

# Immunological Methods for Food Web Analysis in a Soft-Bottom Benthic Community

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## Abstract

Basic to nearly any prediction concerning the behavior and structure of entire communities or of their components is knowledge of trophic connections among species. A major impediment to such understanding of soft-bottom benthos is methodological. Because none of the routinely available methods of food web analysis (e.g. visual gut content analysis, direct observation of feeding, tracer techniques) is generally suitable for examining all trophic interactions of benthic infauna, we sought to evaluate the potential of immunological methods for identifying predator-prey relationships in one typical, estuarine, intertidal sand flat. Whole-organism extracts of individual macro- and meiofaunal taxa were injected into rabbits to produce antisera of varying specificity. Double immunodiffusion precipitin tests of antiserum specificity revealed both phyletic and trophic relationships among 20 taxa. Using relatively unspecific antisera, preliminary analysis of the stomach contents of a few surface deposit-feeders and particle browsers was successful, giving positive identification of several trophic links which would otherwise have gone undetected. The production of taxon-specific antisera is expected to provide the methodological tool necessary to document the breadth of trophic connections in a marine benthic food web.

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## Introduction

The latitude of plausible speculation concerning the factors molding marine benthic communities living in soft substrates (Dayton and Hessler, 1972; Grassle and Sanders, 1973; Smith, 1975; Woodin, 1976) attests to a lack of both quantitative and qualitative information. Our premise is that a major impediment to further understanding of pattern and process in soft-bottom benthic communities is a methodological one. In particular, resource and trophic pathway identification are intertwining problems which must be addressed before even qualitative models of controlling factors (e.g. Levins, 1975) can be applied.

Without this basic knowledge of who eats whom, it is impossible to design experiments which can unequivocally discern whether community structure is pre-

dominantly mediated by predation, by competition, by both, or by neither. Enclosures or enclosures designed for assessing the effects of modified predation pressure may often attract or favor non-target predators. Lack of *a priori* information on these non-target predator effects and the general absence of trophic analyses concurrent with caging experiments result in ambiguity and in weak, *a posteriori* speculations about the causes of observed differences between caged and ambient faunas (Young *et al.*, 1976; Arntz, 1977; Reise, 1977).

Any of several methods might be used to elucidate predator-prey links (Table 1). Each has advantages and disadvantages but few could be systematically applied to reveal the trophic relations of the majority of species in any one soft-bottom benthic community. Direct microscopic examination of gut contents is perhaps the most attractive of the generally applicable methods. It requires no tools more specialized than a microscope and dissecting equipment. The method, however, has obvious inherent

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biases. Some predatory animals masticate or lyse their prey more thoroughly than do others; conversely, some prey lose their morphological identities more readily and rapidly than do others. These biases by no means invalidate the technique of gut content analysis by direct microscopic examination, but the method would be all the more valuable if the extent of these potential sources of error were known (Gaare et al., 1977; Peterson and Bradley, 1978). Although by no means a panacea (cf. Table 1), of the remaining methods that might be generally applicable, immunological techniques appear most attractive.

The most widely used immunological technique in ecological studies of food webs has been the precipitin test, in which antisera to specific prey are reacted in capillary tubes or in gels with the stomach contents or whole-body extracts of suspected predators. The passive haemagglutination inhibition assay is another sensitive immunochemical method useful in food web studies

(Greenstone, 1977), but it is restricted to situations where large quantities of target-organism extracts are readily available. Insects have been examined most frequently [Brooke and Proske, 1946; Dempster, 1960; Loughton and West, 1962; Healy and Cross, 1975 (directed at sheep ticks rather than at insects)], usually to identify prey of species with piercing and sucking mouthparts or predators of economically important pests. Work with the prey of triclad turbellarians (Young et al., 1964; Davies, 1969a, b; Pickavance, 1970, 1971a, b; Reynoldson and Davies, 1970; Young, 1973) demonstrated that antisera could be produced which will differentiate groups of interest in marine soft bottoms. Pickavance (1970), for example, produced antisera specific to the following freshwater taxa: an amphipod species, an isopod species, a species of snail, a bivalve species, copepods as a group, tubificid oligochaetes (as a group distinct from other kinds of oligochaetes), and several insect species.

