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STUDIES OF *Trichoderma* ISOLATES FROM *Mytilus edulis* COLLECTED ON THE SHORES OF CAPE BRETON AND PRINCE EDWARD ISLANDS#†

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Samples of *Mytilis edulis* (mussel) were collected from coastal sites, randomly selected on the south-eastern shores of Prince Edward Island and from similar locations on the southern shore of Cape Breton Island. Cultivation of aliquots of tissue from these animals revealed a diverse fungal flora from which 49 cultures of the genus *Trichoderma* were isolated. The number of propagules from which these cultures were derived was multiplied by a factor to reflect the dry weight of mussel tissue used in the isolation. A random sample of 29 isolates from this theoretical collection was then selected and it was found possible to cultivate 28 of these in the laboratory. All nine isolates of *Trichoderma hamatum*, produced isocyanide metabolites. Improvements in the assay of these compounds are reported.

Des échantillons de *Mytilis edulis* (la moule) ont été collectionnés à locations côtières choisies au hasard sur les côtes sud-est de l'Ile Prince-Edouard et sur des lieux semblables sur la côte sud du Cap Breton. La cultivation d'aliquotes de ces animaux a révélé une flore variée de moisissures desquelles 49 cultures de ce genre ont été isolées. Le nombre de propagules qui ont fait partie de ces cultures a été multiplié par un facteur afin de réfléchir le poids sec du tissu de moule utilisé dans cette isolation.. Un échantillon de 29 isolates de cette collection théorique a été choisi, et on a réussi à en cultiver 28 dans le laboratoire. Tous les neuf isolates de *Trichoderma hamatum* ont produit des métabolirtes de l'isocyanure. On discute des améliorations du titrage de ces composés.

Introduction

In 1987 there occurred 108 incidents of poisoning (including 3 deaths) following human consumption of cultivated *Mytilis edulis* from Prince Edward Island (Bates et al., 1989). As part of the investigation surrounding this toxicity, the fungal floras of mussels from the affected areas were examined. Although no correlation between toxicity and the fungal flora was found, the diverse nature of the flora and, in particular, the predominance of *Trichoderma* spp. (Taylor, 1986) suggested an ecological examination of the populations of this genus present in mussel tissues should be done. Accordingly, the south-eastern coastal region of Prince Edward Island was divided into adjacent 1 Km^2 areas, based on the National Grid and a random sample of such areas selected. For comparison an equal area around Isle Madame, Cape Breton Island, Nova Scotia was divided and sites randomly selected in the same way. All of the areas selected that had a coastal segment were examined in October 1989 and mussels were collected from most of these areas. The fungal floras of the bulked samples were isolated and the *Trichoderma* component examined for its ability to produce antibiotics in the way we have previously described (Brewer and Taylor, 1980). The results of this work are reported.

Materials and Methods

Selection of collection areas The maps (Fig 1 and Fig 2) show the collection areas, the 9 Km^2 grids selected by the random number algorithm, and the areas where

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# This paper is dedicated to the memory of K.A. Harrison

