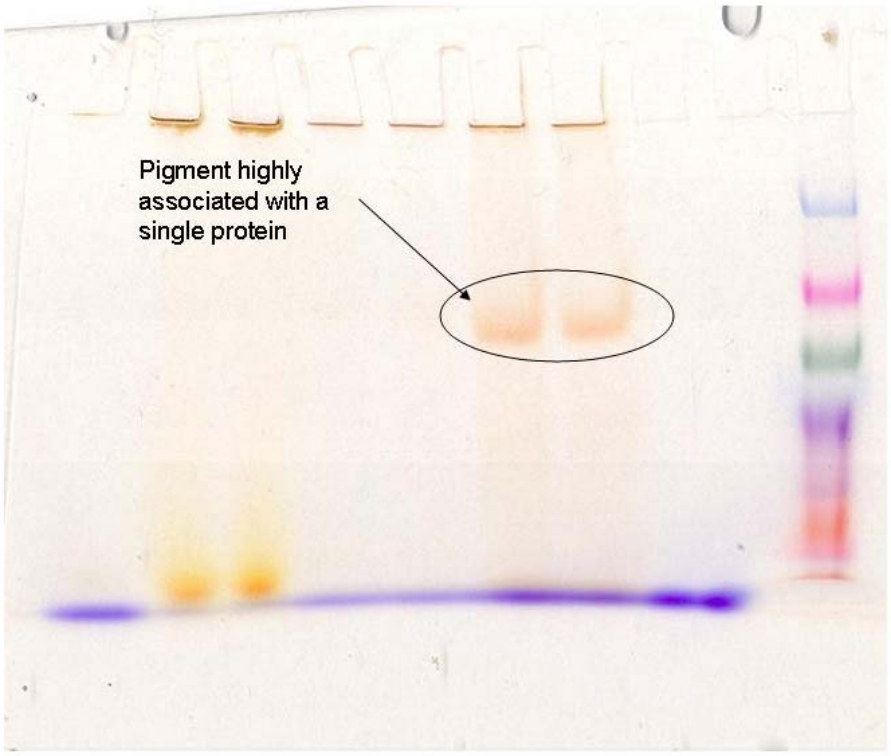


Figure 6. SDS PAGE (Unstained) for WC01-A.

The pigment has been shown to have specific binding to an individual protein at 78kDa (Fig. 7). The association of the pigment to a single protein is very unique in that most molecules with the ability to attach to a protein are generally ubiquitous in most systems (ie. they are generally attached to all proteins present; nonspecific). This binding presumably also allows for the compound to be water soluble in its native environment.