Table 1. Methods for identifying predator-prey links and problems in their application to soft-bottom benthos (modified from Kiritani and Dempster, 1973). L: low; M: moderate; H: high; V: variable

Method	Amount of time required		Expense		Likelihood of artifactual predator or prey behavior	Certainty of specific identity	Viability of prey at time of capture known	Differential digestion a problem	Comments
	Ini- tial	On- going	Cap- ital	On- going					
Direct field observation	H	H	L	L	V	V	Yes	No	Difficult in soft substrates, especially nocturnally
Predator exclusion or inclusion	M	M	L	L	H	L <sup>a</sup>	No	No	Predation difficult to distinguish from disturbance effects
Monitoring of predator and prey abundances over time	M	M	L	L	L	L	No	No	Causal relationship must be established independently
Offering prey in the laboratory	M	M	L	L	H <sup>b</sup>	H	Yes	No	Natural prey availability difficult to duplicate
Monitoring of labeled prey in the field	M	M	M	V	L	H	No	Yes	Recovery of labeled individuals is generally low
Visual analysis of gut contents	M	M	M <sup>c</sup>	L	L	V to H	No	Yes	Highly dependent upon observer acuity and experience
X-ray analysis of gut contents	M	M	M	L	L	V to H	No	Yes	Not useful for soft-bodied prey
Immunological analysis of gut contents	H	L	M <sup>c</sup>	L	L	V to H	?	Yes	See present study
Chemical analysis of gut contents	H	L	V	L	L	V	?	Yes	Sensitivity may be inadequate for small samples <sup>e</sup>
Measuring bio-accumulation of specific chemicals	H	L	V	L	L	V	No	Yes	Transfer efficiencies must be determined independently

<sup>a</sup>Young et al. (1976), Arntz (1977), Reise (1977).

<sup>b</sup>e.g. Ockelmann and Vahl (1970).

<sup>c</sup>Major expense is a microscope for sorting.

<sup>d</sup>If particularly labile, components disappear in prey moribund or dead when eaten.

<sup>e</sup>But note the recent advance of Murray and Solomon (1978).

Wilhelmi's pioneering work (1944) showed that even closely related marine invertebrate species could be distinguished immunologically. Boreham and Ohiagu (1978) have recently presented a thorough and cogent review of studies employing immunological techniques to elucidate prey-predator relationships.

Not being dependent on the retention of morphological identities of prey among gut contents, immunological tools promised to provide valuable information on biases inherent in the usual direct microscopic examination. Below we seek a preliminary evaluation of the potential for routine application of immunological methods to soft-bottom benthic communities in which food web connections are questioned. Specifically, we address the following questions: (1) can antisera of high specificity to soft-bottom benthic species routinely be produced; and, (2) using these antisera, can organisms be detected and reliably identified to the desired taxonomic level in the guts of predators?

## Materials and Methods

### Target Community

The target community selected was that found on the expansive intertidal flat (muddy sand) between the north and south forks of the Skagit River in northern Puget Sound (USA). Although for ease of access we focused on animals found at the +2 m tidal level, the species encountered (Table 2) are found in roughly comparable relative abundances from below mean lower low water (MLLW) to the

+2.5 m level (J.E. Smith, in preparation). The other characteristics which resulted in this choice of sites included relative freedom from human disturbance, minimal large-scale physical heterogeneity, and a relatively short macrofaunal species list, many of whose members are important benthos components in temperate and boreal estuaries worldwide.

### Collection of Organisms

Most organisms for the preparation of antigens were collected at various times during the year from the site of the target community. Collection entailed screening natural sediment from the +2 m tidal level through either a 1 mm or a 350  $\mu\text{m}$  mesh. The screened sediment was transported to the laboratory (100 km) for hand-sorting of animals to species or major taxon. Screened sediment was held at 8°C prior to sorting. Sorted animals were placed into plastic petri dishes with clean seawater (27 to 29‰ S) containing antibiotics (Penicillin-G, 90,000 units  $\text{l}^{-1}$ ; Streptomycin-sulfate, 100 mg  $\text{l}^{-1}$ ), but without food, for 3 to 7 days. This period of isolation was intended to allow animals to clear their guts of foreign proteins. To reduce the likelihood of re-ingestion of fecal material, *Corophium salmonis* and *Macoma balthica* were placed into clean antibiotic seawater with a 5 mm thick substrate of coarse sand which had been boiled for 10 min. All other organisms were allowed to clear their guts without substrate at 8°C.