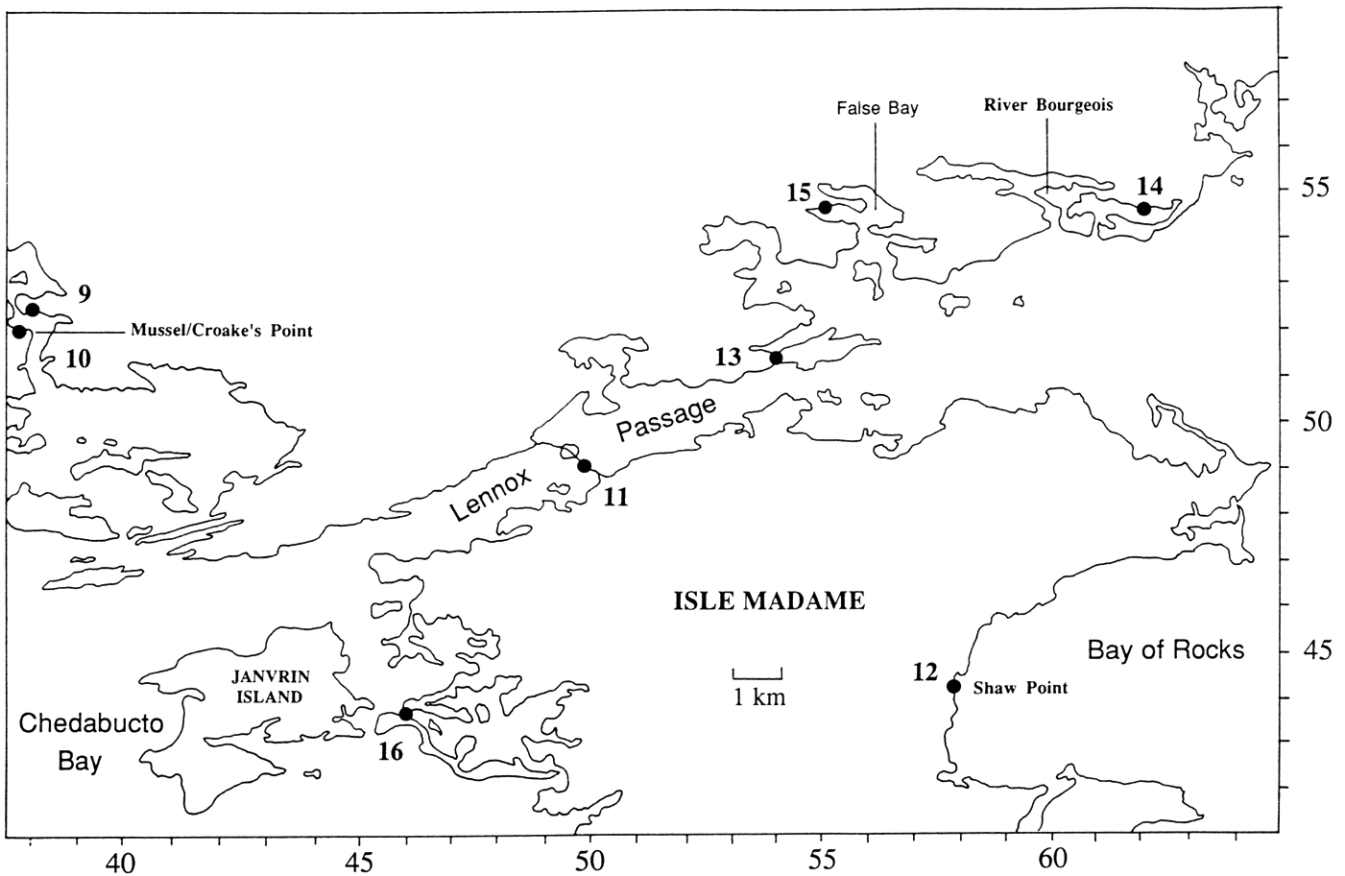

Fig 1 Map of the Southern shore of Cape Breton Island and Isle Madame. Numbers on the axes are 1 km Eastings and Northings of the National Grid. Numbers on the map refer to collection sites (Table I).

