Substrate selection by the archiannelid *Protodrilus rubropharyngeus*

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KURZFASSUNG: **Substratwahl durch den Archianneliden *Protodrilus rubropharyngeus*.** Die Verteilung von Populationen mariner interstitieller Organismen wird durch Auswahl eines geeigneten Substrats seitens der Larven und (oder) Adultformen bestimmt. Entscheidend für die Wahl eines sandigen Substrats sind Korngröße, Sauerstoffgehalt, Temperatur, Lichtdurchlässigkeit etc. sowie die chemischen Eigenschaften der Sandoberfläche. Für *Protodrilus rubropharyngeus* JÄGERSTEN konnte negative Geotaxis sowie eine Bevorzugung von Sandarealen mit relativ hohem Sauerstoffgehalt nachgewiesen werden. *P. rubropharyngeus* reagiert bei starkem Lichteinfall negativ photokinetisch. Vibrationen rufen positive Geotaxis und Verschwinden im Substrat hervor. In Versuchen, bei denen adulte Tiere zwischen Sand mit Tieren und Sand ohne Tiere wählen konnten, bevorzugten sie Sand, in dem sich Artgenossen befanden. Es ließ sich ferner nachweisen, daß eine Substanz, die von adulten Tieren produziert wird, zu „gregariousness" führt. Im Wahlversuch zwischen Sand bestimmter Korngröße und natürlichem, ungesiebtem Sand entschieden sich sowohl Adulte als auch Larven für Korngrößen von 0,5 bis 1 mm. Im natürlichen Biotop dominierten jedoch Sandkörner von 1 bis 2 mm Größe. Dadurch ist das Vorkommen von *P. rubropharyngeus* auf Strandzonen mit Korngrößen von 0,5 bis 1 mm beschränkt. Im Vergleich zu unbehandeltem Sand wurde sterilisierter Sand nur von sehr wenigen Tieren bevorzugt, wenn er in einfachen Wahlversuchen Adulten und Larven angeboten wurde. Dies änderte sich jedoch, wenn sterilisierter Sand mit Sandbakterienkulturen beimpft wurde. Im Wahlversuch zwischen Sanden, die mit verschiedenen Bakterienarten beimpft worden waren, bevorzugten Adulte wie Larven in gleicher Weise bestimmte Bakterienarten. Wenn adulte Tiere zugegen waren, wurde steriler, mit Bakterien beimpfter Sand fast ebenso häufig besiedelt wie unbehandelter Sand. Die streng lokalisierten Populationen der Spezies erklären sich somit aus der Bindung an eine bestimmte Korngröße, dem Vorhandensein eines Oberflächenfilms bakteriellen Ursprungs sowie einer von den adulten Tieren produzierten Substanz.

INTRODUCTION

The localization of populations of marine sand living animals are produced by the selection of a suitable substrate by larval and/or adult stages. The preference of a species for limited areas of a sandy beach seems to be related to the physical properties of the substrate, such as its temperature (GRAY 1965), the depth of light penetration (GRAY 1966a, TAYLOR 1965), its oxygen content (GRAY 1966d), and in relation

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to the chemical properties of the sand grain surfaces (WIESER 1956, MEADOWS 1964, GRAY 1966c).

The importance of the micro-organisms present on the sand grain surfaces in the settlement of the larvae of marine sand-living animals was first suggested by JÄGERSTEN (1940b). By means of various chemical treatments of the substrate he was able to destroy the factor, present in natural untreated sand, which caused the larvae of *Protodrilus rubropharyngeus* JÄGERSTEN to metamorphose. JÄGERSTEN found that the factor causing metamorphosis was irregularly distributed in nature. He suggested that this patchy distribution might be correlated with the irregular distribution of the numerically rich micro-organisms on the sand grain surfaces. Similarly, WILSON (1952, 1953a, 1953b, 1954, 1955) presumed that the presence of micro-organisms in the correct quantity and of a favourable species induced the larvae of *Ophelia bicornis* SAVIGNY to settle. The author (GRAY 1966c) has shown by means of inoculation experiments that *Protodrilus symbioticus* GIARD (now *Protodriloides symbioticus* (GIARD) (see JOUIN 1966) selects a substrate in relation to the surface film, produced by certain favourable species of bacteria, present on sand grains.

As JÄGERSTEN (1940b) regarded his study as a preliminary work, a study of substrate selection by the larvae of *P. rubropharyngeus* seemed a suitable follow-up to the author's work on substrate selection in *P. symbioticus*. The opportunity to carry out this study occurred in July–October, 1965. The animals used in this work were obtained from JÄGERSTEN's locus classicus at Klubban Beach, Fiskebäckskil, on the Swedish west coast.