Since meiofaunal taxa in the target community were not especially abundant

Table 2. Target organisms, their months of collection from North Puget Sound, biomass sorted, time required, and protein concentrations injected to date

Organism	Months of collection	Wet weight (mg)	Sorting time (h)	Protein conc. injected ( $\text{mg/ml}^{-1}$ )
<i>Eteone longa</i>	Apr., May 1977	1751	26	8.0
<i>Hobsonia florida</i>	Feb., Apr., May 1977	3401	44	10.2
<i>Manayunkia aestuarina</i>	Jan. 1978	85	200	2.9
<i>Nereis limicola</i>	Feb., Apr., May 1977	1203	24	6.2
<i>Pseudopolydora kempii japonica</i>	Sept. 1977	76	18	2.1
<i>Pygospio elegans</i>	Apr., May, Sept. 1977	614	34	6.0
<i>Anisogammarus confervicolous</i>	Feb., June 1977	1609	8	8.0
<i>Corophium salmonis</i>	Sept. 1976, Jan. 1977	1662	22	4.1, 0.4, 0.04
<i>Crangon franciscorum</i>	May 1977, Jan. 1978	20293	4	6.3
Tanaid sp.	Feb., Apr., May 1977	1239	28	7.2
<i>Macoma balthica</i>	Sept. 1976, Jan. 1977	14630	18	8.2
<i>Mya arenaria</i>	May 1977	5000	1	7.7
<i>Paranemertes peregrina</i>	Sept. 1977	11990	1	8.0
Nemertea sp.	Feb., Sept. 1977	128	2	3.2
Oligochaeta <sup>a</sup>	July 1977, June 1978	1097	48	3.3
Ostracoda	July 1978	490	75	1.8
Harpacticoida <sup>a</sup>	July, Aug. 1977	1680	175	4.0
<i>Huntemannia jadensis</i> <sup>a</sup>	July 1977	930	100	3.5
Nematoda <sup>a</sup>	July 1977	2530	75	7.5
Turbellaria <sup>a</sup>	July, Aug. 1977	200	20	1.7

<sup>a</sup> Collected at Richmond Beach, Central Puget Sound.

on the Skagit flats ( $10^3$  individuals per  $10\text{ cm}^2$ ), sorting was likely to take an inordinate amount of time. To enhance the probability of success in securing enough material to prepare antisera to these taxa, meiofauna were collected from the mid-tide level at Richmond Beach in central Puget Sound primarily during July and August 1977. This area was known (Feller, 1977) to harbor a rich and abundant complement of meiofaunal taxa, was conveniently located for repeated collection of these small (0.1 to 1.0 mm) animals, and shared all major meiofaunal taxa and several meiofaunal species in common with the target community. Natural sediment from the upper 3 cm at MLLW was placed into a bucket of seawater, swirled by hand, and decanted through a  $350\ \mu\text{m}$  mesh. This material was then backwashed into plastic trays and transported to the laboratory (20 km) for sorting.

Isolation of meiofauna from screened material was greatly facilitated by allowing phototactic organisms to respond to direct illumination from an incandescent bulb. Most harpacticoid copepods could easily be collected with a large-bore pipette from the illuminated end of the plastic trays. These animals were further sorted with a small-bore pipette under a dissecting microscope to insure that the isolated specimens were of the desired taxon. (*Huntemannia jadensis* was among the positively phototactic harpacticoids.) Nematodes, on the other hand, were negatively phototactic. During preliminary sorting trials, one of us (E.D. G.) noticed that aggregations of nematodes appeared on the surface of screened material in response to illumination of the underside of the clear plastic collection trays. Use of cool-light sources elicited the same response, thus the aggregations formed as a result of light, not heat, avoidance. The nematode clumps were easily picked from the sediment surface and isolated as above. Ostracods, oligochaetes, and small turbellarians were pipetted individually.

Many of the harpacticoids in the screened samples were covered with detrital material and epiphytes which we sought to remove before preparation of the organism extract for injection. Therefore, all harpacticoids were isolated in plastic petri dishes containing previously boiled coarse sand. Their movement through this substrate removed essentially all of the visually apparent, potentially contaminating material within 24 h. Seawater in the dishes was changed at least once after isolation at  $8^\circ\text{C}$ . Other meiofaunal taxa were iso-

lated without substrate or food for 3 to 7 days.

Large (1 to 4 cm) polyclad turbellarians were collected from the undersides of intertidal rocks at Lincoln Park, Seattle, because meiofaunal turbellarians were not abundant at the Skagit Flats or Richmond Beach. These polyclads were isolated as above without food for 3 to 7 days before being ground for injection.