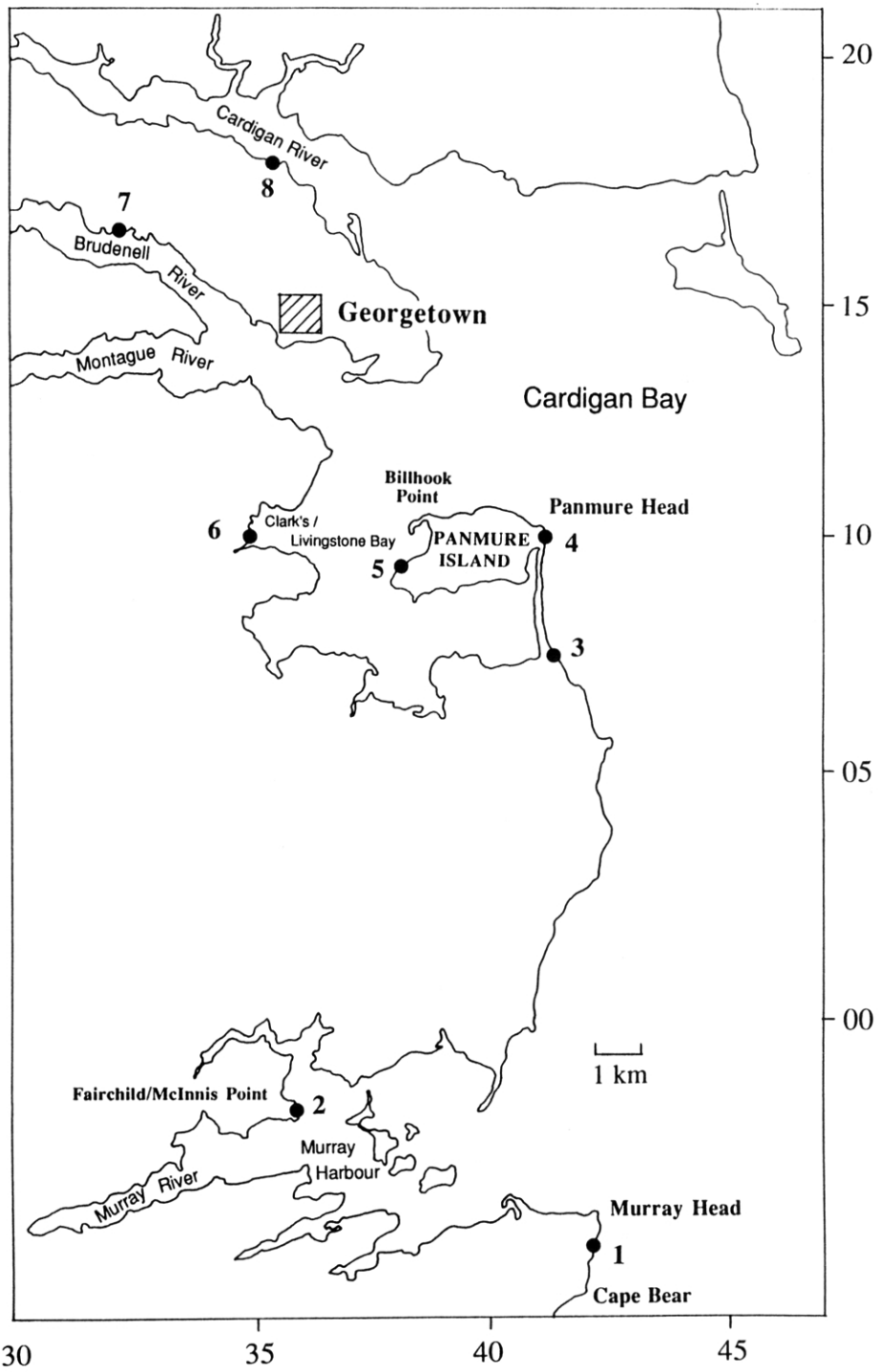

Fig 2 Map of the South-Eastern shore of Prince Edward Island. The axes and collection sites are numbered in the same way as described in Fig 1 and Table 1.

Table I Geographical locations and descriptions of areas in Prince Edward Island and Cape Breton Island where *Mytilus edulis* was collected

Reference		Geographical location	Nature of area of collection	Surrounding area	Day of collection (Oct. 1989)	No. of mussels examined
Grid	Fig 1/2					
Prince Edward Island						
NF422953	1	Between Cape Bear and Murray Head	Rocky shore covered with *Fucus*	Road just above beach then trees and shrubs	24	13
NF359982	2	Fairchild/McInnes Point	Sandy shore - *Zostera* in water	Campground - grass and trees	24	16
NG415074	3	Smith Point	Rocky Shore - with *Fucus*	Cliffs with trees	24	20
NG412099	4	Panmure Head in front of lighthouse cliff	Rocky shore with *Fucus*	Cliff with cliff swallow tunnels	24	13
NG385093	5	Panmure Island between Billhook Point and South West Corner	Sand and pebbles	Road between scrub bush and shore - by a jetty	24	11
NG349098	6	Clarks/Livingstone Bay	Salt marsh (mud & sand)	Bush around salt marsh	24	20
NG323161	7	Brudenell Provincial Park	Sandy beach	Grass and trees	25	20
NG355175	8	East of Morrison Beach across from Ferry Point	Sandy beach	Scrub bush with cottages	25	20
Cape Breton						
PF380525	9	Exit 44 Highway 104 E of Chapel Road	Polluted beach	Road	23	20
PF382521	10	North side of Mussel /Croakes Point	Sandy beach	Camp-site & forest	23	18
PF497492	11	Lennox Passage off causeway between Burnt Is. & Benoit Is.	Side of causeway	Road/Forested island	23	18
PF579445	12	Beach, South of Shaw Point	Marsh & Tidal inlet	Road and bridge	23	21
PF539515	13	Tickle Passage	?	?	23	21
PF619546	14	"Lagoon" at River Bourgeois	Mostly mud and stones	Dirt road	24	15
PF549546	15	False Bay	Estuary; mud and stones	Mixed forest	24	27
PF463437	16	Janvin Island	Polluted and muddy	Bridge and road	24	20

M. edulis were found. In the following these areas are referred to by their National Grid numbers. These grid numbers were also used as the basis for identification of samples and of fungal isolates grown from propagules in the samples. Table I gives a list of the grid references of the areas where mussels were collected, cross references to Fig 1 and Fig 2, the number of animals used for analysis from each area, the date of collection and a general geographic description of the sites.