Both JÄGERSTEN's experiments on the metamorphosis of *P. rubropharyngeus* larvae and my own recorded here, suffered from unexpected inconsistencies on repetition. It appears that in the absence of a suitable substrate the larvae will metamorphose in an unfavourable substrate. Merely recording the percentage metamorphosis in a particular substrate might, therefore, lead to erroneous results. Consequently the majority of experiments recorded are concerned with the choice of the larvae between a treated and an untreated substrate. The latter substrate (termed natural sand in this paper) being that which contained the population of adult worms.

It was found that when two piles of natural sand were offered to *P. rubropharyngeus* (adults or larvae) each pile collected the same number of animals, within experimental limits of 5 %. The ratio of the number of animals in one pile of substrate to the number in the other pile of substrate being unity for natural sand. Thus sand treated in different ways could be compared quantitatively with natural sand in simple choice experiments. The term "attractiveness index" used in this paper refers to the ratio of the number of animals in treated sand compared to the number in natural sand.

This paper shows the behaviour of adult *P. rubropharyngeus* in relation to the physical and chemical properties of the substrate and demonstrates further the influence of bacteria in localizing populations of marine sand-living organisms.

COLLECTION OF ANIMALS

Samples of coarse sand from the water edge at Klubban were collected in plastic bowls and kept at 16° C in the laboratory. In favourable samples, within 1/2 hour from the time of collection large numbers of *P. rubropharyngeus* adults (measuring 8–9 mm long) could be observed on the sand surface, in densities approaching 40^2/sq.m.

Larvae of *P. rubropharyngeus* were obtained between 1. 9. 65 and 20. 9. 65 by towing a fine mesh plankton net (100 μ pore size) in the immediate proximity of the beach at Klubban. Plankton samples taken 3 m from the beach yielded few larvae. Under a low power binocular microscope the *Protodrilus* larvae were easily identified and were removed with a fine pipette.

In the experiments on metamorphosis, metamorphosed larvae are recorded as those in which the four ciliated bands are lost, the tentacles have appeared, the body has elongated and the animal has assumed a benthic from a planktonic existence.

Detailed descriptions of the morphology and larval development of *P. rubropharyngeus* can be found in JÄGERSTEN (1940a, 1952).

BEHAVIOUR OF ADULT *PROTODRILUS RUBROPHARYNGEUS*

Response to oxygen tension, gravity and light

It was thought that the occurrence of *P. rubropharyngeus* on the sand under laboratory conditions might be due to a preference of the animals for areas of high oxygen tension, as occurred with *P. symbioticus* (GRAY 1966d). This proved to be the case.

Glass tubes, stoppered at one end were filled with seawater and half filled with natural beach sand. Approximately fifty animals were added to each tube and they were then completely filled with sand. The stopper in two tubes was replaced by a glass wool plug thus allowing oxygen to diffuse in at both ends of the tube. In two tubes the bottom of the tube was plugged with glass wool, the top remaining stoppered with a rubber bung, thus allowing oxygen to enter only from the bottom of the tube. Finally two tubes were stoppered with a bung at the bottom and were open to oxygen diffusion at the top. The six tubes were submerged vertically in a tank of seawater at 18° C and left for 24 hours in darkness. The tubes were then removed and divided into six equal regions. The sand and animals from each region were placed in separate petri dishes and the animals counted under a low power binocular microscope. The results based on duplicate experiments are shown in Table 1.

Experiment A shows that where oxygen could enter at both ends of the tubes the animals are negatively geotactic moving to the sand surface (the observed/expected ratio exceeds 1 at the surface only). B and C show that the animals move to areas of sand having the greatest oxygen tension (observed/expected ratio of 2.5 in experiment B); even reversing their geotactic movement in response to the high oxygen tensions at the bottom of the tubes in experiment C (obs./exp. ratio 1.7). In nature these reactions would tend to keep the animals near the sand surface where oxygen

Table 1

Position of *P. rubropharyngeus* in an oxygen gradient

Region of tube	Oxygen gradient	Total observed number of animals in each region	Observed No./ expected No.
1	20 %	A 49	1.9
2		13	
3		5	0.5
4		10	
5		6	0.6
6	20 %	14	
1	20 %	B 82	2.5
2		10	
3		8	0.4
4		6	
5		6	0.2
6	0 %	0	
1	0 %	C 13	0.9
2		11	
3		6	0.4
4		5	
5		12	1.7
6	20 %	34	

tensions are greatest and the normal negative geotaxis would lead them. However, *P. rubropharyngeus* is extremely susceptible to vibration, and if the containing vessel is moved even slightly the animals immediately burrow below the surface by rapid anguilliform swimming movements.

Observations on the behaviour of the animals in light and shade have shown that by means of a photonegative klinokinesis *P. rubropharyngeus* adults avoid high light intensities. Thus the animals will tend to avoid the sand surface at times when they are likely to be washed away by wave action (due to the vibration response), and also when the light intensity is too great.

In the interactions of these responses controlling the vertical distribution of the animals *P. rubropharyngeus* behaves in a similar manner to *P. symbioticus* (GRAY 1966a, d).

