THE SEQUENTIAL COMPARISON INDEX—A SIMPLIFIED METHOD FOR NON-BIOLOGISTS TO ESTIMATE RELATIVE DIFFERENCES IN BIOLOGICAL DIVERSITY IN STREAM POLLUTION STUDIES

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Over the last 20 yr there have been developed a number of methods which use changes in the aquatic biota as a means of assessing pollution (1) (2) (3) (4) (5) (6) (7) (8) (9) (10). Though the degree of sophistication in these and other methods varies, all will work well only when the investigator has a basic background in biology—and many require advanced or graduate training. This is understandable since an ecosystem is a complex affair, and often difficult to analyze for even the most experienced investigator. With increasing numbers of fish kills and greater stress on all hydrologic systems, there is need for a rapid method by which a layman can assess the biological consequences of pollution and express the results numerically. Such a method also would be useful to professional biologists on preliminary surveys or when results of some sort must be made available without delay. It should be emphasized that this technique would not replace the more elaborate and accurate techniques already available but should be used only when sophisticated techniques are not appropriate.

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While this and similar methods (11) may not reflect the phylogenetic relationships of the organisms being classified, their use can be justified because even the most primitive societies use simple systems of biological classification based on differences in structure, color, size, etc. By utilizing this ability to recognize such differences a crude means of assessing the biological effects of pollution, usually characterized by a reduction in diversity, is derived.

Methods and Procedures

A modification of the sign test and theory of runs of Dixon and Massey (12) appeared to fill the needs previously described. Using this modification the current specimen need only be compared with the previous one. If it appears similar it is part of the same "run"; if not, it is part of a new run. The more runs for a given number of specimens, the greater the biological diversity.

To illustrate the counting process, suppose a collection of microorganisms has been gathered from a stream. These are placed on a slide and examined sequentially with a microscope (Figure 1). Counting in alphabetical order from left to right in row 1, it appears that both *b* and *c* look like the preceding specimen and therefore are part of the same run. Specimen *d*, however, obviously is not like *c* and therefore is the start of a new

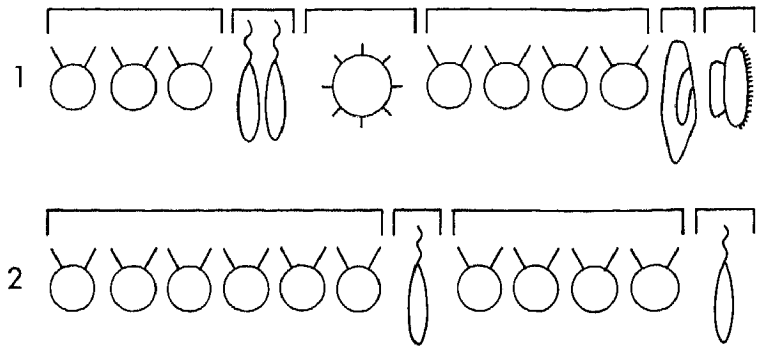

FIGURE 1.—Comparison of each specimen with the previous specimen is made to determine the number of runs.

run. Specimen *e* is like *d* and therefore is part of run 2. Specimen *f* is not like *e* and therefore is part of a new run; but since *g* is not like f, the latter is a run in itself. In row 1 there are 6 runs for 12 specimens. Row 2 shows what would happen if a few of the species in row 1 were eliminated by pollution. Twelve specimens now provide 4 runs.

If conclusions were to be based on the specimens visible in a single field—a risky practice even for gross differences—a mere estimate of species present would suffice and one's powers of observation and memory would not be strained. However, in a count of several hundred specimens involving many microscopic fields, the process of estimating diversity is simplified greatly if one need compare only two specimens side by side to see if they differ.

In order to test these assumptions, the junior authors collected and examined diatoms and macroscopic invertebrates from a polluted stream. Two hundred specimens were used as the basis for the comparison of relative diversity, since this number can be collected and counted readily, yet is large enough to give comparatively reliable results in estimating gross differences. The area selected for study was the east branch of Tauy Creek, which flows through Baldwin City, Kansas, and receives the wastewater effluent from this city. Station 2 was selected first, approximately 20 yd (18.3 m) below the outfall, to discern any immediate effects of the effluent. The habitats at this site were noted carefully, since each station must include similar habitats in order for the comparison of diversity to be valid. Control station 1 was set approximately 85 yd (77.7 m) upstream from the outfall, to insure that it would not be affected by the effluent. Station 3 was placed 400 yd (365 m) downstream in order that the secondary effects of the effluent (e.g., lowered DO) might be apparent. Station 4 was located about 4,400 yd (4,020 m) below the outfall to delimit any effects detected at stations 2 and 3. Stations 1, 3, and 4 each included habitats similar to those of station 2 and essentially were comparable to it.