Isolation of fungi The mussels (Table I) were placed in polypropylene containers and were submerged in water from the collection site. Within 48 h they were transported to the laboratory where they were cooled to 4°C, opened and the tissue and fluid within the shell mascerated at 4°C for 30 s. The mascerate (10 mL) was diluted with sterile distilled water (90 mL) and 3, 10-fold dilutions prepared from this primary dilution. Each of the 3 suspensions (1 mL) was used to inoculate 10 Petri plates containing potato-dextrose agar (Difco), supplemented with yeast extract (Difco, 0.3% w/v) and rose bengal (0.0035% w/v). The plates were incubated at 25°C for 7 days, when sub-cultures of the fungal colonies on the plates were made onto a medium containing (g L^{-1}): molasses (20), dextrin (30), fish meal (15), Pharmamedia (Traders Protein Division, Fort Worth, Texas, 15) and agar (20).

Random selection of Trichoderma *isolates for antibiotic analysis* The frequency of particular *Trichoderma* isolates in mussel tissue was calculated from the number of colonies appearing at a dilution where all could be counted on 10 plates. The frequency was then defined as ((number of colonies on 10 plates x dilution)/dry weight of 10 mL of homogenate). Thus each of the 49 *Trichoderma* isolates was multiplied by this frequency, and from the theoretical collection obtained 50 isolates were selected at random. As described in a preceding paper (Brewer and Taylor, 1980), several selections were made and each selection was scored on the basis of greatest diversity of collection sites, and species, and more or less even distribution between Cape Breton and Prince Edward Islands. The selection scoring highest by these criteria had 29 different isolates from 11 collection areas, 6 from Cape Breton and 5 from Prince Edward Island.

Fermentation, Harvesting and Extraction of Trichoderma *cultures* The isolates were grown in groups of 5, together with a control culture of *Trichoderma hamatum* (HLX 1379; Brewer et al., 1982). Inocula (48 h old, 1 mL) were prepared from the isolates grown on malt agar as described in an earlier paper (Brewer et al., 1982) and were added to production medium (100 mL) containing: glucose (4 g); potassium nitrate (0.1 g);, L-tyrosine (0.1 g); sodium chloride (0.25 mg); magnesium sulfate heptahydrate (35 mg); ferrous sulfate (16 mg); zinc sulfate heptahydrate (2 mg); copper sulfate pentahydrate (2 mg); phosphomolybdic acid (3 mg); o-phosphoric acid (5.4 g) and water (conductivity <10 Ω) The solution was treated with potassium hydroxide solution to pH 5.4. Twenty such flasks for each isolate and the control were shaken in a horizontal plane at 220 r min^{-1}, each culture describing a circle of radius 3.81 cm. The culture flasks (500 mL capacity) in both fermentation stages were randomly distributed on the shaker. After 70 h at 25°C, like cultures were combined, cooled to 4°C, their pH adjusted to 8.0 when the cultures were filtered through a pad of washed 'Celite 535' from which most of the fine material had been removed by decantation. The filtrates (500 mL) were treated at 4°C with concentrated hydrochloric acid to pH 4.5 and were then extracted with ethyl acetate (3 x 150 mL). In the majority of cases the aqueous phase separated cleanly leaving an emulsoid organic phase. The 3 emulsions were combined and, if necessary, centrifuged at 1500 r min^{-1} for 30 min at 0°C. In a few cases the phases did not separate under gravity and these extractions were completed by centrifugation as described. In all cases, the combined centrifuged ethyl acetate extracts were kept at

-15°C for 18 h, when the ice that had separated was filtered and the clear filtrate (200 mL) used for analysis

Analytical methods

The dry weight of the mascerated mussel tissue was determined by drying 10 mL of the mascerate at 105°C for 24 h. Salinities of water samples were measured using an American optical hand-held refractometer; the instrument being calibrated using standard sea-water samples (Culkin and Smed, 1979). Phosphate was determined by the method of Parsons et al., (1984). All mean values are given with their standard deviations.

Antibiotic assays of fungal isolates were obtained using plugs from fungal colonies as described by Brewer et al., 1972, using *Micrococcus luteus* (HLX 701) as the assay organism.