Gregarious behaviour

Whereas *P. symbioticus* showed little evidence of gregarious behaviour (GRAY 1966d), *P. rubropharyngeus* is highly gregarious. A small pile of natural sand (1 cm diameter) was placed in each of seven small petri dishes containing sea-water. Ten animals were added to each dish and the animals were allowed to enter the piles of sand. Another pile of natural sand was then added to each dish, followed by approximately twenty animals. After 3 hours the sand and animals contained in each pile of sand were transferred by pipette to separate petri dishes and the animals were counted. If the animals were gregarious more would be expected to be found in the piles of

Table 2

Gregarious behaviour of *P. rubropharyngeus*. Choice between A (sand containing animals) and B (sand only)

	Sand A (containing animals)		Sand B (no animals)
Experiment number	Total number of animals in sand	Actual number in the experiment	Number of animals in sand
1	22	12	9
2	31	21	2
3	35	25	6
4	33	23	9
5	41	31	13
6	34	24	10
7	26	16	8
Observed total		152	57
Expected total if not gregarious		104.5	104.5
Observed/expected		1.45	0.55

Control	Both sands contained no animals		
		Number of animals	
		A	B
	Observed/expected	33 1.0	33 1.0

sand originally containing animals. Table 2 shows that this is the case. The animals move into the sand containing animals (sand A) in preference to sand with no animals (sand B), giving an observed/expected ratio of 1.45 for sand A, compared to 0.55 for sand B. In the control experiment with sand lacking animals the observed/expected ratio was unity. Thus *P. rubropharyngeus* is highly gregarious.

It was thought that this gregarious behaviour might be due to the animals themselves rendering the sand attractive. This hypothesis was tested by simple choice experiments. One sample of natural sand was treated by cleaning in concentrated H_2SO_4, and another by drying at 20^0 C. Each treated sand was then divided into two halves. To one half, 500 animals were added for 12 hours, and the other half was left without animals. After 12 hours the animals were removed from the sand in which they had been contained. One pile of treated sand which had contained animals and one pile which had contained no animals were placed in each of three small petri dishes containing seawater. Approximately twenty healthy specimens of *P. rubropharyngeus* were added to each dish and the dishes were left for 3 hours. Thus the animals had a choice of two similar types of sand, one of which had originally contained animals and the other which had contained no animals. At the end of each experiment the sand and animals contained in each pile were transferred by means of a pipette to separate petri dishes, and the animals were counted under a low power binocular microscope. Further experiments were conducted in a similar manner but giving the animals a choice of treated sand with or without the addition of animals, against natural untreated sand. The results are shown in Table 3.

Table 3, experiment 1 shows that the addition of the adult animals to acid-cleaned sand for 12 hours increased its attractiveness fourteen times (end column experiment 1) compared to acid cleaned sand which had not contained animals. This

Table 3

Gregarious behaviour of *P. rubropharyngeus*. Choice of animals between two types of sand

Experiment number	Type of sand in each experiment	Number of animals in each sand	Attractiveness index
1	Acid cleaned + 500 animals for 12 h	84	14.0[1]
	Acid cleaned no animals	6	
2	Acid cleaned + 500 animals for 12 h	21	0.33
	Natural sand	66	
3	Acid cleaned no animals	6	0.11
	Natural sand	54	
4	Dried sand + 500 animals for 12 h	21	0.18
	Natural sand	116	
5	Dried sand no animals	9	0.07
	Natural sand	125	

[1] This is not a true attractiveness index as a natural sand control was not used in this experiment.

acid cleaned sand to which animals had been added had an attractiveness index only one third that of natural sand (end column experiment 2). However, the attractiveness index was three times as great as acid cleaned sand which did not contain animals (end column of experiment 2 is 0.33 compared to 0.11 for experiment 3). Similarly, dried sand to which animals had been added (experiment 4) was two and a half times as attractive as dried sand without animals (0.18/0.07). The gregarious behaviour of *P. rubropharyngeus* is therefore due to a chemical substance produced by the presence of the adult animals which remains in the sand when the animals are removed, and yet serves to attract other members of the species.

GRANULOMETRY

BOADEN (1962) has shown in field experiments that *Protodrilus chaetifer* REMANE prefers sand of 210 to 150 μ diameter particles, whilst *P. adhaerens* JÄGERSTEN prefers particles coarser than 500 μ.

In simple choice experiments between graded sand and ungraded sand GRAY (1966b) found that *P. symbioticus* GIARD preferred an optimum grain size of between 200 to 300 μ.

The preference of both larvae and adults of *P. rubropharyngeus* for a specific grade of sand was tested. Sand from a favourable habitat of *P. rubropharyngeus* was separated into grades by passing it wet through a series of wire mesh sieves. The portion retained by each sieve was labelled and kept for use in a series of choice experiments. A quantity of unsieved sand was also kept and was used as a control.

