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THE ROLE OF MICRO-ORGANISMS IN THE SETTLEMENT OF *OPHELIA BICORNIS* SAVIGNY

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During the course of earlier work with larvae of *Ophelia bicornis* Savigny it became increasingly clear that a major factor in stimulating these larvae to settle and metamorphose is the presence, on sand grains of suitable size, of living micro-organisms such as bacteria, in numbers neither too many nor too few (Wilson, 1954). It had been shown that an acid-cleaned sand (which is neutral or almost so) kept in sea water becomes increasingly attractive to these larvae with time. It had been admitted, however (*loc. cit.*, p. 366), that these results were derived from a comparison of experiments not designed to this end, and that further tests, planned for the purpose, were desirable.

Methods were identical with those of 1953 (Wilson, 1954), except that the relative abundance of the unmetamorphosed, metamorphosing and metamorphosed larvae in each sample as expressed in words in the tables was in 1954 based on random partial counts, giving a probable slight increase in accuracy of assessment. Unmetamorphosed larvae were not counted except when sticking to a sand grain, while metamorphosing larvae were recorded as in early, mid or late metamorphosis. These stages are not usually shown in the tables but are referred to in the text wherever the stage of metamorphosis is a matter of interest.

Fertilizations and experiments were mostly in a mixture of sea water from the International Hydrographical Station E 1, and from the Clyde (by kind co-operation of the Millport Marine Station). Except when otherwise stated, all sea waters were passed through Berkefeld filter candles before use. The larvae used in the tests were always 5 days old.

The Experiments

In preparation for the experiments some Bullhill Bank sand which had been collected on 10 June 1952 was thoroughly cleaned in hot concentrated sulphuric acid on 21 April 1954, washed, and stored in distilled water. Such acid-cleaned sand is almost white in colour. On various dates subsequently, as noted in the description of each experiment, a quantity was removed from the distilled water, washed with filtered sea water and strewn thinly over the bottom of one or more covered glass dishes filled with filtered sea water. These were left on a bench shielded from direct light from the sky. The water in some dishes was changed a number of times at intervals of days or weeks; in others it was not changed. To a few dishes a little peptone sea water (Spencer, 1952, p. 98) was added from time to time to encourage bacteria. Further quantities of sand were similarly soaked for varying periods in unfiltered water from the laboratory circulation (tank water), sometimes mixed with mud from the bottoms of the tanks.

Each experiment was divided into two sections. Each section comprised a set of sands tested together in the same free-choice dish and often separately in conical vessels as well. The ability of the larvae to metamorphose was checked for each experiment by a control dish strewn with fresh Bullhill Bank sand. In these control dishes metamorphosis was invariably almost 100%.

Experiment 1 (*Table I*)

Section A. Four samples of acid-cleaned sand soaked in sea water for different lengths of time and one sample soaked in tank water were tested against a sample of the same acid-cleaned sand which had been stored in distilled water throughout the experiment (see Table I for dates and other details). It will be seen from the table that the longer the sand had soaked in sea water the bigger the settlement which was obtained. These soaked sands were observed to have numbers of unidentified organisms on and among their grains. Some of the organisms were almost certainly algal; bacteria would not be seen at the magnification used. All the sands were clean, but that soaked since 11 June 1954 (sample 4 in Table I, section A) had more 'algal' growths than the others. The sand from distilled water was perfectly clean.

Section B. The acid-cleaned sand samples tested here had been soaked in tank water, in one instance with the addition of tank mud, or in sea water to which peptone had been added. Some of these waters and media were changed from time to time. All the sands were discoloured by organic growths, the tank water sands being various shades of brown owing to algal growths, including diatoms. The sand from sea water with peptone medium was grey, even after washing well, and showed fluffy growths on some grains. While all these sands (after rinsing well in sea water) attracted more larvae to settle than did the sand straight from distilled water it does not appear that they were as effective as some of the cleaner sands in section A. A direct comparison with that section cannot, however, be made.