Analysis of isocyanides in Trichoderma *fermentations* The dry ethyl acetate extract of the fermentation broth (see above, 200 mL) was treated with an ethereal solution (10 mL) of diazomethane (10 mg), the solution was kept 30 min at room temperature when the excess diazomethane was blown off with a stream of nitrogen in an efficient fume-hood. The solution was then concentrated to 20 mL and the concentrate treated with a solution (2 mL) of bis(η^5-pentamethylcyclopentadienyl)di-μ-thiocyanato-dithiocyanatodirhodium(III) (Hanson et al., 1985; hereinafter $(\eta^5\text{-}C_5Me_5Rh(SCN)_2)_2$; 15 mg) in methylene dichloride. The orange solution was kept 3 h at room temperature, was evaporated to dryness and the residue dissolved in methyl alcohol to give a solution of volume 2 mL. This solution (diluted as necessary, 1 mL) was injected onto a reversed phase chromatography column (Merck, C_{18}, 20 x 0.2 cm). The column was developed at 22°C with methyl alcohol at a flow rate of 1 mL min^{-1}. The column effluent was directed to a Waters UV detector set at 254 nm and the signal from the detector was integrated and plotted by a Hewlett-Packard recording integrator (Model 3854A). The remainder of the apparatus has been described previously (Russell, 1988). Standard solutions of the η^5-$C_5Me_5Rh(SCN)_2$ complexes of methyl 3-isocyanocyclopent-2-enylideneproprionate, methyl 3-(3-isocyano-1,5-epoxycyclopentyl)-prop-2-enoate, and 1-(2,3,4,5-diepoxy-1-hydroxycyclopentyl)ethanol (Boyd et al., 1991, and references therein) in methyl alcohol were chromatographed in the same way and the relations between their concentrations and peak areas obtained from the integrator were calculated. These relations were then used to calculate the yield of these three metabolites in the usual way.

The presence of isocyanide antibiotics was confirmed by thin layer chromatography on commercially prepared silica gel plates (Merck). The parent isocyanide metabolites in the esterified ethyl acetate extract were separated using the solvent acetic acid-diethyl ether-petroleum ether 0.1:29.9:70 as the developing solvent. The η^5-$C_5Me_5Rh(SCN)_2$ complexes present in the methyl alcohol solution were separated using the solvent methyl alcohol-dichloromethane 1:24 (Boyd et al., 1991).

Ultraviolet spectra were obtained using a Cary 14 spectrophotometer.

Solvents and reagents Methylene dichloride was distilled from calcium hydride before use; methyl alcohol was glass distilled and of the quality designated "for high pressure liquid chromatography". Diazomethane was prepared from *p*-toluene-sulfonylmethylnitrosamide (de Boer and Backer, 1963). All other reagents were used as received.

Table II Air temperatures and physical characteristics of water at sites of collection of *Mytilus edulis* in Cape Breton and Prince Edward Island.

Grid reference	Temperature Air (°C)	Temperature Water (°C)	pH	Salinity ‰	% Dry Wt .of mussel tissue	Phosphate µM
NF422953	12	11	7.1	28	9.9	>11
NF359982	12	11	7.2	27	13	3.8
NG415074	12	11	7.1	27	13.6	5.9
NG412099	12	11	6.7	28	11.3	>11
NG385093	12	9	7.0	27	12.5	4.1
NG349098	10	12	7.3	27	9.0	0.7
NG323161	8	7	7.0	26	10.3	1.4
NG355175	8	9	7.1	26	14.2	2.3
PF380525	6.5	8.5	6.9	19	9.4	3.3
PF382521	7	8.5	6.9	19	8.1	3.3
PF497492	8	9.4	6.8	27	9.4	7
PF579445	5	10			8.0	
PF539515			6.7	27	12.9	10.7
PF619546	7.5	10	6.8	27	8.1	4.4
PF549546	6	6.5	7.6	0	8.3	3.1
PF463437	10	10.5	7.9	23	11.2	7.6

Results

A rectangle of 360 Km^2 was selected based on latitude 45° 30' 00" N and longitudes 61° 14' 46" W, and 60° 54' 3" W. The area is shown in Fig 1 and comprised most of Isle Madame, Lennox Passage and the southern shore of Cape Breton Island from Port Hawkesbury to St. Peters. This area was divided into forty, 9 Km^2 square sections based on the National grid and numbered 1-40. Sixteen of these sections were selected at random and 7 of these that had no coast-line were rejected. No mussels were found on one of the 9 sections with a coast-line.