At each station pairs of small synthetic "sponges" measuring $1 \times 3 \times 4$ in. ($2.5 \times 7.6 \times 10.2$ cm) were submerged to provide an artificial substrate for diatoms. These were anchored several inches above the stream bed to avoid siltation and left in the stream for two weeks to allow adequate growth to occur (9). The sponges then were removed from the stream and the fluid and diatoms associated with each were squeezed into a quart jar containing preservative. The latter consisted of 6 parts water, 3 parts 70-percent ethyl alcohol, and 1 part formalin (a 5-percent solution of formaldehyde works as well). In the laboratory, two-drop aliquots of thoroughly mixed

sample were distributed evenly on haemocytometer slides. Actually, ordinary slides will serve as well if care is taken not to examine each area more than once. It is important to get the organisms sufficiently near each other * for comparison and yet far enough apart so that one specimen does not interfere with the evaluation of another. For practical purposes it will be sufficient to have a substantial majority of specimens so arranged. Counts were limited to 200 specimens from each of the 4 stations for each counter. The first diatom observed was designated "x." If the second looked like the first another "x" was recorded; but if it looked different, an "o" was recorded. Thus, in the sequential counting, two "x's" or two "o's" together designated two adjacent diatoms which were alike. This process was continued until 200 diatoms had been examined. The number of runs then was determined by counting the number of alternate groups of "x's" and "o's."

During examination of unsettled samples it was found that the concentration of the diatoms affects the number of runs obtained. In one sample, the diatoms were dispersed so widely that often only a single diatom, and sometimes none, was visible in a field. This led to a high number of runs from one counter, and a low number of runs in the case of another. Probably this is because the observer's memory of the appearance of the previous specimen is taxed, and mistakes are made. Therefore it is recommended that diatoms be concentrated by allowing them to settle and then removing samples from the bottom of the jar. Unsettled samples will work equally well if the specimens are sufficiently near each other for easy comparison.

Macroscopic invertebrates also were collected, and since the east branch of Tauy Creek is a small stream with rather rigorous environmental conditions and a sparse invertebrate fauna, all 3 collectors worked as a group until at least 200 specimens were obtained at each of the 4 stations. (It is important that the relative amounts of effort applied to the various habitat types be the same at each station.) Only those macroscopic invertebrates living in the substrate were used in this study. These were processed with a graded series of 4 wire screens 8.5 in. (21.6 cm) in diam with frames 2 in. (5.1 cm) high and a mesh size of 4–18/in.† Roughly one quart at a time of substrate material was placed in the top (largest mesh) screen, and washed through with several gallons of water. Specimens then were picked out of the screens with forceps and dropped into 70-percent ethyl alcohol. Approximately equal volumes of mud (containing some sand and gravel) and organic debris (mostly rotting leaves) were used at each station. Once a scoop of material was washed, *all* specimens were taken, even if the number exceeded 200, because it was difficult to remove them from the screens in random order. The collections for each station were kept separate and preserved. The specimens were arranged in rows on a large enamel tray for sorting into groups on the basis of gross appearance. It should be emphasized that while all investigators involved were biologists, no person directly involved in the counting process was familiar with the detailed classification of the organisms studied. After the sorting, numbers were assigned to the specimens (Table I). For example, if there were 37 worm-like specimens with black heads, these (Group A) were assigned numbers 1 through 37; for 14 "horn-of-plenty" snails (Group B), 38 through 51 were assigned; for 6 small white-shelled clams (Group C), 52 through 57; and so forth. Numbers

* Concentration of specimens by settling or centrifugation may be necessary.

† General Biological Supply House brass testing sieves 105A59.

TABLE I.—Sorting of Specimens into Groups with Assigned Numbers in Preparation for Random Drawing

Number of Specimens		Organism	Group Letter	Numbers Assigned
Station 1	1	leech	A	1
	1	insect "a"	B	2
	2	insect "b"	C	3–4
	3	insect "c"	D	5–7
	1	insect "d"	E	8
	2	insect "e"	F	9–10
	1	insect "f"	G	11
	5	"limpet"	H	12–16
	1	flat snail	I	17
	18	sprial snails	J	18–35
	2	white clams	K	36–37
	3	orange larvae	L	38–40
	4	insect "g"	M	41–44
	45	pincher "worms"	N	45–89
	144	worms	O	90–233
Station 2	1	worm "a"	A	1
	5	worm "b"	B	2–6
	620	worms "c"	C	7–626
	69	spiral snails	D	627–695
Station 3	1	insect "a"	A	1
	1	insect "b"	B	2
	5	spiral snails	C	3–7
	3	worm "a"	D	8–10
	3	worm "b"	E	11–13
	334	worm "c"	F	14–347
Station 4	23	insect "a"	A	1–23
	1	insect "b"	B	24
	11	"shrimp"	C	25–35
	25	clams	D	36–60
	4	worm "a"	E	61–64
	2	worm "b"	F	65–66
	2	leeches	G	67–68
	10	pincher "worms"	H	69–80
	100	worm "c"	I	81–180
	1	flat snail "a"	J	181
	1	"limpet"	K	182
	11	spiral snail	L	183–193
	1	insect "c"	M	194
	1	insect "d"	N	195
	1	insect "e"	O	196
	17	insect "f"	P	197–213