Experiment 2 (*Table II*)

Section A. It is known from earlier work that natural sands after washing and drying, or after sterilization in water at about 100° C, lose any attractive property they may have had in the fresh state, and some of them become repellent. In this series of tests acid-cleaned sands which had become attractive after soaking in sea water for several weeks (samples 2 and 5) were likewise shown to lose their acquired attractiveness by these treatments, and one of them after treatment (sample 6) seems to have become repellent. This sand from tank water and mud (sample 5), had, before washing and drying, unidentified flocculent growths on the sand grains, which were visibly more overgrown than the grains of the sand which had been soaked in clean filtered sea water (sample 2) and which appeared to be clean, except for a few small growths seen on some grains before treatment. After treatment these had disappeared, although a few dead growths were still to be seen on the grains comprising samples 6.

Section B. These tests showed that washing and drying had little or no effect on acid-cleaned sand from distilled water, so far as larval settlement is concerned, but again demonstrated that acid-cleaned sand which had acquired some degree of attractiveness by soaking in sea water lost this property after washing and drying.

Experiment 3 (*Table III*)

Section A. It was again shown that acid-cleaned sand gains in attractiveness during soaking in sea water but that this soaking has to be prolonged for several weeks to be effective. The gain after only 1 week (sample 2) was very slight, after 3 months (sample 3) very marked. Treatment with alcohol (sample 4) or formalin (sample 5) destroyed the attractiveness (the fixatives were well washed out first with fresh water, then with sea water), but there is no evidence that this particular sand had been made repellent by these treatments.

Section B. Fresh Bullhill Bank sand, collected a few days before the experiment began, was soaked for 4 days, 2 days and 1 day respectively in sea water to which peptone had been added. The peptone induced heavy growths of bacteria, especially so after 4 days, and all these sands had a pronounced smell which disappeared during rinsing in sea water before the tests were made.

From Table III it will be seen that the longer the sand had been in peptone sea water, the less attractive it was. This is even more evident if the state of the metamorphosing larvae in the conical vessels be considered. Most of those in sample 2, which had been 4 days in peptone sea water, were early and mid-metamorphosis stages, but in sample 3, which had been 2 days in peptone sea water, there was a smaller proportion of early stages and a decidedly larger proportion of late stages. In sample 4, exposed in peptone sea water for only 1 day, the metamorphosing larvae were almost without exception late stages.

Table III also shows that the fresh Bullhill Bank sand retained its attractiveness more effectively in sea water than when stored (in a stoppered jar) in its natural moist state, as collected at low tide. It is known from earlier work that the attractive property gradually diminishes when such sand is so stored.

Experiment 4 (*Table IV*)

Section A. Samples of acid-cleaned sand were soaked in sea water containing peptone, some of them for several weeks. One sample was kept sterile, in all the others bacterial growths took place and were heavy in those immersed for more than a day. Sample 2, which had been soaking longest with changes of the medium, was discoloured dark grey. This sand had also some flocculent brown growths, not present in the other sands which were relatively clean after rinsing before use although small organisms on the grains could be seen here and there.

The settlements obtained with these sands, after rinsing in sea water, are shown in Table IV. All (samples 2–5) attracted more larvae than the sand from sterile concentrated peptone sea water (sample 1), but except for sample 3 which had soaked for 10 days the settlements were not really heavy. Sample 3 produced a good settlement but the sands immersed for longer or shorter periods did not. Moreover, the metamorphosing larvae in sample 3 in the conical vessel experiment were mainly mid and late metamorphosis stages; while in all other samples, including 6, the metamorphosing larvae had a higher proportion of early stages and a smaller proportion of late. From this it would seem that acid-cleaned sand in peptone sea water increases in attractiveness for a time and then decreases (but compare Expt. 5 B). This result should also be compared with that obtained for fresh Bullhill Bank sand (Expt. 3 B) kept in peptone sea water; only a decrease in attractiveness was then recorded.

Another portion of sand from sample 2 was washed in tap water and sterilized by heating almost to boiling-point (sample 6). The attractiveness was less than the sand not so treated, but it did not become repellent. The sand grains were cleaner than the unsterilized grains in sample 2, in particular the flocculent growths were no longer visible. In the conical vessel test of this sand most of the metamorphosing larvae were relatively early stages, there being very few late stages and a smaller proportion than in sample 2.