A similar rectangle of 405 Km^2 based on latitude 46° 00' 00" and longitudes 62° 21' 00" and 62° 23' 7" was selected. The area is shown in Fig 2 and comprised the SE coast of Prince Edward Island from south of the entrance to Murray Harbour, north to Bruce Point. This area was divided into forty five 9 Km^2 square sections of which 5 (15 x 3 Km) on the SE edge were entirely oceanic. These were eliminated leaving an area the same size as that selected in Cape Breton. These areas were numbered 13-54 and 16 of these selected at random. There was no coast line on 7 of these sections, and it proved difficult to gain access to one, thus leaving 8 areas where mussels were collected.

The grid references to the nearest 100 m of the 9 Km^2 sections where mussels were found are given in Table I, and is indicated approximately in Fig 1 (numbers 1-8, Table I) and Fig 2 (numbers 9-16, Table I). Included in Table I are names of the nearest geographical location on the 1:50000 map, brief descriptions of the collection area and its surroundings, the date of collection of each sample and the number of mussels used for examination from each site. In many cases this was less than the number collected, because only shells that contained apparently healthy animals were used.

In Table II the temperatures, acidities and salinities of the water where the mussels were collected are recorded. In Cape Breton, by contrast to the Prince Edward Island

sites, the water temperatures were higher than the air temperature. Temperature data were not collected for the specimens from PF539515 because this site was on private land and we were not allowed access. However the owner provided a sample which we assume was collected at this location. The water sample from site PF579445 was lost.

There appeared to be no correlation between the dry weight of mussel tissue and the pH, or the salinity, or the phosphate content of the water where they were growing. The mean dry weight of the 8 samples of mussel tissue (Table II) from Prince Edward Island was 11.7±1.8 g 10 mL^{-1} and that of the 8 samples from Cape Breton 9.4±1.8 g 10 mL^{-1}, the difference, using Student's 't' test being significant at the 90% confidence level.

In Table III calculations of the total number of fungal propagules cultivated from each gram dry weight of mussel tissue are given. The mean value of all fungal propagules on this basis cultivated from the Prince Edward Island samples was 1139±881 g^{-1}, and from the Cape Breton samples 4546±4787 g^{-1}. The very large variance in these statistics probably arises from the presence of sporing hyphae in some samples. The difference is, however, significant at the 90% confidence level. Values are also given in Table III of the numbers of propagules of 5 common *Trichoderma* spp. and in 3 cases of isolates that have been classified only to the genus level. The mean value of the frequencies of all *Trichoderma* spp. collected on Prince Edward Island was 103±113 g^{-1} and on Cape Breton 1207±1820 g^{-1}. This difference may be significant ($P<0.1$). The values obtained for *Trichoderma harzianum* (Table III) were 44 g^{-1} and 814 g^{-1} for this species in the two areas; these figures include abnormally high numbers for locations NF 422953 and PF380525. If these two values are omitted from the calculations, the mean value for the remaining 7 samples become 14±12 g^{-1} for Prince Edward Island and 224±247 g^{-1} for Cape Breton and these two means are significantly different ($P < 0.05$).

Table III Fungal propagules $gram^{-1}$ dry weight of tissue of *Mytilus edulis* collected on the shores of Cape Breton and Prince Edward islands.

Grid reference	All fungi	*T. hamatum*	*T. koningii*	*T. harzianum*	*T. viride*	*Trichoderma polysporum*	*Unidentified Trichoderma*
NF422953	819		21	256	21		
NF359982	2282	20	32	2	2		
NF415074	456			18			2
NG412099	540	32		32			16
NG385093	723	34	17	5			
NG349098	2789	239		26			
NG323161	806	4	2	12			
NG355175	697		28	1			
PF380525	9859	235	305	4947			
PF382521	2642	247	183	148			
PF497492	1638	23		181	21		213
PF579445	1275		50	28			
PF539515	519	23	1	23	110		
PF619546	376		2	45		20	
PF549546	7229		72	505	553	48	
PF463437	12831	594	358	636	80		

A blank indicates that none of this species were found in the samples; dry weights of mussel tissues are given in Table II.

Antibiosis of Trichoderma *spp. isolated from mussel tissues* The values for the *Trichoderma* spp. given in Table III allow a theoretical collection to be assembled based on the total weight of mussel tissue collected and the frequency (number of propagules g^{-1}) of each species in each sample. This collection then represents all 49 (Table III) of the *Trichoderma* species collected and enables a random sample of this collection to be selected. The advantages and pitfalls of this procedure have been discussed (Brewer and Taylor, 1980). In this case several samples of 50 isolates were made and each was scored as described in the Experimental section. The sample chosen for study consisted of 29 different isolates, seven from five Prince Edward Island sectors and twenty-one from six Cape Breton sectors (Table IV). One of the isolates (T. *harzianum*) in the chosen sample was contaminated with a *Penicillium* sp. and was discarded. Three of the isolates of *T harzianum* in the sample could be readily distinguished from the other isolates of *T. harzianum* by the strong yellow-green pigmentation of the medium when grown on malt agar.