#### *Preparation of Whole-Organism Extracts*

After isolation and starvation, organisms were picked from their petri dishes, rinsed in distilled water to remove excess salts, blotted dry, and quick-frozen on dry ice. Organisms from several different days' collections and sortings were combined and weighed to the nearest mg (frozen weight) before being ground with buffer solution in a hand-operated tissue grinder. Grinding was done in an ice bath to prevent thermal denaturation of proteins. Since the protein concentration of each organism or taxon was unknown, and it was desirable to produce an extract of high protein concentration, grinding was performed using as little buffer solution as possible. The buffer solution, TES-saline, consisted of the following: 5 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid], 30 mM NaOH, and 150 mM NaCl at pH 7.3. After the animal tissues had been solubilized in TES-saline, the suspension was centrifuged for 10 min at  $700 \times g$  and the supernate stored at  $-20^\circ\text{C}$ . Protein concentration of the extract was determined spectrophotometrically according to the method of Lowry et al. (1951), using bovine serum albumin as reference standard and TES-saline as a blank. Extracts were typically diluted 1:1000 (v/v) with TES-saline before protein analysis to achieve the desired range of absorbance at 750 nm wavelength.

#### *Preparation of Antisera*

Extracts of known protein concentration (Table 2) were injected into pairs of New Zealand white female rabbits, 2.5 to 3.0 kg in body weight, which had been maintained on normal laboratory diets for 2 weeks. The rabbits had no previous immunization history. One day prior to the start of the immunization sequence, approximately 20 ml of whole blood was collected from a marginal ear vein of each rabbit. This pre-immunization blood was allowed to clot for 24 h at room

temperature (ca. 20°C), centrifuged for 10 min at 1600 x *g*, and the serum was then collected and stored at -40°C. This pre-immunization serum was always tested against the extract to be injected (see below) as a check that no antibodies to the extract were present before immunization.

We employed the immunization schedule described by Kenny (1971) except that, in general, less total protein was injected into each rabbit. Briefly, extracts of known protein concentration were emulsified in an equal volume of Freund's incomplete adjuvant (Difco). This mixture, 1 ml extract plus 1 ml adjuvant, was injected intramuscularly, 1 ml per flank per rabbit (i.e., 2 ml total per rabbit) on Day 1 of the immunization schedule. Incomplete rather than complete adjuvant was used to avoid possible interference by antibodies produced against Mycobacteria. On Day 21 each rabbit was given 0.1 ml of the full-strength extract (without adjuvant) intravenously via a marginal ear vein. On Day 24, 0.2 ml; on Day 27, 0.3 ml; and on Day 30, 0.4 ml of full-strength extract were administered in the same way. Finally, the rabbits were anesthetized and bled by cardiac puncture on Day 37. The approximately 125 to 150 ml of whole blood collected from each rabbit was allowed to clot at 4°C for 24 h, centrifuged for 10 min at 1600 x *g*, and the serum was then stored in 5 to 10 ml lots at -40°C. Approximately 50 to 75 ml of serum were obtained from each rabbit.

#### *Specificity Testing*

The specificities of antisera were tested against target-community organism extracts of known protein concentration using a micromethod of double diffusion in agar (Ouchterlony, 1968). Microscope slides (25 x 75 mm) were pre-coated with 0.5% agarose in distilled water (1 ml per slide) and baked for 1 h at 100°C. Melted agarose (0.5% in 8 mM veronal, 40 mM sodium veronal, 0.25% Triton X-100, 0.01% sodium azide) was pipetted evenly onto pre-coated slides, 1.2 ml per slide, and allowed to gel at room temperature. A plastic template, 25 mm square and 3 mm thick, was placed on the gelled agarose surface. The template had 4 small (20 µl) depressions (wells) surrounding a central well and serving as point sources for the diffusion of organism extracts and antisera through the agarose. To determine whether an immunization series was successful in producing antiserum to the target organism extract (i.e., if the resultant serum con-

tained antibodies to antigens from the extract), pre-immunization and post-immunization sera from each pair of rabbits were placed separately into the 4 principal wells surrounding the center well containing the extract. This juxtaposition allowed direct comparison of the reactivities of antisera produced by each rabbit of the pair as well as detection of any antibodies present in the pre-immunization serum.