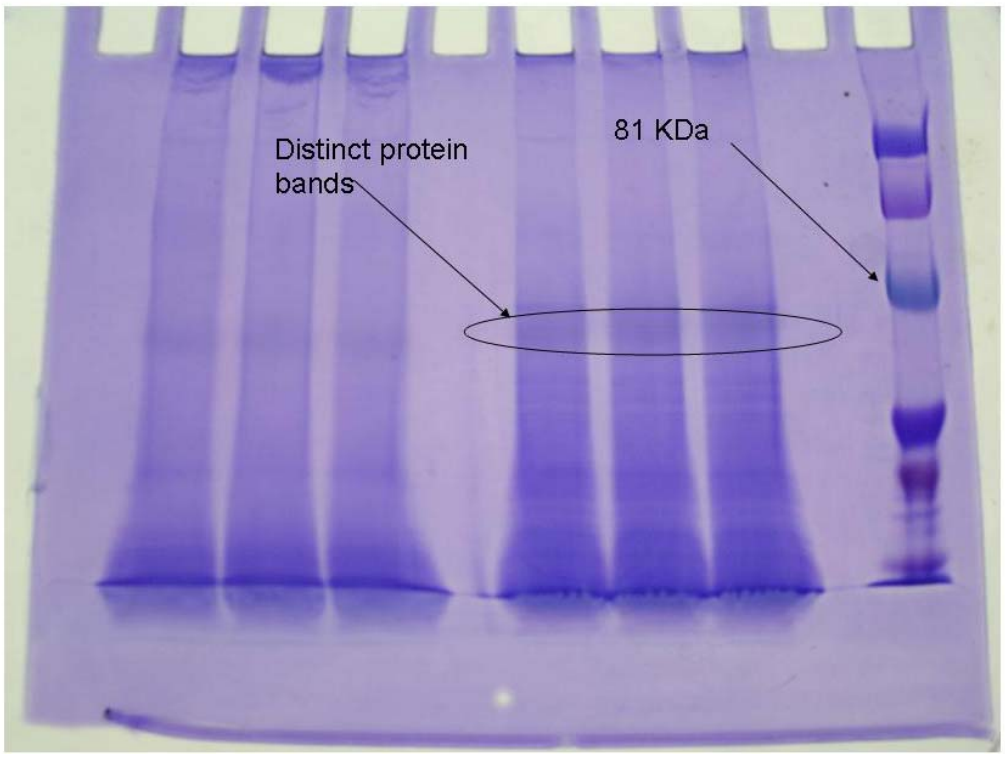


Figure 7. SDS PAGE (Coomassie Stain) 78kDa associated protein for WC01-A.

Figure 8 Preparative Thin Layer Chromatography for WC01-A from *Watersipora subtorquata*.

The organic crude extract is purified by preparative TLC (Fig 8) Note the extreme intensity of the red chromophore which is unusual in marine species (543nm chromophore)

Separation by NP/RP/Ion Pairing HPLC yielded poor results with very low resolution. Most solvent systems produce smeared wide banding. It is also noteworthy that the compound is highly reactive which may also contribute to possible breakdown on silica or other chromatographic substrates.