Table IV Frequency and antibacterial activity of a random sample of isolates of *Trichoderma* from tissues of *Mytilus edulis*

Isolate number#	Species	Frequency prop. g^{-1}	Activity zone diameter (mm)*	Trichoviridin mg L^{-1}	Dermadin mg L^{-1}	270 mg L^{-1}
NF422953-4	*harzianum*	21				
NG415074-4	*harzianum*	15	12			
NG412099-2	*harzianum*	32	8			
NG412099-20	*sp.*	16	8			
NG349098-1	*hamatum*	222	17	22	3	8
NG349098-11	*hamatum*	9	20	35	4	10
NG323161-2	hamatum	4	22	90	3	16
PF380525-1	*harzianum*	4705				
PF380525-2	*harzianum*	235				
PF380525-3	*koningii*	235				
PF380525-24	*hamatum*	235	9	10	17	66
PF382521-2	*koningii*	173			2	9
PF382521-3	*harzianum*	123			0.1	
PF382521-10	*harzianum*	25	8			
PF382521-22	*hamatum*	247	14	107		
PF497492-2	*hamatum*	9	N.D.†			
PF497492-3	*harzianum*	64	17			
PF497492-4	*harzianum*	43	13			?0.05
PF497492-8	*hamatum*	21	22	48	12	46
PF539515-23	*hamatum*	11	14	55		30
PF549546-2	*viride*	481				0.4
PF549546-6	*viride*	72				
PF463437-2	*harzianum*	198	8			
PF463437-3	*hamatum*	198	20	96	17	20
PF463437-4	*koningii*	198				
PF463437-8	*harzianum*	198				
PF463437-9	*harzianum*	198			?0.3	?0.7
PF463437-12	*harzianum*	40	11			

* *Micrococcus luteus* was used as the assay organism; blank spaces indicate that no inhibitory activity was found

\# Characters to the left of the dash are grid references to place of isolation of the mussel sample.

† Not determined.

All of the remaining 28 cultures were then examined for their ability to produce metabolites that inhibited the growth of *Micrococcus luteus* and the diameter of the zones of inhibition around the plugs of fungal tissue (Brewer et al., 1972) are given in Table IV. None of the isolates of *T. viride* or *T. koningii* produced antibiotics under these conditions and only seven of thirteen *T. harzianum* isolates inhibited the growth of *M. luteus*. By contrast all 9 isolates of *T. hamatum* produced antibiotics and the mean zone diameters (16±5 mm) was greater than those observed for the active isolates of *T. harzianum* (11±3 mm, Table IV). Six of the seven Prince Edward Island isolates were active; eleven of twenty-one Cape Breton isolates were inhibitory.

Production of isocyanide antibiotics by Trichoderma *isolates from* M edulis The fermentation of isolates of *Trichoderma* from *M. edulis* and the isolation of their metabolites followed the procedures described by Brewer et al. (1982). The random sample of isolates given in Table IV were grown in five groups of 5 and one group of 3. In all six cases a control culture of *T. hamatum* HLX 1379 was included.