from 1 to 200 (the highest number used) then were selected at random. If a number selected represented a specimen of the same group as the previous selection, it was part of the same run; if not, it began a new run. Part of a "drawing" or selection might look like the data in Table II.

A modification of the "sequential comparison" method was used by Robert A. Jordan to check the effects of a severe lowering of pH on the protozoan population in a small side channel of a large stream. Despite counting problems resulting from the use of living material, a striking difference was detected between exposed and control areas. However, protozoans are difficult to use even when their movements are slowed by methyl cellulose or polyvinyl alcohol.

TABLE II.—Labelling Stream Specimens

Sequence of Selection	Number Selected	Group	
1	127	H	
2	189	H	1 run
3	153	H	
4	176	H	
5	17	A	1 run
6	24	A	
7	105	F	1 run
8	91	F	
9	55	C	1 run

TABLE III.—Number Runs/200 Diatoms at Each of the 4 Sampling Stations

Station	No. of Runs			
	1	2	3	4
Trial* A	157	104	149	151
Trial* B	132	105	151	158

* Trials A and B were counts by different workers.

Samples with unequal numbers of specimens might be compared by using the relationship (diversity index) between the number of specimens and the number of runs as a basis of comparison.

$$\text{Diversity Index} = \frac{\text{number of runs}}{\text{number of specimens}}$$

In order to check the diversity index and also to see if the system of counting used for diatoms also would be useful with invertebrate collections, populations were obtained from two areas of the Kansas River near

TABLE IV.—Number of Runs/200 Invertebrates at Each of the 4 Sampling Stations

Station	No. of Runs			
	1	2	3	4
Trial* A	113	45	16	145
Trial* B	121	42	14	138

* Trials A and B were separate drawings of random numbers after the specimens were sorted.

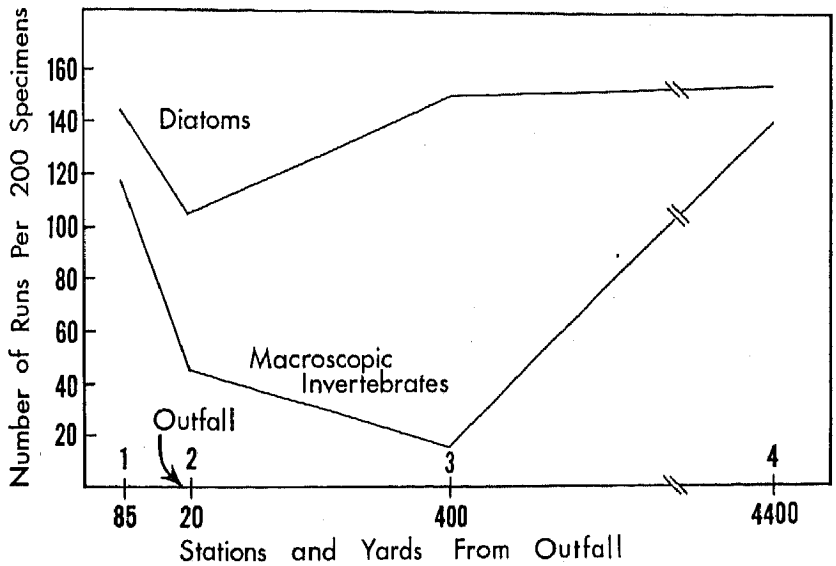

FIGURE 2.—Variation in the number of runs at various stations on the east branch of Tauy Creek relative to the Baldwin City sewerage outfall. Each curve represents the average of results in Tables III and IV.

Lawrence, Kansas, by two students, David N. Kohrmann and Frank D. Holland III. Invertebrate collections were obtained by anchoring pairs of wire mesh containers each with several cubic feet of driftwood and dry rocks (both free of aquatic invertebrates) in the river for three weeks. One pair was placed above the dam at Lawrence and one below. One container from each pair was placed "inshore" a few feet from the bank and the other "outshore" in about 8 ft (2.4 m) of water. Invertebrates were removed from each "trap," preserved in 70-percent alcohol, and then shaken up and poured on a grid. If any clumps were noted, alcohol was poured on these so that each specimen was apart from its neighbors. Counts then were made in the fashion described for diatoms.