Section B. The four sand samples tested here comprised two distinct and unrelated tests. In the first test an acid-cleaned sand soaked in sea water for about a month (Table IV B, sample 1) was compared with a similar acid-cleaned sand soaked in sea water plus 'B.B. water' (see Wilson, 1954, p. 363) for over a year (sample 2). The latter sand had been used in the 1953 series of experiments, but had been kept since then with only a single addition of fresh filtered sea water, in March 1954. Both these sands, compared with the control sample 4 from distilled water, were attractive to the larvae but that which had been soaked for a year was much more attractive than that which had been in sea water for only a month. This year-old sand was seen to have minute diatoms, other algae, a few flagellates, and apparently bacterial slimes on the grains, whereas the other sands were clean. It was not as dirty, however, as sample 2 in section A of this experiment, and was not noticeably discoloured.

The second comparison was between an acid-cleaned sand (sample 4) and similar sand (sample 3) which had been enclosed within a bolting silk envelope and buried in Salthouse Lake (Station II) sand which had previously been heated in tap water near to boiling point to ensure maximum repellence. The test was to determine if any part of the repellent property of a repellent sand can be transferred to a neutral sand in close proximity though not in contact with it. Some earlier experiments had indicated that such transference, if it occurs at all, takes place more readily when physical contact between grains is allowed (Expt. 43, Wilson, 1953*a*) and doubtfully so when the two sands are separated by bolting silk (Wilson, 1953*b*). But in these earlier experiments the sands used were fresh and unsterilized, and the attractive factor would therefore be present as well. In the present test this would not be so. The result (Table IV B) gives no indication that any part of the repellent factor was transferred. In the free-choice dish no significant difference was discernible, while comparison of the conical vessel tests suggests that sample 3 was a little more attractive than the control (sample 4). This result is explicable if it be assumed that the repellent factor consists of organic matter, which after heating is too adherent to the grains to be passed through the meshes of bolting silk. Bacteria would be able to pass through the meshes, and it is possible that the neutral sand within the silk envelope would receive bacteria from those that would multiply on this organic matter after the heated sand, heaped around the silk envelope, had been in sea water for a short time. Thus the neutral sand after receiving adherent living bacteria, and perhaps other organisms, would be slightly attractive.

Experiment 5 (*Table V*)

Section A. This was a repetition of Expt. 3 B after the sands had been kept for another 6 days. The result (see Table V) was much as before, no further marked decrease in attractiveness of the originally fresh Bullhill Bank sand having taken place, perhaps because the grains in the earlier experiment already carried as many micro-organisms as could be accommodated on their surfaces. However, the metamorphosing larvae in sample 4 from the conical vessel were not almost all late stages as they had been in the corresponding sample in Expt. 3 B, and in general there was detectable a further slight decline in attractiveness.

Section B. Some of the acid cleaned sands tested in Expt. 4 A after immersion for varying periods in peptone sea water (medium not changed) were re-tested after a further period had elapsed. The result (Table V B) is much as before (Table IV A), there not being any definite increase in attractiveness of sample 3, and especially sample 4, such as might have been expected. Sample 2 was, as before, more attractive than either. These results indicate that acid-cleaned sand kept in peptone sea water does not necessarily show a regular increase in attractiveness with time when kept in this medium, as seemed to be implied by Expt. IV A. It is not known if the organisms encouraged by the peptone were the same species for all samples and the observed difference in settlement between the various sand samples could relate to a specific difference between the organisms grown, or to their proportional abundance. It may be noted that all sands looked nearly quite clean, only a few unidentified growths being noted on some grains. The sand of sample 4 definitely had fewer visible growths on the grains than did samples 2 and 3. No such growths were seen on any grains from sample 1.

Discussion

A consideration of the 1954 experiments taken in conjunction with those of previous years makes it clear that the factor most active in inducing settlement of *Ophelia bicornis* larvae is the presence, on the sand grains, of living micro-organisms. The organisms must be neither too few nor too abundant. Presumably for a sand to attain maximum attractiveness they must be of particular species in certain relative abundance. In the experiments in which acid-cleaned sands were soaked in filtered sea water it is most unlikely that the latter requirements were even approximately fulfilled; indeed it seems probable that the species of organisms which grew under those conditions were mostly not the same and were not in the same relative abundance as those present on sand grains freshly gathered from the surface of the Bullhill Bank. None the less, after several weeks of soaking in sea water, they did impart a very considerable degree of attractiveness to acid-cleaned sand. No other explanation of the observed results seems possible.