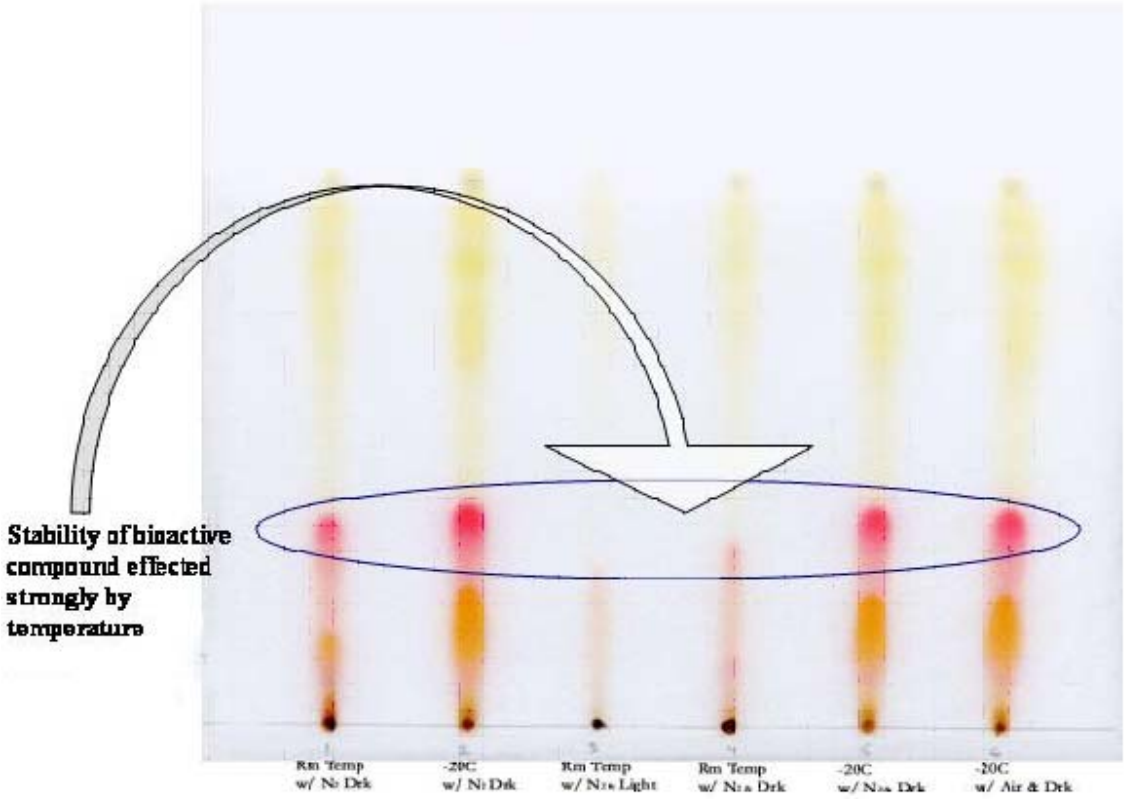


Figure 9. Stability Experiment with extract from *Watersipora subtorquata*.

The largest difficulty faced in the bryozoa study has been the instability of the natural product. This difficulty has made it very challenging to obtain pure quantities of the natural product for NMR analysis. The typical 2D HMBC NMR has relative sensitivity ~0.1%. Therefore time required to obtain data is directly proportional to the amount of analyte available. Since the analyte is relatively unstable it is quite difficult to achieve large quantities for analysis and has been a major point of difficulty in obtaining structure. Early in the process of bioassay guided fractionation, questionable stability began to be evident. (Fig 9) Stability experiments performed with variables such as light, temperature, and N2 (g). The experiment showed that qualitatively via TLC after 14 days that the compound was affected most predominantly by temperature. Note that all conditions resulted in decomposition of the

compound. An increase in temperature only increased this effect.

Infrared spectra for the compound confirmed a sulfur conjugation but did not show characteristic aromaticity which should also be present in the sample. The presence of hydroxyl groups was also evident on the IR spectra.

^{1}H Assignments (DMSO) Proton			^{13}C Assignments (DMSO) Carbon	
#	d	multiplet	#	
1	6.72	d	1	107.17
2	6.77	d	2	108.4
3	7.57	dd	3	109.67
4	7.63	d	4	110.05
5	9.36	d	5	113.37
6	13.75	s	6	116.86
7	14.49	s	7	131.68
			8	132.14
			9	134.58
			10	139.91
			11	161.73
			12	163.18
			13	168.28
			14	184.59
			15	184.93

Figure 10. NMR Peak list for WC01-A.

The proton and carbon NMR peak lists (Figure 11) show the compound to have few protons with high conjugation. The bioactive compound (WC01-A) was shown to have composition as follows: Carbon = 70.06%, Hydrogen = 7.25%, Nitrogen = <.05%, Sulfur = 3.8%, Oxygen = 19.35%. Ultra-Violet Data in isopropyl alcohol gives data as follows; 204nm (1.5);247nm (1.1); 543nm (.6) The accurate mass of the compound using negative ion electrospray mass spectroscopy gave an accurate mass of for the m/z [-H] = 311.0000; The molecular formula has been confirmed by a calculated mass of C16H7O5S = 311.0014.

Conclusions

Evidence at this time indicates that the red pigment (WC01-A) is a compound which has not been previously described before in the literature. The compound shows a high degree of bioactivity in the sea urchin embryo assay and in other human cell lines (not shown (L. Wilson) which makes it a viable candidate for further cell division studies. Further analytical study of derivatives may provide key information in the characterization of the complete compound.

CHAPTER 3. Genetic diversity of mtDNA in *Bugula neritina* reveals a new cryptic species.

Scott A. Hodges

Abstract

Two cryptic species have previously been identified in the morphological species, *Bugula neritina*, and these species differ in whether they associate with a bacterium, *Endobugula sertula*, that produces bryostatin compounds. We sought to determine if there were further cryptic species within *Bugula neritina* by sequencing larger and more variable regions of mtDNA. Using an approximately 1550 bp mtDNA region between the 16s and COI genes, we identified a new cryptic species that is very closely related to the cryptic species that associates with *E. sertula*. We also surveyed both natural reefs and two OCS oil platforms for the types of *B. neritina* they harbored. Very little population structure was identified though samples from the two oil platforms were members of a single subclade suggesting limited dispersal. The new cryptic species was rare in our samples and was only obtained from Santa Cruz Island.