Typically, 5 to 10 µl of undiluted antiserum were added to each well, and organism extracts were added in similar quantities at full strength (i.e., the same concentration at which they were injected). Diffusion of extracts and antisera proceeded for 48 h in a humidified chamber at room temperature. Templates were removed at the end of this period, and the microscope slide was immersed in TES-saline for 24 h to remove unprecipitated proteins. The slides were then placed into distilled water for 4 h to remove salts, and then dried overnight at 37°C. Precipitated antigen-antibody complexes (precipitin lines between wells) were stained in a solution of Coomassie Brilliant Blue R (0.5% in destaining solution) for approximately 7 min and then destained for 5 to 15 min in 95% ethanol:distilled water:glacial acetic acid (45:45:10 by volume). After destaining, the slides were rinsed in distilled water and dried at 37°C. When dry, the slides may be stored indefinitely. Staining and washing the microscope slides, however, often results in accidental loosening or loss of the agarose slab if the slides are not pre-coated.

Once it was determined that an antiserum to one of the target organism extracts had been successfully produced, i.e., that at least one precipitin line had formed between the wells containing target organism extract and post-immunization serum, the degree to which it also reacted with extracts of all other organisms (cross-reacted) was tested in the same manner; extracts from various other target organisms were placed in peripheral wells with the test antiserum in the central well (Fig. 1). All specificity tests were run at least in duplicate, using two plastic templates per microscope slide.

Precipitin lines between wells were examined and counted using a Wild M5 dissecting microscope with darkfield base. Darkfield microscopy was most useful for determining whether precipitin lines were present, as some lines may be nearly invisible when viewed with the naked eye. Lines were further classified as to staining intensity and relative

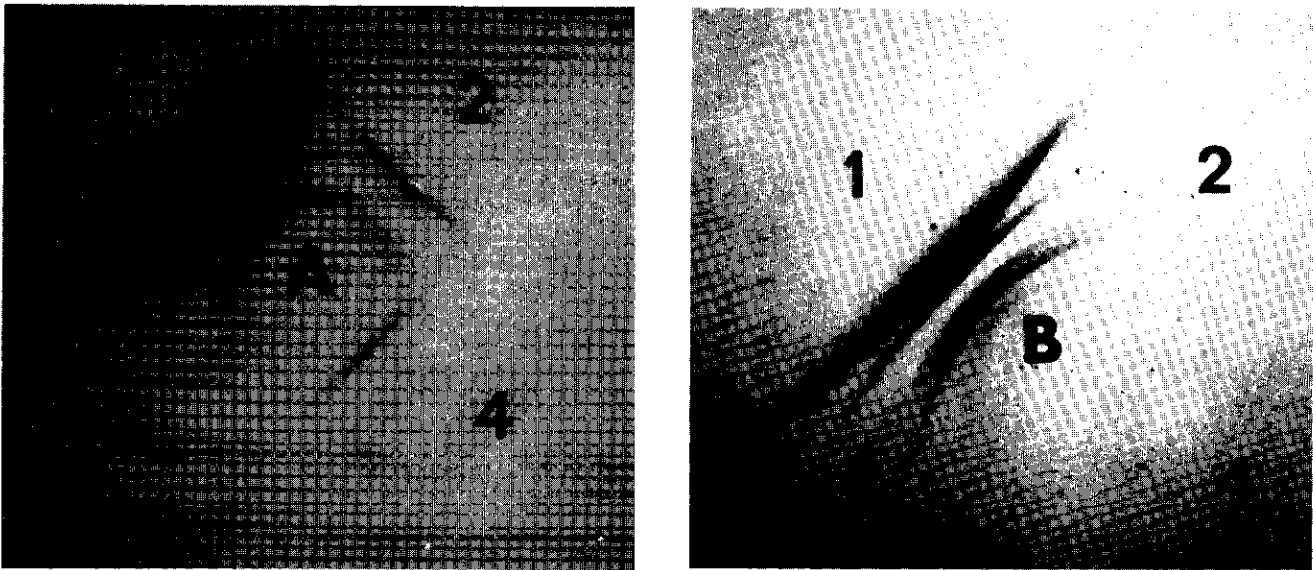


Fig. 1. Representative double-immunodiffusion specificity tests. (A) *Mya arenaria* antiserum surrounded by extracts of *M. arenaria* (1), *Macoma balthica* (2), the tanaid sp. (3), and *Nereis limnicola* (4). (B) *Corophium salmonis* antiserum surrounded by extracts of *C. salmonis* (1), the tanaid sp. (2), *Crangon franciscorum* (3), and *Anisogammarus confervicolous* (4)

position between wells using the diffuse white background lighting provided in the Wild M5 darkfield base.