Several improvements in the analysis (Feicht and Taylor, 1982) of isocyanide metabolites were used taking advantage of recent improvements of our knowledge of the chemistry of these compounds. It was found that the $\eta^5C_5Me_5Rh(SCN)_2$ complexes of the metabolites (Hanson et al., 1985) had high extinction coefficients at 251 nm (1-(2,3,4,5-diepoxy-1-hydroxy-3-isocyanocyclopentyl)ethanol: ε_{251} 18500 dm^3 mol^{-1} cm^{-1}; methyl 3-(3-isocyano-1,5-diepoxycyclopentyl)-prop-2-enoate: ε_{251} 40100 dm^3 mol^{-1} cm^{-1}; and methyl 3-isocyano-cyclopent-2-enylidenepropionate: ε_{251} 12000 dm^3 mol^{-1} cm^{-1} (shoulder; λ_{max} 290 nm, ε 14200), that the methyl esters of the acidic metabolites could be prepared by the less hazardous method recently described (McAlees et al., 1990) and that these complexes (henceforth referred to by the trivial names: trichoviridin, dermadin and 270, respectively) were readily separated using a simple high pressure reversed phase chromatography system. Under the conditions described in the Experimental section, during a period of 6 months, the mean elution times observed for the 3 metabolites were: trichoviridin, 5.05±0.09 min; dermadin, 9.14±0.39 min and 270 12.20±0.4 min (n=20). All three complexes were detected and the area under the eluted peaks integrated when 20 ng of the compound was injected onto the column and in all three cases a linear relationship between weight applied to the column and integrated area was obtained (r^2 values: trichoviridin, 0.96; dermadin, 0.98; 270, 0.99). The analyses for trichoviridin were complicated when 1-(2,3-epoxy-1-hydroxy-3-isocyanocyclopent-4-enyl)ethanol (Boyd et al., 1991) and congeners were produced in the fermentation in quantities equal to or greater than trichoviridin. The former complex was eluted from the column at 4.78 min and was not completely separated from the latter. No attempt was made to find chromatographic conditions that achieved a complete separation, but the integrator was programmed to calculate the theoretical area of the trichoviridin peak. Thus the analyses given in Table IV for trichoviridin are less accurate than those for dermadin and 270 where complete separations and Gausian shaped peaks were always obtained.

The mean values obtained for the control *T. hamatum* (HLX 1379) cultures in the 6 experiments were: trichoviridin, 27±27 (range 0-80) mg L^{-1}; dermadin, 17±18 (range 0.7-56) mg L^{-1} and 270, 30±26 (range 4-92) mg L^{-1}. These values are, of course for the weights of the complexes and must be multiplied by 0.34, 0.32 and 0.306 respectively to obtain the yields of the metabolites; this also applies to the values given in Table IV. The latter values are, in general, the mean of two analyses on the same extract, but in certain cases (marked with an asterisk in Table IV) a duplicate fermentation was done. In all cases, thin layer chromatography of the concentrated ethyl acetate extract (Feicht and Taylor 1982) revealed the presence of isocyanide by their polymerization with

nickelous chloride. Similarly the prepared $\eta^5C_5Me_5Rh(SCN)_2$ methyl alcohol solutions were examined by thin layer chromatography (Boyd et al., 1991); the presence of yellow/orange spots of the correct R_f confirming the presence of the three isocyanide (and many other) metabolites.

Discussion

With a base of so few examples it is untenable to draw firm ecological conclusions on relationships between *Mytilus edulis* and its fungal burden. However all samples had a fungal flora which had a striking resemblance to the soil flora from permanent pasture (Brewer and Taylor, 1980). There also seems to be some ground for concluding that the fungal populations borne by the animals from Cape Breton were greater than those from Prince Edward Island and that this may be correlated with their lower dry weight and generally less healthy appearance. Reinforcing this supposition is the fact that the samples bearing the greatest fungal populations (PF380525 and PF549546) were from the (visually) most polluted sites.

The presence of these fungal populations implies, but does not prove, a toxic potential if these animals are used as a source of food. We have made a preliminary assessment of this potential by taking a random sample of the *Trichoderma* population which is known to produce toxic metabolites *in situ* (Taylor 1986) and we found that 60% of these isolates and 100% of the isolates of *T. hamatum* produced growth inhibiting substances. These proportions are much greater than those found in isolations from soil (Brewer and Taylor 1981).

One common group of metabolites produced by this genus is characterized by an isocyanide functionality and the results of analyses of the random sample of isolates for metabolites of this type are given in Table IV. The values given and the proportions of the three compounds are only a rough guide to the production potential of these fungi - due to their great variability. This variability is exemplified by the results reported on the control culture, *T. hamatum*, HLX 1379. Whilst these results compare well with those reported about 10 y ago (Brewer et al., 1982), despite many sub-cultivations and a few selections of single spore cultures for improved production ability, it is clear that many fermentations are required to establish the mean yield of these metabolites by these isolates. Despite this proviso, it appears that only isolates of *T. hamatum* produce isocyanide metabolites. Three of the isolates of *T. harzianum* may have produced traces of these metabolites (Table IV) but subsequent repeat experiments failed to confirm this supposition. It is therefore possible that the antibacterial activity of these *T. harzianum* isolates (Table IV) is due to another group(s) of mycotoxins. This result is at variance with that reported by Fujiwara and his co-workers (1982) who found that most of the *Trichoderma* spp. given in Table III produced isocyanides.

The results suggest that when locating a site for the cultivation of *M. edulis* for human consumption the proximity of the site to roads, human habitation and sources of eroded agricultural land should be taken into consideration.

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