Approximately forty healthy adult animals were placed in each of three petri dishes (7 cm diameter) containing seawater at 16° C ± 1° C. A pile of graded sand and a pile of natural sand (each 1 cm in diameter) were then added to each dish. After 3 hours in natural light the sand and animals contained in each pile were transferred to separate petri dishes and the animals counted. This experiment was repeated in duplicate for each grade of sand. The ratio of the number of animals in the graded sand to the number of animals in natural ungraded sand was determined for each grade and was termed the attractiveness index. In the case of the larvae, the experiments were left for 24 hours and at the end of the experiment the numbers of metamorphosed animals found in the graded and in the natural sand were compared as above to give an attractiveness index.

A quantity of natural sand was dried at 60° C for 48 hours and graded on standard wire mesh sieves. The portion retained by each sieve was weighed and expressed as a percentage of the total weight.

In Figure 1A the shaded histogram bordered by a solid line shows the percentage dry weight of the natural sand, whereas the upper histogram bounded by a broken line shows the attractiveness index for each grade of sand to the adult animals.

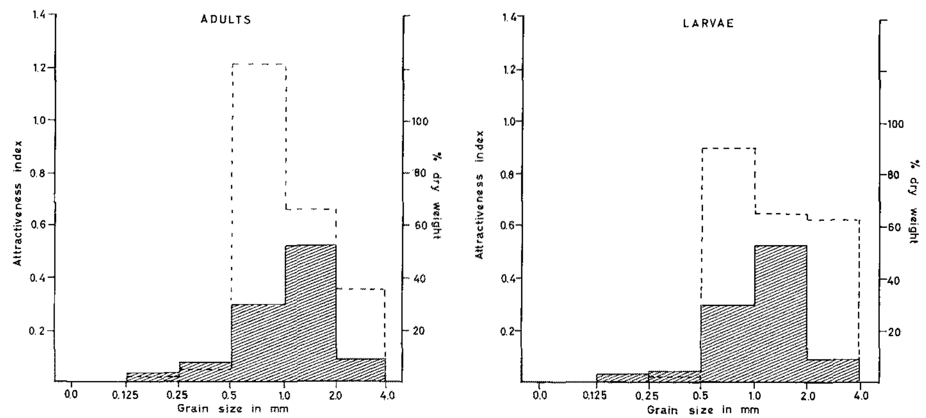

Fig. 1: Attractiveness of graded sand to *P. rubropharyngeus* compared to the granulometry of natural sand. Broken line: attractiveness index of each grade of sand. Solid line with shading: percentage dry weight of each grade of sand

Figure 1B shows the attractiveness index of each grade to the larvae, again compared to the percentage dry weight of each grade of sand. In each case, both adults and larvae chose a grade of sand between 0.5 to 1 mm diameter particles. Unlike *P. symbioticus* (Gray 1966b), which prefers the dominant grain size in its habitat, *P. rubropharyngeus* prefers a 0.5 to 1 mm grade to the dominant 1 to 2 mm grade. This choice preference will exclude the species from beaches with sands composed of particles finer than 0.5 mm diameter. To the author's knowledge *P. rubropharyngeus* near Fiskebäcksil is restricted to beaches with fairly coarse sand.

ATTRACTIVENESS OF A SUBSTRATE

Although *P. rubropharyngeus* prefers a grade of sand between 0.5 and 1 mm, within a substrate of uniform characteristics it is nevertheless localized in narrow areas. These localisations were presumed to be due to the presence of an attractive factor in the sand. By means of a series of simple choice experiments the nature of this attractive factor was determined.

Destruction of attractiveness

Quantities of substrate collected from an area of sand containing many *P. rubropharyngeus* were treated in various ways, washed in ten changes of sea-water and used in simple choice experiments. A quantity of treated sand was transferred by means of a pipette to each of three petri dishes (7 cm dia.) containing sea-water to form two piles (1 cm dia.) in each dish. Two similar piles of natural untreated substrate were then added to each dish, thus giving an equal choice of treated and untreated substrate. Approximately thirty healthy animals were added to the petri dishes and were left for 3 hours to select a substrate. After 3 hours the sand and animals contained in each pile of substrate were transferred by means of a pipette to separate petri dishes and the animals were counted. The total number of animals in the treated substrate was then compared to the total number of animals in the untreated substrate to give an attractiveness index for each sand.

In the case of the larvae the experiments were conducted in a similar manner except that smaller numbers of animals were used and they were left to choose and metamorphose in a substrate for 24 hours. In addition to the attractiveness index for each type of sand, the percentage metamorphosis of the larvae in each treated substrate was tested in a separate experiment. A number of larvae were placed in embryological cups containing seawater and substrate. After 24 hours the number of metamorphosed larvae were counted.

Table 4A shows that the attractiveness index of the treated substrates to the adults is reduced from one to almost zero by all the treatments. Distilled water, however, does not alter the attractiveness index appreciably. In Table 4B the attractiveness of the treated substrate to the larvae is reduced, but never to the low levels shown by the adults. Either the adults are more discriminating in their choice of substrate or the smaller number of animals used in the larval experiments did not show a true result. In both tables the attractiveness of natural sand is appreciably reduced by the various treatments.