Introduction

Knowledge of the population genetics of marine organisms will be imperative for developing marine biotechnology. Genetic markers allow the accurate identification of species, the determination of genetic diversity, both within and between populations, the determination of the degree of gene flow among populations and the identification of processes such as hybridization. Each of these aspects of population genetics could be important for the successful development of marine biotechnology. The development of marine biotechnology is not only dependent on the identification of useful compounds that organisms produce, but also on the accurate identification of the organisms that produce them. Without accurate identification, sampling of individuals may result in variable yields of target compounds or worse, no yield at all. It has become increasingly clear that molecular-genetic markers can be extremely useful for the identification of taxa of marine organisms (Knowlton 2000, Palumbi 1994). Using molecular markers, the identification of cryptic marine sibling species (species indistinguishable by morphology) has increased and the general conclusion has been that the current systematic treatments of many groups is characterized by excessive ‘lumping’ rather than excessive ‘splitting’ (Knowlton 2000).

One example of why identifying cryptic species is important for marine biotechnology is the bryozoan, *Bugula neritina*. Though identified by morphological assessment as a single cosmopolitan species, mtDNA has revealed that *B. neritina* is actually at least two distinct species (Davidson and Haygood 1999). Furthermore, the anticancer drug candidate Bryostatin 1 is found in only one of these two species. This compound is produced by a symbiotic bacterium and the two species of *Bugula* each harbor a different strain of the bacterium (Davidson and Haygood 1999; Lopanik et al. 2006). The two species are separated by depth in natural populations with the species from deeper waters containing Bryostatin 1. Thus,

these species offer an excellent opportunity to begin assessing of the genetics of organisms with potential for biotechnology. Because both species of *Bugula* are found in southern California waters (Davidson and Haygood 1999) previously sequence the cytochrome c oxidase subunit 1 (CO I) gene of the mtDNA to identify the two species and determine their spatial distributions. The two species differ by 9.1% over 638 base pairs of this gene (Davidson and Haygood 1999). However, only very little sequence variation was found within either of the two types identified. We sought to obtain mtDNA sequence that would enable us to identify finer phylogentic divisions and thus new lineages that could contain novel bryostatin compounds.

Also important for developing marine biotechnology resources is the identification of how sources of materials can be obtained without damaging the environment (e.g., Devries and Beart 1995). Harvesting of organisms in sufficient quantities for applied uses could potentially have significant ecological impacts, particularly for those species inhabiting natural reefs (e.g., Schaufelberger et al. 1991; Thorpe et al. 2000). For instance, isolation of multigram quantities bryostatin 1 from *Bugula neritina* required approximately 10,000 gallons of wet animal (Schaufelberger et al. 1991). Thus, we also sought to determine if lineages of B. neritina that contain bryostatins inhabit OCS oil platforms and their likely source populations.

Methods

Sample collection

Samples of *Bugula neritina* were collected from both natural populations, from Santa Barbara County and from Catalina Island as well as from OCS oil platforms, Hogan and Houchin. Samples were brought to UCSB where they were sorted, identified and cleaned of any contaminating organisms.

DNA isolation and PCR amplification

DNA was isolated using standard Qiagen DNA prep kits and quantified. A subset of samples was first amplified for the COI gene using the primers and conditions described by Davidson & Haygood (1999). All PCR ampli cations were done in a total volume of 25 L, with 20 L Promega PCR Master Mix (2X: 50 units per mL of Taq polymerase, 400 M dATP, 400 M dGTP, 400 M dCTP, 400 M dTTP, 3 mM MgCl2), 3 L of forward and reverse primers (10 M each), and approximately 20 ng of DNA template. PCR products were then purified and used directly in simultaneous bi-directional sequencing following the protocol for the Thermosequenase kit (Amersham). Sequencing was conducted on a Li-Cor 4200 sequencer with 41 and 66 cm gels composed of 5.5 and 3.75% acrylamide, respectively.