We elected to summarize our data on a "worst case" basis with respect to possible cross-reactions. The full-strength extracts used far exceeded the protein concentrations likely to be found in preparations of predators' guts for immunoassay. Furthermore, in contrast with the techniques used by Kenny (1969), the maximum (rather than the minimum) number of precipitin lines observed for any particular extract-antiserum test pair formed the data base for determining immunological affinities. The maximum number of precipitin lines resulting from reaction of an antiserum with the extract from which it was prepared was designated the identity or self-reaction and had (by definition) a self-similarity of 1.00. The number of lines formed by a non-self extract with that same antiserum divided by the number of self-reaction lines gave a proportional measure of the immunological similarity of the extract-antiserum pair. For example, the reaction of *Eteone longa* extract with *Pygospio elegans* antiserum produced a maximum of 3 lines, suggesting a "scaled similarity" of three-tenths or 0.30 between this pair (Fig. 2). *Nereis limnicola* extract produced 2 lines with that same antiserum for a similarity of 0.20.

Each antiserum was used to define an axis in multidimensional space. The scaled similarity measure described

above was used to define the position of each taxon's extract along the axes defined by each antiserum. We next clustered the extracts of all taxa according to the Euclidean distance between the extracts' locations in this multidimensional antiserum space (weighted pair-group method using arithmetic averages of Sneath and Sokal, 1973). The results of this clustering were summarized in dendrogram form (Fig. 3).

#### Preliminary Gut Contents Assays

Using antisera to all organisms in the target community, preliminary analyses of gut contents were made in replicate for 11 taxa collected on 13 February 1978. Animals were screened (1000, 350, or 150  $\mu\text{m}$  mesh) gently from surficial sediments in the field and frozen intact on dry ice within a few minutes of collection. Frozen organisms were lyophilized, sorted to desired taxon in the dry state, and then ground individually or as a group of individuals in 0.1 to 0.2 ml TES-saline with mortar and pestle on an ice bath. Aliquots (5 to 10  $\mu\text{l}$ ) of a solubilized gut extract were placed in 10 separate control template wells, each surrounded by four different antisera in peripheral wells. The following taxa were ground for preliminary analysis: single individuals of *Nereis limnicola*, *Nemertea* sp.; 2 batches each of *Eteone longa* (7 and 8 individuals), *Hobsonia florida* (2

WHOLE-ORGANISM EXTRACTS

	ETEONE	HOBSONIA	MANAYUNKIA	NEREIS	PSUDOPOLYDORA	PYGOSPIO	ANISOGAMMARUS	COROPHIUM	CRANGON	TANAID	MYA	PARAMERMES	NEMERTEA SP.	OLIGOCHAETA	OSTRACODA	HARPACTICOIDA	HUMBERTMANNIA	NEMATODA	TURBELLARIA
ETEONE	<b>5</b>	3	1	3			1	1	1	2	2	1							
HOBSONIA	4	<b>8</b>	4	2	3		4		1	2	3	3	1	3	4				
MANAYUNKIA	4	7	<b>3</b>	2				1	2	4	1	4							
NEREIS	3	1	7	<b>1</b>			1	1	1	6	2	4	1						1
PSUDOPOLYDORA	3	1	1	5	<b>4</b>		1	1	2	1	1	2							1
PYGOSPIO	3	3	1	2	4	<b>10</b>	1	2	1	2	3	1	2						1
ANISOGAMMARUS						8	<b>1</b>	1	1	1	1	1	2	2					
COROPHIUM						3	1	<b>7</b>	1	1		1	3	1					
CRANGON	1	1	2			3	2	12	<b>2</b>	1		2	2	3	2	1			
TANAID SP.						2	4	5	15	<b>3</b>	3	3	4	3	4	4			1
MACOMA	4		3	2				1	8	5	2	3	1	2					3
MYA	2	1	3	4	1	2		3	1	5	9	6	5	4	2	4	1	2	3
PARAMERMES	1	2	5	3			2	2	4	14	7	2	1	1	1	1	2		
NEMERTEA SP.										1	3	<b>6</b>							
OLIGOCHAETA	2	1	2	4	2	1	1	1	2	2	3	1	10	1	1	1	1	1	1
OSTRACODA								2	3	2	2	1	2	7	1	1	1	1	1
HARPACTICOIDA			1					2	1	1	3	1	1	1	9	6	3		
HUMBERTMANNIA	2	1			1			1	1	2	2		1	9	12	2			
NEMATODA	2				1		1	1	2	1	1	2		1	2		13	1	
TURBELLARIA			1	1	1	1	1	1	3	1							1		6