Surprisingly the larvae metamorphosed in all the treated sands varying from 66 % in autoclaved sand to 100 % in alcohol – and formalin-treated sand. In the control with no sand no larvae metamorphosed whilst 96 % metamorphosed in natural

Table 4

Destruction of the attractiveness of sand to *P. rubropharyngeus*

Treatment of substrate	Number of animals used in test	Attractiveness index after treatment	% Metamorphosis after 24 h in this sand
Adults			
Alcohol (96 %) for 5 min	103	0.07	
Formalin (5 %) for 5 min	90	0.35	
Boiling for 10 mins in seawater	107	0.05	
Conc. H_2SO_4 for 5 min	137	0	
C.T.A.B. for 10 min	145	0.01	
Drying at 20° C	98	0.01	
Autoclaving at 1.5 Kgm/cm² for 15 min	110	0.04	
Conc. H_2SO_4 for 1 hr	105	0	
Incinerating	124	0	
Distilled water for 10 min	131	0.96	
Larvae			
Conc. H_2SO_4 for 5 min	16	0.33	96
Alcohol (96 %) for 5 min	19	0.20	100
Autoclaving at 1.5 Kgm/cm² for 15 min	25	0.35	66
Formalin for 5 min	20	0.30	100
Drying at 20° C	22	0.13	87
Incinerating	20		0
Conc. H_2SO_4 for 1 hr	20		0
Control with no sand	20		0
Control with natural sand	20		96

sand. Sand which had been incinerated and sand which has been treated with concentrated H_2SO_4 for 1 hour did not bring about any metamorphosis.

Reconstitution of attractiveness

Sand rendered highly unattractive by treatment with concentrated sulphuric acid and by drying at room temperature (with attractiveness indices of 0 and 0.01 respectively) was soaked in different media for 2 weeks. The simple choice experiments of treated sand against natural control sand were then repeated. Table 5A shows the results for the adult worms. With dried sand the attractiveness is completely returned after two weeks' soaking, becoming more attractive than natural sand. This may be due to the natural sand declining in attractiveness during storage.

In the case of the acid cleaned sand, however, only soaking in normal seawater brought about a complete restoration of attractiveness.

Table 5B shows the effect on the restoration of attractiveness to larval *P. rubropharyngeus* of soaking three different substrates in different media. Once again dried sand completely regained its attractiveness even on being soaked in distilled water.

Table 5

Reconstitution af attractiveness of sand to *P. rubropharyngeus*

Type of sand and treatment	Number of animals used	Attractiveness index before treatment	Attractiveness after treatment	% Metamorphosis before treatment	% Metamorphosis after treatment
Adults					
Dried at 20° C soaked in : –					
distilled water for 2 wk	129	0.01	5.1		
normal sea water for 2 wk	70	0.01	2.9		
boiled sea water for 2 wk	70	0.01	7.9		
Acid cleaned sand soaked in: –					
distilled water for 2 wk	68	0	0.5		
normal sea water for 2 wk	100	0	2.7		
boiled sea water for 2 wk	113	0	0.3		
Larvae					
Dried at 20° C soaked in : –					
distilled water 4 wk	17	0.13	1.12	87	100
autoclaved sea water 4 wk	11	0.13	1.20		
Acid cleaned soaked in: –					
distilled water 4 wk	13	0.33	0.18	96	100
autoclaved sea water 4 wk	20	0.33	0.54		
Autoclaved soaked in: –					
distilled water 4 wk	18	0.35	0.38	66	100
autoclaved sea water 4 wk	19	0.35	0.36		

However, acid-cleaned sand and autoclaved sand do not regain their full attractiveness on soaking in either distilled water or autoclaved seawater for 4 weeks. The metamorphosis of the larvae was 100 % in dried, acid cleaned and autoclaved sand soaked in distilled water.

INOCULATION OF BACTERIA TO STERILE SAND

Cultures of natural sand bacteria were obtained by shaking 1 gm of natural sand with 10 ml of sterile seawater for 10 minutes. Small samples of the solution obtained were smeared on the surface of agar plates (1.2 % Agar 0.3 % peptone in sterile seawater). These plates were incubated for 48 hours at 22° C, by which time the plates were covered with a bacterial colony.

20 gms of sand was added to each of nine 250 ml flasks with tight cotton wool plugs, containing 100 mls of seawater. The flasks were the autoclaved at 1.5 kg/cm^2 for 30 minutes and allowed to cool. Seven were inoculated with a wire loopful of bacterial culture, the remaining two acting as controls. The flasks were incubated at 22° C in complete darkness.

The sand from one flask was taken and tested for attractiveness to adult *P. rubropharyngeus* in a simple choice experiment against natural sand following the method described earlier. The sand in the remaining flasks was tested for attractiveness at daily intervals. The controls were tested on the first and last day of the experiment.