Similar protocols were used to amplify and sequence the large fragment between 16s and the COI gene.

Sequence Analysis

Sequences from an individual were aligned and manually corrected using Sequencer 4.6. All sequences were then aligned using CLUSTAL and manually aligned. Sequences were searched using BLAST to identify genes.

Results

Cloning of a large mtDNA fragment

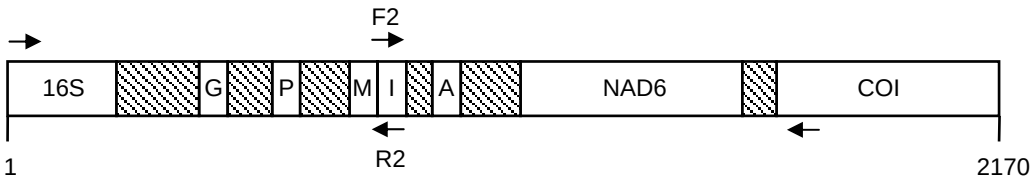
Direct sequencing of COI PCR products and sequence comparisons revealed members of the both the 'shallow' and 'deep' clades identified by Davidson and Haygood (1999). After identifying samples that belonged to the 'shallow' and 'deep' clades, we designed PCR primers at the 5' and 3'ends of the COI and 16s genes (Table 1).

Table 1. Primers used to amplify and sequence an approximately 1500 bp region of the mtDNA of *Bugula neritina*.

Primer Name	Primer Sequence 5'-3'
16s-forward	cttatcgacaagaaggcttgcgacctcgatgttg
F2	agtgaattaagctgatacctt
R2	aaggtatcagcttaattcact
COI-reverse	cctctaccaattatrccagctcatagtccaa

We then attempted PCR using all four combinations of the primers from these two genes to determine if they would produce a large PCR product. The COI-reverse and 16s-forward primers produced a PCR product of approximately 1550 bp from both 'shallow' and 'deep' clades. These PCR products were then sequenced. Sequence analysis revealed the NAD6 gene and a number of tRNA genes were contained within this sequence along with several intergenic regions (Fig. 1).

Figure 1. Inferred gene structure of a portion of mtDNA from *Bugula neritina*. Specific primers were designed to the 16S and COI genes (indicated by arrows) and then the intervening region was amplified using PCR from both a 'shallow' and a 'deep' type individual based on their COI sequence. The PCR product was approximately 1550 bp in length and sequencing followed by BLAST searches identified that it contained the NAD6 gene. We also identified tRNA genes for glycine (G), proline (P), methionine (M), isolucine (I) and arginine (A). Shaded boxed indicate non-coding sequences. Total length in base pairs is noted.



Sequence analysis of samples

We used the COI-reverse and 16s-forward primers to amplify the large mtDNA fragment from a wide range of samples including those from natural reefs as well as those from OCS oil platforms Hogan and Houchin. We sequenced these PCR products using the amplification primers and two internal primers (F2 and R2, Table 1, Figure 1) designed from our initial sequencing of both 'shallow' and 'deep' individuals of *Bugula neritina*. Sequence divergence between 'shallow' and 'deep' samples for this region were between 10 and 12%. Sequence divergence among members of the 'deep' lineage ranges from 0 to 2.2%. Phylogenetic analysis of these data revealed that there were two distinct clades within the 'deep' lineage, which we call 'Deep-1' and 'Deep-2'. A large majority of the 'deep' samples were members of the Deep-1 clade, only four individuals of the Deep-2 clade were found. All members of the Deep-2 clade were found from Santa Cruz Island. Within the Deep-1 clade there appeared to be some suggestion of structure. Notably, a clade with 62% support was found that included all samples from OCS oil platform Hogan and Houchin.