Fig. 2. Maximum number of precipitin lines observed in extract-antiserum double-immunodiffusion tests. Bold-face numbers on diagonal refer to self-reactions. Full specific names are given in Table 2

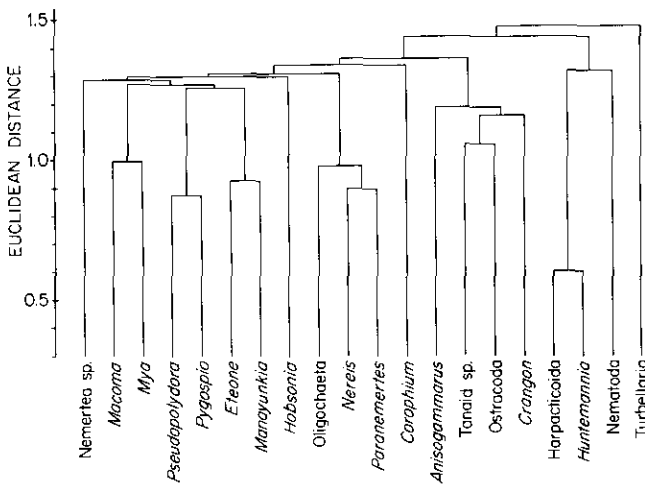


Fig. 3. Dendrogram of immunological similarities among target taxa, based upon maximum number of precipitin lines observed in double-immunodiffusion specificity tests. Relative distance partially reflects ability of antisera to discriminate among target taxa. Full specific names are given in Table 2

and 3), *Manayunkia aestuarina* (35 and 50), *Macoma balthica* (2 and 5), *Mya arenaria* (1 and 2); 3 batches each of *Anisogammarus confervicolous* (2, 5, and 8), and a tanaid sp. (10, 10, and 25); 4 batches of *Corophium salmonis* (5, 5, 10, and 15); and 12 *Crangon franciscorum* (singletons). The guts of *Macoma balthica*, *Mya arenaria* and *Crangon*

*franciscorum* were excised for grinding; the other species were ground whole. Results were lumped for all size classes and sexes of predators examined. "Predator" and "prey" are used in the purely operational sense of the presence of the latter in the extract of the former.

The mere presence of precipitin lines between antiserum of prey Species A and the gut extract of predator Species B does not necessarily confirm a trophic link. Antiserum to Species A may also produce one or more lines with the predator's gut tissues and/or with extracts of other organisms in the predator's gut besides Species A, i.e., cross-reactions alone might account for the lines observed using the Species A antiserum. Such a possibility required construction of a standardized algorithm to eliminate from consideration all reactions which could have been due strictly to cross-reactions.

The initial step in our algorithm for confirming trophic links was to divide the maximum number of precipitin lines observed in the reaction between a predator extract and each taxon's antiserum by the number of precipitin lines for that taxon's self-reaction (the principal diagonal of Fig. 2). The taxa were ranked on the basis of this ratio, with the highest ranked taxon corresponding to the largest ratio. The next step in the algorithm was to eliminate all of the reactions which might have been due solely to cross-reactions with the predator being examined. The number of precipitin lines which could have been due to cross-reactions with the predator (obtained from the predator's extract column in Fig. 2) were subtracted from the previously noted maximum for each antiserum reaction. The numbers resulting from this subtraction were used in all subsequent steps of the algorithm. If the result of this subtraction was zero or negative, then the presence of that particular taxon in the gut of the predator could not be determined unambiguously, and that taxon was eliminated from further consideration.

The subsequent steps in the algorithm were designed to eliminate all taxa whose reactions could have been due solely to cross-reactions with other taxa in the gut. We assumed that the reaction of the highest ranked taxon in the original ranking was the least likely to have been due to cross-reactions. The number of precipitin lines which might have been due to cross-reactions with this highest ranked taxon (obtained from that taxon's column entries in Fig. 2) was subtracted from the number of lines remaining for each predator ex-