Results

Results for the diatom studies are given in Table III and for the invertebrate studies in Table IV. Figure 2 shows graphically the results for both diatoms and macroscopic invertebrates at all 4 stations.

Results for the Kansas River study are given below:

"Inshore" Above Dam	"Outshore" Above Dam
41 specimens	12 specimens
37 runs	12 runs
Diversity Index $=\frac{37}{41}=0.9$	Diversity Index $=1$
"Inshore" Below Dam	**"Outshore" Below Dam**
36 specimens	24 specimens
14 runs	11 runs
Diversity Index $=0.39$	Diversity Index $=0.46$

In between the "above dam" and "below dam" sampling sites was a milk plant and a paper mill which may have affected the diversity index. Note, however, that the diversity indices for each area are fairly close for a crude estimate, particularly when far fewer specimens were available than normally would be used for the calculation.

No physical or chemical parameters were measured at any of the stations for two reasons. First, these give the conditions existing only at the time of the test, whereas biological parameters represent a summation of the

past and present environmental circumstances. To get meaningful relationships, a comparatively enormous effort would have had to be made to collect physical-chemical data. Second, and most important, it is best to test an ecological hypothesis with as few preconceived ideas regarding the condition of the test environment as possible. Essentially this project was to use a biological diversity assessment method with two different groups of organisms to determine whether wastewater from Baldwin City, Kansas, had biological effects.

Discussion of Results

If there were two large rooms with floors covered with different colored marbles, but room no. 1 had only 3 different colors while room no. 2 had 20 colors, it is evident that in a random collection of 200 marbles from each room the marble colors from room no. 2 probably would vary more than those from room no. 1. If each series of 200 marbles were examined sequentially, it is highly probable that those from room no. 2 would be more likely to differ in color from their preceding neighbor than those from room no. 1. Unfortunately communities of aquatic organisms are varied and specimens often elusive. Theoretically, fish and diatoms might be compared since estimating the diversity of all kinds of organisms from various sampling areas has great merit. However, sampling effectiveness is quite important and comparable collections of organisms are more likely to be obtained from groups with less motility than from those with more movement. Best results should be obtained if organisms collected by similar methods and equal effort are compared. Square foot bottom samples or artificial substrates have many advantages in this respect.

An additional advantage of this method is that the problem of evaluating "rare" specimens is reduced considerably. In a simple total of the number of species present, those with small populations are not distinguished from those with enormous populations. The "sequential comparison" assessment reduces distortions of this type.

To check the reliability of the runs obtained with this technique, the methods described by Sokal and Rohlf (13) were used to compute means, standard deviations, and 95-percent confidence limits for the means, for stations 1 and 2. The difference between the two means then was tested. Numbers representing individual organisms were placed in a hat, 200 were drawn at random, and the number of runs determined. Ten trials were made for each station (Table V). For station 1 the mean number of runs was 117.3, the standard deviation 5.438, and the upper and lower 95-percent confidence limits for the mean, 121.19 and 113.41, respectively. For station 2 the mean was 40.6 runs, the standard deviation 7.919, and the 95-percent confidence limits for the mean, 46.26 and 34.94. These are compact distributions for biological data and indicate that the method should be reliable. The difference between the means was tested with the t-test and the experimental t-value (25.249) was significant ($P < .001$) indicating that the mean number of runs at stations 1 and 2 was significantly different.

TABLE V.—Results of 10 Successive Drawings of the Numbers Representing the Organisms Collected at Stations 1 and 2 (Table I).

Drawing	Station 1	Drawing	Station 2
1	111	1	32
2	126	2	34
3	113	3	52
4	123	4	43
5	121	5	43
6	110	6	26
7	117	7	45
8	113	8	42
9	121	9	49
10	118	10	40

Conclusions

The method described is adequate for estimating relative differences in biological diversity, and is designed for non-biologists. Since there is a high degree of subjectivity, differences in diversity probably will be significant (*a*) only if obtained over a short time span by a single operator or a group working as a team, (*b*) if the exposed area differs substantially from the control, and (*c*) collections are non-selective (i.e., species collected as found with no preference for a particular group).

This method was tested several times by the senior author with freshwater protozoan communities maintained indoors in plastic troughs with a constant flow of lake water (14).

These communities were exposed to high temperatures and pH shocks. In addition to the diversity changes recorded by conventional species counts it was possible to obtain similar results with the method described in this paper. This was not, however, included in the species restoration manuscript (14) since the research project was at that time in its preliminary stages, and the means of administering the shocks had not been perfected.

The "sequential comparison" index also appears practical for biologists with no previous experience in the classifications of aquatic organisms. It has not been tested by non-biologists; in fact, one of the purposes of this paper is to encourage such use in a variety of situations.

Acknowledgments

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