0.5 gm of sand were taken from each flask at daily intervals and added to 10 ml of sterile sea-water in a screw capped bottle. The bottle was shaken vigorously for 10 minutes and 1 ml of the resulting suspension added to 9 ml of sterile seawater. This serial dilution of 1 ml of solution to 9 ml of sterile seawater was repeated six times. From each dilution 0.05 ml were taken by means of a sterile pipette and smeared onto a sterile agar plate (0.3 % peptone 1.2 % agar). These plates were incubated for 48 hours at 22° C. Assuming one visible colony had grown from one bacterial cell contained in the original inoculated sand, the number of bacteria per gm of sand could be calculated by counting the individual colonies on each plate.

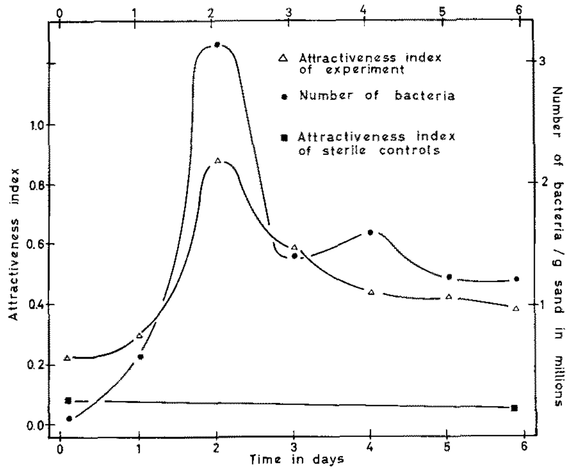

Fig. 2: Increase in attractiveness of autoclaved sand to *P. rubropharyngeus* produced by inoculating with a culture of natural sand bacteria

Figure 2 shows the results of this experiment. The triangles show the increase in attractiveness and the points increase in bacterial numbers. The squares represent the controls which contained no bacteria. The increase in attractiveness correlates closely with the increase in bacterial numbers. However the attractiveness index never approaches 1.

It was not possible to repeat this experiment using the larvae as they were very scarce at this time.

As the animals were previously found to be highly gregarious it was thought that the increase in the attractiveness index of a substrate produced by inoculating unattractive sand with bacteria might be increased further by adding adult animals to inoculated sand. This in fact did increase the attractiveness slightly.

Sand autoclaved at 1.5 kgm/cm² for 20 minutes was inoculated with bacteria and cultured for 3 days at 22° C. Half of this sand was then tested for attractiveness against natural sand in two simples choice experiments using adults in one experiment and larvae in the other experiment. Approximately 500 adult animals were then added

Table 6

Increase in attractiveness on adding adult *P. rubropharyngeus* to sand inoculated with a culture of natural sand bacteria. Attractiveness index of sand

Animals	Inoculated sand	Inoculated sand + 500 animals for 24 h
Adults	0.69	0.77
Larvae	0.52	0.65

to the remaining half of the inoculated sand. After 24 hours the animals were removed from the sand. This sand was then tested for attractiveness against natural sand using both adults and larvae in two experiments. The results are shown in Table 6 and show that adding adult animals to sand inoculated with a culture of natural sand bacteria does increase the attractiveness index of the sand.

SPECIFICITY OF BACTERIA

As the types of bacteria present in marine sand are almost unknown, cultures of known species were obtained from the National Collection of Marine Bacteria, Torry Research Station, Aberdeen. These were *Serratia marinorubra* (NCMB No. 4), *Pseudomonas* sp (NCMB No. 129), *Corynebacterium erythogenes* (NCMB No. 5) and *Flavobacterium* sp (NCMB No. 411). Each culture was inoculated onto sand in seawater previously sterilised at 1.5 kgm/cm^2 for 20 minutes. A culture of natural sand bacteria was inoculated onto a further sterilised sand sample to act as one control and one flask was kept sterile to act as the other control. After incubating for 3 days, samples of sand from each flask were placed in piles (1 cm diameter) in each of three petri dishes (10 cm diameter) containing seawater. Approximately 70 healthy adult animals were added to each dish. After 3 hours under artificial illumination the sand and animals contained in each pile were transferred to separate petri dishes and the animals were counted. The numbers of bacteria contained in the sand in each flask were estimated using the serial dilution method described earlier. Table 7A shows the results from two experiments using adult animals. Both experiments gave closely similar results. Natural sand bacteria (1.0 on the index scale) render the sand twice as attractive as *Pseudomonas* (0.34 and 0.59) and *Flavobacterium* (0.41 and 0.48) and three times as attractive as *Serratia marinorubra* (0.23 and 0.31). The latter three species however render the sand more attractive than *Corynebacterium erythogenes* (0.06 and 0.04) and the sterile control sand (0.01 and 0.02).