The period needed to impart a marked attractiveness to acid-cleaned grains was very considerably shortened by adding peptone sea water to the filtered sea water in which they were immersed, but this never made the sand as attractive as did some of the prolonged soakings in filtered sea water without the addition of nutrients. Presumably the grains after immersion in the peptone sea water would become coated with micro-organisms different from those that grew slowly in pure filtered sea water, and they may well have become too abundant to attract very many larvae.

It was observed that fresh Bullhill Bank sand after immersion in peptone sea water became less attractive than before, especially after a heavy growth of bacteria had taken place. These observations support the conclusion that the quantity and quality of the living organic film on the grain surface is of first rate importance to the settling *Ophelia* larva. To the identity of the species concerned and their actual abundance there is at present no clue.

This series of studies has been another demonstration of the influence of the minutest micro-flora and fauna on the distribution and activities of larger microscopic animals and ultimately on the macro-fauna itself. They suggest that more attention could profitably be paid to the most minute living constituents of bottom deposits and their interrelations with the larger organisms subsisting in or on those soils.

The organic films left on natural sand grains after washing and drying are undoubtedly to a large extent responsible for the floatation properties of dried grains, discussed in previous papers (see especially Wilson, 1952). Less than one per cent of acid-cleaned grains stored in distilled water float when sprinkled dry on the surface of water, but during the course of the experiments recorded in this paper it was found that after some days of immersion in sea water a markedly higher percentage floated (after washing and drying). After several weeks or months the percentage of floatable grains was still higher and in one instance was over 90%. The percentage floatability of acid-cleaned sand rose steeply and more quickly during immersion in peptone sea water, and the floatation of fresh Bullhill Bank sand was also raised by immersion in peptone sea water. That flotation can be caused by electrical charges produced on the grains by rubbing is probably also true, but is likely to have little bearing on problems concerned with settlement reactions.

It seems certain that dead organic matter coating sand grains is repellent to *Ophelia bicornis* larvae and renders many sands such as Salthouse Lake sand (Stations I and II), after the silt has been washed away, distasteful to these larvae. The repellent effect is likely to be most marked if there are no living micro-organisms present as well, as when sands have been sterilized in water at 100° C, or after fixation or drying. Sands will thus vary in attractiveness and repellence according to the number and kinds of living micro-organisms, and according to the amount and quality of the non-living organic materials coating the grains. Grain size, and perhaps also shape, is another and lesser factor influencing larvae about to settle, but is not the main factor as was earlier thought (Wilson, 1948). That grade does exert some influence has often been demonstrated (Wilson, 1953*b*).

Summary

Experiments in the summer of 1954 made it clear that the factor most active in inducing metamorphosis and settlement of *Ophelia bicornis* Savigny larvae is the presence on sand grains of living micro-organisms, such as bacteria, and that these should be neither too few nor too abundant. It would appear likely, and for this there is some evidence, that certain species are more effective in promoting settlement than are others, though there is at present no clue to the identities of the species concerned. Dead organisms and non-living organic matter are not attractive and may indeed be repellent, as a consideration of some of the results suggests.

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TABLE I. EXPERIMENT 1

Begun 5. vii. 54 with larvae 5 days old. Results on 7. vii. 54

Section A

Acid-cleaned Bullhill Bank sand soaked in

	(1) Distilled water since 21. iv. 54	(2) Filtered sea water, unchanged since 23. iv. 54	(3) Filtered sea water, changed 4 times since 23. iv. 54	(4) Filtered sea water, changed twice since 11. vi. 54	(5) Filtered sea water, unchanged, since 26. vi. 54	(6) Unfiltered tank water unchanged since 26. vi. 54
	Settlements after 2 days in free-choice dish					
Metd	None	Many	Many	Fair number	Very few	Very few
Meting	1 or 2	Many	Fair number	Many	Very few	Few
Unmet.	Very few	Fair number	None	Good number	Several	Fair number
	Settlements after 2 days in conical vessels					
Metd	Few	Very many	Very many	Fair number	—	—
Meting	Several	Many	Good number	Very many	—	—
Unmet.	Fair number	Several	Several	Good number	—	—