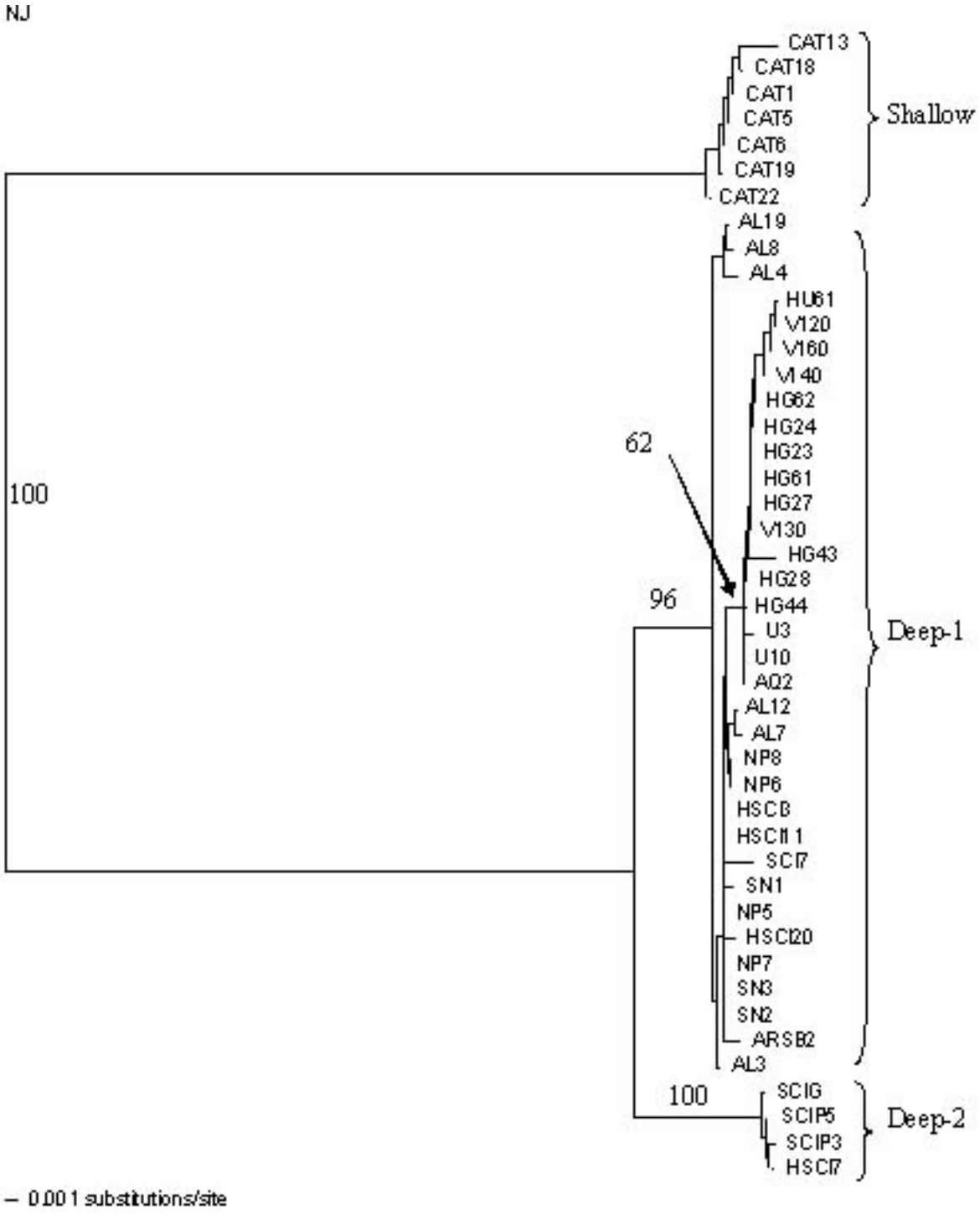


Figure 2. Neighbor-joining analysis of 45 individuals identified as *Bugula neritina*. Substantial sequence divergence was identified between 'shallow' and 'deep' samples. Among 'deep' samples, two highly supported clades were identified, Deep-1 and Deep-2. Numbers indicate bootstrap values (100 replicates). The clade containing samples from OCS oil platforms was supported at 62%.