Consideration of the numbers of bacteria per gm of sand in relation to the attractiveness indices of each sand does however indicate a positive correlation. So the differences in the attractiveness indices might be due to quantitative rather than qualitative differences between the different sands. However the numbers of bacteria per gm of sand do not differ appreciably whereas the attractiveness indices are widely different. Gray (1966c) has shown that in establishing a surface film on sterile sand grains there is initially a positive correlation between the increase in attractiveness

Table 7

Choice of *P. rubropharyngeus* between sands inoculated with different species of bacteria

Sand inoculated with bacterial species	Attractiveness index relative to natural sand bacteria	Number of bacteria/gm	Attractiveness index relative to natural sand bacteria
Adults			
Serratia marinorubra	0.23	1.5×10^6	0.31
Pseudomonas sp.	0.34	7.7×10^6	0.59
Corynebacterium erythogenes	0.06	2.5×10^5	0.04
Flavobacterium sp.	0.41	6.2×10^5	0.48
Natural sand bacteria	1.0	1.7×10^7	1.0
Sterile control	0.01	0	0.02
Larvae			
Serratia marinorubra	0.13		
Pseudomonas sp.	1.26		
Corynebacterium erythogenes	0.51		
Flavobacterium sp.	1.34		
Natural sand bacteria	1.0		
Sterile control	0.21		

and the increase in the number of bacteria present in the sand. Once the surface film is established this correlation disappears and the differences between bacterial types are then of greater importance than differences in number of bacteria. Thus it is possible in this case that the surface film on the sand grains is not completed and the preference for certain species of bacteria is masked by small quantitative differences in the numbers of bacteria present. Further experiments would have been desirable to clarify this point.

Table 7B shows the preferences of the larvae in a similar experiment. Here the larvae selected both *Pseudosmonas* (1.26) and *Flavobacterium* (1.34) in preference to natural sand bacteria (1.0). Consideration of the choice preferences of the adults and larvae together, by ranking the attractiveness indices of both experiments, shows a correlation at the 7 % level of significance (using Spearmans Rank Correlation).

Thus it seems likely that both adults and larvae of *P. rubropharyngeus,* like adult *P. symbioticus* (Gray 1966c), select substrates in relation to the number and species of bacteria present in the sand.

THE RELATIONSHIP BETWEEN *PROTODRILUS RUBROPHARYNGEUS* AND THE SUBSTRATE

Like *P. symbioticus* (Gray 1966a, d), *P. rubropharyngeus* moves to the sand surface by means of a negative geotaxis and shows preference for areas of sand with the highest oxygen tension, whilst avoiding the immediate surface layers under strong illumination. In the case of *P. rubropharyngeus* a positive geotaxis on stimulation by vibrations (by wave action in nature), further contributes to keeping the animals just below the surface at a depth compatible with their reactions to oxygen tension, gravity and light.

P. rubropharyngeus adults and larvae prefer a grade of sand between 0.5 and 1 mm diameter particles. As was the case with *P. symbioticus* (Gray 1966b) this preference will tend to limit the horizontal distribution of the species. However, within a substrate predominantly of the 0.5 to 1 mm grade *P. rubropharyngeus* is localised in narrow areas of the beach. This localisation of a population is probably achieved by selection of the substrate in response to an attractive property either by larvae or adults or in part by both.

Jägersten (1940) found that only boiling the natural sand for 3 to 4 hours or keeping it in fresh water for three weeks destroyed the property of inducing metamorphosis, which was present in natural sand. He found that treating natural sand with alcohol, formalin and concentrated acids did not change the metamorphosis inducing character of the sand.

My experiments on metamorphosis (although not as complete as is desirable due to the scarcity of the larvae) agree fairly well with those of Jägersten. Concentrated H_2SO_4 for 5 minutes, alcohol and formalin treatments had little or no effect on the ability of the sand to induce metamorphosis. However, incineration and concentrated acid treatment for 1 hour, unlike the results obtained by Jägersten, destroyed the ability of the sand to induce metamorphosis. Autoclaving sand for 15 minutes at 1.5 kg/cm^2 reduced the percentage metamorphosis by 33 %. The validity of the metamorphosis experiments does, however, seem doubtful, as the larvae will metamorphose in an unfavourable substrate if a favourable substrate is not present. The more reliable results obtained from giving the larvae a choice between treated and untreated sand (Table 4B) show clearly that concentrated H_2SO_4, alcohol, formalin, autoclaving and drying treatments reduce appreciably the attractiveness of the sand to the larvae. The same treatments (Table 4A) render the sand even less attractive to the adults. Thus the attractive factor to both larvae and adults is a property of the sand grain surfaces. The experiments on the restoration of attractiveness (Table 5A and B) suggest that as dried sand can be restored to its original level of attractiveness by merely soaking in distilled water the attractive factor is organic and can be rehydrated. When the attractiveness was completely destroyed by acid-cleaning, only normal sea-water could reconstitute the attractiveness. The response of *P. symbioticus* to the same chemical treatments of the substrate gave results similar to those recorded above (Gray 1966c).