Section B

Acid-cleaned Bullhill Bank sand soaked in

	(1) Distilled water since 21. iv. 54	(2) Unfiltered tank water, changed 4 times since 23. iv. 54 (sand dark brown)	(3) Unfiltered tank water, unchanged since 23. iv. 54 (sand pale brown)	(4) Unfiltered tank water and mud, changed 4 times since 23. iv. 54 (sand brown)	(5) Filtered sea water containing peptone, changed 3 times since 23. iv. 54 (sand grey)
	Settlements after 2 days in free-choice dish				
Metd	None	Few	Fair number	Fair number	Several
Meting	Very few	Several	Several	Few	Several
Unmet.	Several	Good number	Good number	Several	Fair number
	Settlements after 2 days in conical vessels				
Metd	See A 1 above	Very few	Several	Good number	Many
Meting	—	Few	Fair number	Fair number	Many
Unmet.	—	Several	Many	Several	Few

Metd = metamorphosed; meting = metamorphosing; unmet. = unmetamorphosed.
Scale of words used to express number in Tables I–V:

Very few (sometimes also expressed in numbers 1–4)
Few
Several
Fair number
Good number
Many
Very many
Multitude
Great multitude

TABLE II. EXPERIMENT 2

Begun 10. vii. 54 with larvae 5 days old. Results on 12. vii. 54

Section A

	Acid-cleaned Bullhill Bank sand soaked in					
	(1) Distilled water since 21. iv. 54	(2) Filtered sea water, unchanged since 23. iv. 54 (sand almost white)	(3) Sand as (2) washed and dried	(4) Sand as (2), sterilized at about 100° C	(5) Unfiltered tank water and tank mud, changed 4 times since 23. iv. 54 (sand dark brown)	(6) Sand as (5), washed and dried
	Settlements after 2 days in free-choice dish					
Metd	None	Many	None	None	Good number	None
Meting	None	Many	None	Very few	Good number	None
Unmet.	Fair number	Several	Fair number	Good number	Fair number	Very few
	Settlements after 2 days in conical vessels					
Metd	Very few	Many	Very few	Few	Many	None
Meting	Few	Many	Few	Very few	Fair number	None
Unmet.	Fair number	Fair number	Fair number	Fair number	Fair number	Very few

Section B

	(1) Acid-cleaned Bullhill Bank sand in distilled water since 21. iv. 54	(2) Sand as (1), washed and dried	(3) Sand as (1) in filtered sea water, unchanged since 26. vi. 54	(4) Sand as (3), washed and dried
	Settlement after 2 days in free-choice dish			
Metd	None	One	Several	None
Meting	None	One	Several	None
Unmet.	Fair number	Fair number	Fair number	Several
	Settlements after 2 days in conical vessels			
Metd	See A 1 above	None	Several	None
Meting	—	Several	Several (early to late)	Several (all early)
Unmet.	—	Fair number	Fair number	Fair number

TABLE III. EXPERIMENT 3

Begun 21. vii. 54 with larvae 5 days old. Results on 23. vii. 54

Section A

	Acid-cleaned Bullhill Bank sand soaked in				
	(1) Distilled water since 21. iv. 54	(2) Filtered sea water, unchanged since 14. vii. 54	(3) Filtered sea water, changed 4 times since 23. iv. 54	(4) Sand as (3) fixed in alcohol for 1 h	(5) Sand as (3) fixed in neutral formol for 1 h
	Settlements after 2 days in free-choice dish				
Metd	None	None	Many	Very few	None
Meting	None	Very few	Many	Very few	Very few
Unmet.	Fair number	Good number	Fair number	Good number	Several
	Settlements after 2 days in conical vessels				
Metd	Very few	Several	Very many	None	Few
Meting	Fair number	Several	Good number	Fair number	Many
Unmet.	Many	Many	Several	Many	Fair number

Section B

	Fresh Bullhill Bank sand (collected 15. vii. 54) soaked in				
	(1) Filtered sea water since 17. vii. 54	(2) Filtered sea water containing peptone, unchanged since 17. vii. 54	(3) Filtered sea water containing peptone, unchanged since 19. vii. 54	(4) Filtered sea water containing peptone, unchanged since 20. vii. 54	(5) Fresh Bullhill Bank sand kept in natural moist state since 15. vii. 54
	Settlements after 2 days in free-choice dish				
Metd	Multitude	Few	Very few	Several	Very many
Meting	Good number	Few	Fair number	Many	Fair number
Unmet.	Several	Few	Several	Fair number	Few
	Settlements after 2 days in conical vessels				
Metd	Great multitude	Several	Good number	Multitude	Great multitude
Meting	Very few	Multitude	Great multitude	Multitude	Few
Unmet.	None	Good number	Very few	None	None