Discussion

We identified a new cryptic species of by sequencing a large region of the mtDNA. *Bugula neritina*. Previously two major clades within the morphological species had been identified (Davidson & Haygood 1999) though little variation was found within either of these clades. We were able to identify a region of the mtDNA that was more than twice as large as that previously utilized and contained a number of intergenic regions, allowing us to identify a greater degree of genetic variation. Using this region we identified two distinct lineages within the previously identified 'deep' clade. Using the standard molecular clock of 1% divergence per million years, this suggests that these clades have been diverged for about 2 million years. Cryptic species may be particularly common in organisms that occupy habitats that impose strong constraints on morphology such as aquatic environments for plants (Knowlton 2000; Whittall et al. 2004). This study suggests that with additional refinement of molecular markers, many distinct lineages may likely be found. We only sampled a small range of the cosmopolitan *B. neritina* and thus many more cryptic species may exist.

Because the 'deep' clade of *Bugula neritina* is associated with the endosymbiont, *Endobugula sertula*, which produces bryostatins (Lopanik et al. 2006), the new cryptic species may contain novel bryostatins. Because the two clades of 'deep' *B. neritina* have likely been separated for 2 million years, it is quite possible that the endosymbiont has also diverged and thus produce novel compounds. This hypothesis depends on whether the association between *B. neritina* and *E. setula* occurred prior to the split between the two 'deep' clades or after. Samples of the new 'deep' clade need to be analyzed both for the presence of E. sertula and for the production of bryostatin compounds. Unfortunately, this clade was quite rare in our samples and analysis for bryostatin compounds was not feasible.

Our data also indicate that dispersal to OCS oil platforms may be a relatively rare event. All of the samples from platforms Hogan and Houchin clustered within a single subclade of Deep-1. These platforms are near each other and only 5 to 7 miles offshore. The closeness of the platforms to one another suggests that they may have received colonists from a similar source though other members of this clade are from a variety of natural reefs. The low sequence variation within this clade makes it impossible to make inferences on a particular source population.

We found no members of the 'shallow' clade of *Bugula neritina* on either OCS platform. This may have been due to sampling only at moderate to deep depths though Davidson and Haygood (1999) found members of this clade at depths up to 10 meters. The very high frequency of Deep-1 clade members on the platforms suggests that collections of *B. neritina* from these artificial habitats would contain high yields of bryostatins, undiluted from members of the Shallow clade that does not contain them.

ACKNOWLEDGEMENTS

Chapter 1
We thank J. Bram, M. Newnham, and A. Willis for assistance in the field and laboratory, and M. Nishimoto and D. Schroeder for discussion. We also thank Venoco, Inc., Pacific Operators Offshore, LLC., and Nuevo Energy for access to their platforms and the Minerals Management Service, US Department of the Interior, and the Coastal Marine Institute, University of California, Santa Barbara (UCSB) for support.

Chapter 2
We thank C. Culver, B. Mardian, M. Newnham, and M. Page for assistance with field collections of invertebrates for this study. We also thank F. Kuepper, J. Cooper, L. Delo, L. Hensic, M.A. Jordan, D. McLaren, H. Miller, C. Carlson and S. Goldberg for assistance with laboratory analyses and project development. We gratefully acknowledge Venoco, Inc., Pacific Operators Offshore, LLC., and Nuevo Energy for access to their platforms and the Minerals Management Service, US Department of the Interior, and the Coastal Marine Institute, University of California, Santa Barbara (UCSB) for support. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies either expressed or implied of the US government.

Chapter 3
I wish to thank Brian Counterman for assistance in the laboratory, and Douglas Bush, Deborah Kaska, Justen Whittall and Ji Yang for discussions. I thank Mark Page, Carrie Culver, Brent Mardian and Matt Kay for collection of samples and Greg Dosier for additional laboratory assistance. In particular, I thank the Minerals Management Service, US Department of the Interior, and the Coastal Marine Institute, University of California, Santa Barbara for financial and logistical support.

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The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

The Minerals Management Service Mission

As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Royalty Management Program** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.