Reports

Incidence of Marine Fungi in Relation to Wood-Borer Attack

The destruction of wood, when it is submerged continuously or intermittently in the sea, by isopod gribbles and teredine borers is well known. Various investigations have been made of the dynamics of attack and of the individual organisms involved. However, there is comparatively little information about the biological and physical agents that act upon the submerged wood substrate prior to the borer attack. One group of marine organisms that warrants serious consideration in this respect is that of the wood-inhabiting fungi, especially the Ascomycetes and the Fungi Imperfecti.

While our knowledge of marine fungi in general has increased considerably within the past 10 years, no intensive investigation has been made of the possible association of these fungi with the activity of wood-boring animals. However, it has been postulated that fungi may participate in a "conditioning" of the wood before attack by borers (*1*).

Since 1952, the Marine Laboratory of the University of Miami has been engaged in marine mycological studies (*2*), including numerous investigations of the distribution of fungi in Biscayne Bay, Florida, the Caribbean Sea, the Bahamas, the Gulf of Mexico, and at more than 63 stations throughout the United States, Canada, Alaska, Nova Scotia, Newfoundland, and the Canal Zone.

The collections and studies have involved different woods (primarily southern yellow pine and basswood), various seasons of exposure and lengths of submergence, and selected samplings within specific localities. The period of tests in boreal and northern temperate areas included the winter months, primarily from October through February, when borer activity is negligible and often completely absent. Hence, over this submergence period, with the factor of borer damage naturally limited, we have been able to examine the occurrence and extent of fungal infestation operating independently of marine borers. Two common gribbles, *Limnoria lignorum,* a boreal form, and *L. tripunctata,* an inhabitant of temperate areas, both have maximum activity at seasons of elevated temperature, usually beginning in the early spring (*3*). Similarly, the spawning of *Teredo navalis,* a widely distributed teredine borer, is activated at 11° or 12°C (*4*).

The following pertinent observations should be noted especially. (i) Fungal infestation of wood occurs at all our test localities, varying in intensity and in different genera and species involved. Species of *Lulworthia* and *Helicoma* are extremely abundant, colonizing wood at the majority of the stations. (ii) Vigorous attack upon submerged wood in boreal and northern temperate areas during winter months is accompanied by no, or very slight, borer damage. A similar situation occurs in subtropical localities, however, with a considerably shorter period of fungal attack prior to borer infestation. In Biscayne Bay, Florida, vigorous sporulation by ascomycetous fungi occurred on wood that had been submerged for approximately 2 to 3 weeks.

In addition to being manifested by the presence of surface and imbedded ascocarps and conidia, fungal infestation is also manifested through (i) softening and disintegration of the outer wood tissues, often to a depth of several millimeters, (ii) proliferation of the fungal hyphae throughout the wood, including ramification within the lumina of the tracheids and the wood rays, and (iii) direct penetration through the walls of the wood elements. In the latter process, a noticeable constriction of the hypha occurs as it passes through the cell wall, a condition common also among terrestrial wood-destroying fungi. Similar unsubmerged samples of yellow pine and basswood showed no fungal infestation.

Currently, it is not possible to evaluate completely the role of marine fungi in the deterioration of wood. However, the attack by these fungi upon the physical structure of wood is obvious. In our laboratory, pure culture studies of many marine genera indicate a definite growth-wise affinity for wood and wood products. Uniclonal attack upon wood, in standing and shaking sea-water aquaria, has been demonstrated repeatedly.

The vigorous fungal infestation of submerged wood prior to borer attack represents a biological phenomenon that investigators of marine wood destruction should not ignore. In northern areas, winter fungal infestation of wood is evident. Hence, in the early spring, when borer activity increases rapidly, the animals have available a wood substrate thoroughly infected by a variety of marine fungi. The interrelationships within this biota are being studied in our laboratory (*5*, *6*).

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5. These studies were supported by contract Nonr 1811(00), Microbiology Branch, Office of Naval Research. This article is contribution No. 188 from the Marine Laboratory, University of Miami.
6. A complete summarization of the fungal infestation at all our test localities is in preparation.

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Coumestrol, a New Estrogen Isolated from Forage Crops

Natural estrogens in plants have assumed considerable importance since the demonstration of their presence in forages and the suggestion of their possible beneficial effects on milk production (*1*) and meat production (*2*) and of their probable responsibility for the infertility in sheep grazing on such pastures (*3*). Included among the forages from which estrogens have previously been isolated are subterranean clover (*4*) and red clover (*5*). Other forages which have also been reported to be estrogenic, but from which no estrogens have been isolated to date, include alfalfa, *Medicago sativa*; ladino clover, *Trifolium repens*; strawberry clover, *Trifolium fragiferum*; orchard grass, *Dactylis glomerata*; rye grass, *Lolium perenne*; and blue grass, *Poa pratensis* (*6*).

All the estrogens previously isolated from forage crops have proved to be isoflavones. These include genistein, biochanin A, and formononetin (*7*). Genistein and daidzein in the form of their glucosides have also been isolated from soybean oil meal, a generally used feed ingredient. The estrogenic activity of subterranean clover has been attributed to

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Table 1. Uterine response to orally administered estrogen.