TABLE IV. EXPERIMENT 4

Begun 24. vii. 54 with larvae 5 days old. Results on 26. vii. 54

Section A

Acid-cleaned Bullhill Bank sand soaked in

	(1) Sterile concentrated sea water peptone since 1. v. 54	(2) Filtered sea water containing peptone, changed 5 times since 23. iv. 54	(3) Filtered sea water containing peptone, unchanged since 14. vii. 54	(4) Filtered sea water containing peptone, unchanged since 21. vii. 54	(5) Filtered sea water containing peptone, unchanged since 23. vii. 54	(6) Sand as (2) washed in tap water and sterilized at about 100° C
	Settlements after 2 days in free-choice dish					
Metd	None	Very few	Fair number	Very few	Very few	Very few
Meting	Several	Fair number	Good number	Few	Several	Few
Unmet.	Fair number	Good number	Fair number	Fair number	Fair number	Fair number
	Settlements after 2 days in conical vessels					
Metd	Very few	Very few	Many	None	Few	Very few
Meting	Fair number	Very many	Very many	Very many	Very many	Good number
Unmet	Several	Many	Fair number	Very many	Many	Fair number

Section B

Acid-cleaned Bullhill Bank sand

	(1) Soaked in filtered sea water, unchanged since 26. vi. 54	(2) Soaked in 'B.B. water' plus an equal volume of filtered sea water since 10. vii. 53. Some filtered sea water added in March 1954	(3) In bolting silk envelope in filtered sea water, amid Salthouse Lake (St. II) sand sterilized at about 100° C, since 16. vii. 54	(4) Soaked in distilled water since 21. vi. 54
	Settlements after 2 days in free-choice dish			
Metd	Fair number	Many	Very few	Very few
Meting	Fair number	Good number	Very few	Very few
Unmet.	Fair number	Many	Several	Fair number
	Settlements after 2 days in conical vessels			
Metd	Fair number	Multitude	Fair number	None
Meting	Good number	Multitude	Fair number	Fair number
Unmet.	Very many	Many	Fair number	Fair number

TABLE V. EXPERIMENT 5

Begun 27. vii. 54 with larvae 5 days old. Results on 29. vii. 54

Section A

Fresh Bullhill Bank sand (collected 15. vii. 54) soaked in

	(1) Filtered sea water unchanged since 17. vii. 54	(2) Filtered sea water containing peptone, unchanged since 17. vii. 54	(3) Filtered sea water containing peptone, unchanged since 19. vii. 54	(4) Filtered sea water containing peptone, unchanged since 20. vii. 54	(5) Fresh Bullhill Bank sand kept in natural moist state since 15. vii. 54
	Settlements after 2 days in free-choice dish				
Metd	Very many	Several	Few	Fair number	Very many
Meting	Fair number	Several	Few	Good number	Many
Unmet.	Very few	Few	Several	Fair number	Very few
	Settlements after 2 days in conical vessels				
Metd	Great multitude	Fair number	Fair number	Very many	Great multitude
Meting	Few	Many	Very many	Very many	Fair number
Unmet.	None	Very few	Very few	Very few	None

Section B

Acid-cleaned Bullhill Bank sand soaked in

	(1) Distilled water since 21. vi. 54	(2) Filtered sea water containing peptone, unchanged since 14. vii. 54	(3) Filtered sea water containing peptone, unchanged since 21. vii. 54	(4) Filtered sea water containing peptone, unchanged since 23. vii. 54
	Settlements after 2 days in free-choice dish			
Metd	Few	Fair number	Very few	None
Meting	Few	Fair number	Several	Few
Unmet.	Fair number	Good number	Good number	Fair number
	Settlements after 2 days in conical vessels			
Metd	Several	Multitude	Very many	Several
Meting	Several	Multitude	Many	Multitude
Unmet.	Very many	Few	Good number	Very many