Inoculation of bacteria onto unattractive sand was shown to increase the attractiveness of the substrate to both *P. symbioticus* (Gray 1966c) and *P. rubropharyngeus* (Fig. 2). Whereas, in the case of *P. symbioticus* the full attractive property of unattractive sand could be restored simply by inoculating it with natural bacteria, this was not the case with *P. rubropharyngeus. P. rubropharyngeus* is highly gregarious (Tables 2 and 3) and when in contact with sand the animals render the sand attractive to other members of the species. Thus, a combination of sand inoculated with bacteria and the presence of the adult animals gives an almost complete restoration of the attractive property of unattractive sand, (Table 6). The experiments on bacterial specificity suggest that, as in the case of *P. symbioticus* (GRAY 1966c) only certain types of bacteria can render the sand attractive.

Selection of a substrate by *P. rubropharyngeus* therefore occurs in both larval and adult stages. Sand grains predominantly of 0.5 to 1 mm diameter, a favourable number of bacteria of the right type forming a film on the sand grain surfaces and the presence of the adult animals themselves attracts *P. rubropharyngeus* to narrow areas of a beach. Thus *P. rubropharyngeus* and *P. symbioticus* (GRAY 1966c) select substrates in very similar ways and the larvae of *Ophelia bicornis* Savigny (WILSON 1955), *Nassarius obsoletus* Say (SCHELTEMA 1960) and adult *Corophium* (MEADOWS 1964) are probably attracted to substrates in the same manner.

SUMMARY

1. *Protodrilus rubropharyngeus* JÄGERSTEN, a marine interstitial archiannelid, was found to move to the surface layers of sand in response to a negative geotaxis and preference for areas of highest oxygen tension.
2. Strong light and vibrations tend to keep the animal just below the sand surface except on calm days.
3. The adults were found to be highly gregarious.
4. Both adults and larvae showed a preference for the 0.5 to 1 mm grade of sand.
5. The localisation of high numbers of animals in narrow areas of a uniform beach seems to be related to the presence of a localized surface film on the sand grain surfaces. This film is produced by certain favourable species of bacteria, and together with a chemical produced by the animals themselves, attracts other members of the species to this sand.

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Discussion following the paper by GRAY

PANDIAN: You have shown that there is a direct relationship between the bacterial content and the index of attractiveness of the polychaete. If I remember correctly, T. SRINIVASAN (1965) in his Ph. D. thesis (Madras University, India) has shown that isoleucine and leucine present in the excretory material of the adult attracts the larvae to settle. In view of this work, will you be led to think that some substances secreted by the bacteria may enhance the index of attractiveness of the polychaete?

GRAY: I did not know of this work and I am grateful to you for bringing it to my notice. I cannot say if a substance produced by the bacteria does attract the adults and larvae or not, as here substrate selection was always exhibited after contact with the sand. If the bacteria are removed from the sand, the sand remains attractive suggesting that the surface film is still attractive. This is undoubtedly a chemical attraction.

CRISP: Can you explain why in some of the inoculation experiments, the attractiveness increased rapidly but subsequently fell to a lower value? In natural sands the attractiveness is presumably a permanent feature.

GRAY: If the surface film on the sand grain surfaces has not been fully established, then a relationship between the attractiveness index and the number of bacteria is apparent. The falling level of the attractiveness index in my graph, which subsequently rises, is I think due to the fact that the surface film is not yet fully established, and the curve thus follows closely the curve for the numbers of bacteria in the sand.

WEBB: It may be important that the presence of different microscopic animals on the sand grain surface profoundly alters the spatial organization of the grains and in thus affecting permeability could influence the availability of oxygen.

GRAY: Yes, I agree it is possible that the packing of the substrate and its oxygen content may be altered by placing it in a petri dish. However, I feel that the large differences in the attractiveness index shown by the different chemical treatments are more important than small differences in oxygen content. The point you make is an interesting one, which I should consider.

B.-O. JANSSON: What is your opinion of grain size as an environmental factor in regard to its room-restricting properties? Do you think it is of greater importance than, for example, temperature, salinity or oxygen?

GRAY: I have not made any observations on the importance of grain size preferences in association with other factors such as temperature or light. I cannot give an answer to your question I am afraid.

BOADEN: Is there exploratory behaviour? Do the animals enter the treated sands if unsuitable or do they proceed directly to the attractive sands?

GRAY: On contacting unattractive substrates the animals do not enter the substrate but sample it with the anterior end and then withdraw the head and try in another direction. With attractive substrates the behaviour is entirely different and the animals enter the substrate gliding over it and do not leave it.

WIESER: Is the attractiveness of bacteria entirely due to *Protodrilus* feeding on them or is there some other chemoreceptor mechanism involved?

GRAY: Certainly *P. rubropharyngeus* feeds on bacteria, but it is very difficult to assess whether the attractiveness of a sand is solely related to the numbers of bacteria. If sand is shaken and most bacteria are removed leaving the film behind, the attractiveness index is not altered. So it appears that the attractive property acts chemically rather than nutritionally.