Test material and total amount fed per mouse	No. of mice	Av. uterine wt. (mg)
None (control)	5	10
Alcohol extract from 3.5 of dried ladino clover meal	5	41
Crystalline ladino clover estrogen (0.5 mg)	5	34
Crystalline ladino clover estrogen (0.75 mg)	5	61
Genistein (15 mg)	4	28
Diethylstilbestrol (0.2 μg)	5	47
Mentzer's M84* (1.0 mg)	5	101

* 3-(*p*-hydroxyphenyl)-4-*m*-propyl, 7-hydroxycoumarin (*9*).

the presence of genistein, although it has also been reported that the treatment of clover "chloroplast" with alkali yielded a small amount of a second estrogen with at least 10 times the activity of genistein (*8*). No information on the chemical nature of this second estrogen was presented, however.

A crystalline compound possessing estrogenic activity has recently been isolated at this laboratory from ladino clover. We have also found estrogenic activity in several alfalfa samples as well as in a sample of fresh strawberry clover; the activity in these samples appears to be attributable to the same estrogen. The compound is the predominant estrogen in strawberry clover, ladino clover and alfalfa, and it appears to be a coumarin derivative rather than an isoflavone. Because of the coumarin structure of the molecule, we propose the name *coumestrol* for the estrogen. The effectiveness of coumestrol as an estrogen has been demonstrated by feeding it to immature female mice and measuring the effect on uterine weight increase. The results of one such assay are presented in Table 1. Genistein and diethylstilbestrol were also included in this study for purposes of comparison. From the data in Table 1 it can readily be seen that coumestrol is considerably more potent than the estrogenic isoflavone, genistein, although it is much less active than diethylstilbestrol.

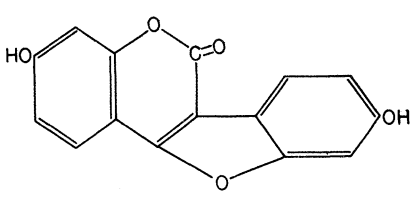

Fig. 1. Proposed structure of coumestrol.

For the bioassay, the estrogen, incorporated in the stock diet of the animals, was fed to 18-day-old immature female mice for a period of 7 days. The mice were killed and the weights of the freshly excised uteri were determined.

The estrogenic compound was isolated by means of solvent extraction of the dried meal, followed by several countercurrent distributions, with final recrystallization from methanol.

Coumestrol has a bright blue fluorescence in neutral or acid solution, turning to a greenish yellow in strong alkali. This characteristic greatly facilitated its isolation. It melts with slight decomposition at 385°C (Kofler block). Its ultraviolet absorption spectrum (measured in methanol) shows maxima at 208, 243, and 343 mμ. Alkali fusion of the compound yielded resorcinol and beta-resorcylic acid but no other identifiable phenols or phenolic acids.

Coumestrol has the empirical formula, $C_{15}H_8O_5$. Among the derivatives that have been prepared are the diacetate, $C_{19}H_{12}O_7$ (melting point, 234°C) and the dimethyl ether derivative, $C_{17}H_{12}O_5$ (melting point, 198°C), indicating that two free hydroxyl groups are present. There are no methoxyl groups in the compound.

Treatment of coumestrol with dimethyl sulfate under strongly alkaline conditions gave a trimethyl ether-monomethyl ester, $C_{19}H_{18}O_6$ (melting point, 98°C). Mild alkaline hydrolysis of this compound gave the trimethyl ether acid, $C_{18}H_{16}O_6$ (melting point, 178°C). The formation of an acid by this means confirms the presence of a coumarinlike structure in the molecule. Titration of this acid indicated a minimum molecular weight of 331. Decarboxylation of this acid derivative of coumestrol yielded a compound having the empirical formula $C_{17}H_{16}O_4$ (melting point, 82°C). Ozonolysis of this decarboxylated product yielded a number of degradation products, two of which have been identified as 2-hydroxy-4-methoxybenzoic acid and 2,4-dimethoxyzenzoic acid. The close agreement between the analytical data obtained and the theoretical data expected from the above derivatives has led us to propose the structure shown in Fig. 1 for coumestrol.

Mentzer *et al.* (*9*), on purely theoretical grounds, have synthesized a number of coumarin derivatives that showed estrogenic activity. Their most active estrogen was 3-(*p*-hydroxyphenyl)-4-propyl-7-hydroxycoumarin. Except for the alkyl side chain at position 4, a striking similarity exists between our proposed structure for coumestrol and the synthetic coumarin derivative. A recent article (*10*) reports the isolation of a lactone from *Wedelia calendulacea* with basic structure similar to our proposed structure, differing however, in number, position and type of substituent groups. Therefore, it would seem that if the proposed structure proves to be correct, coumestrol represents an estrogenic compound not previously reported in the literature.

Note added in proof: We have confirmed the proposed structure of coumestrol and also the estrogenic effectiveness of the synthetic material.

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Wet Freeze-Drying of Muscle

It was found that acetone, at Dry-Ice temperature, does not denature proteins but still is capable of dissolving water to some extent. It was also found that chlorated paraffins do not harm contractile proteins at room temperature. This allows one to dehydrate muscle without destroying its molecular structure and contractility.

Fresh frog sartorius muscles or thin strips (1 to 2 mm) of freshly isolated rabbit's psoas were tied to applicator sticks at rest length and immersed in acetone cooled in Dry Ice. The frozen muscle is kept at this low temperature in acetone for a week, after which time the acetone is exchanged with fresh precooled anhydrous acetone in which the muscle is left for a fortnight. We have used 50-ml test tubes as containers and have kept these test tubes in thermos bottles filled with Dry Ice. The acetone slowly dehydrates the frozen muscle. Instead of exchanging the acetone, one can also bind the water extracted from the muscle with granulated $CaCl_2$.

After the water has thus been extracted, the dehydrated muscle is transferred into precooled ethyl chloride and