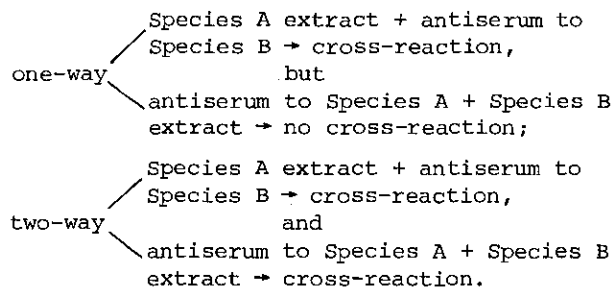
tract-antiserum reaction. Again, if the subtraction resulted in zero or a negative number for a taxon, it was eliminated. The algorithm proceeds by iteration, with the column entries of successively lower ranked taxa being subtracted. If, after subtracting the column entries of a lower ranked taxon, a higher ranked taxon was eliminated, then the lower ranked taxon was placed ahead of the higher ranked taxon in the original ranking, all eliminated taxa were reinstated, and the algorithm was begun anew. The list of confirmed prey obtained from the algorithm, i.e., those not eliminated, accounts for the number of precipitin lines observed between the predator extract and each of the antisera, if one makes the extremely conservative assumption that the row entries in Fig. 2 are additive (i.e., that the number of precipitin lines between an antiserum and a mixture of two or more cross-reacting taxa is equal to the sum of the lines observed when each cross-reacting taxon is tested separately). We know of exceptions to this assumption, but its use is necessitated by the general impracticality (see "Discussion") of routine identification of "reactions of identity" among the numerous precipitin bands of Fig. 2. Thus, the number of confirmed prey for a given predator is a minimum estimate of the true number of prey taxa consumed.

## Results

We have successfully prepared antisera to all 20 target taxa in the mudflat community. A large number of hours was spent preparing organism extracts. Initial sorting of animals from screened sediment necessitated by far the greatest expenditure of time (Table 2), but, as we became more experienced (e.g. the nematode phototaxis), less time was required. The most abundant polychaete on the mudflat, *Manayunkia aestuarina*, was the most difficult to isolate in quantity because of its small size and habit of hiding inside its tube. Specificity tests of the polyclad flatworm antiserum revealed extensive cross-reactions but few precipitin lines with the target meiofaunal turbellarians. Thus, our attempt to circumvent tedious sorting of these small animals by producing an antiserum to a phylogenetically related and easily collected group was a failure. Precipitin reactions with the polyclads isolated from Lincoln Park will not be discussed further.

Using the worst-case criterion of maximum number of precipitin lines observed, cross-reactions were observed in

224 (58.9%) of the 380 possible reactions (20 antisera times 20 extracts minus 20 self-reactions). This number includes both one- and two-way cross-reactions, which we define for any two species A and B as:



Recall in comparing Fig. 1 with Fig. 2 entries that the number given in Fig. 2 was the maximum observed in several trials and that many of the cross-reactions of Fig. 2 were so weak as to be detectable only under microscopic examination.

The maximum number of precipitin lines observed in self-reactions ranged from 5 to 15; for cross-reactions, from 1 to 9 (Fig. 2). Antiserum to *Huntemannia jadensis*, a harpacticoid copepod, produced 9 cross-reacting lines with the general harpacticoid extract, which contained *H. jadensis* protein as well as that of other harpacticoid species.

Some examples of two-way cross-reactions include those of *Mya arenaria* with *Macoma balthica*, with the tanaid, and with *Nereis limnicola* (partially shown in Fig. 1A), and *Corophium salmonis* with *Crangon franciscorum* (Fig. 1B). Slightly less than 69% of the total number of cross-reactions were two-way, and the antisera of just 6 taxa (*Pygospio elegans*, the tanaid sp., *Macoma balthica*, *Mya arenaria*, *Paranemertes peregrina*, and *Oligocheata*) account for nearly half of all two-way cross-reactions. The number of cross-reactions may appear high, but by comparing the number of lines involved in any of the cross-reactions for a given antibody with the number of lines in the self-reaction for that particular antibody, the severity of the cross-reactions is more realistically portrayed. For example, 63% (12 of 19) of the possible cross-reactions occurred with *Crangon franciscorum* antiserum. Since 12 precipitin lines were observed in the *C. franciscorum* self-reaction, at least 12 times 19, or 228 cross-reacting lines were possible. However, only 22 lines (9.6%) of the 228 possible were observed among the 12 taxa which cross-reacted with *C. franciscorum* antiserum. Of these 12 taxa, the largest number of cross-reacting lines (3 each) occurred with *Anisogammarus confervicolous* and Ostracoda. Thus, al-

though many cross-reactions occurred, on the basis of the number of lines which might theoretically have appeared in these cross-reactions, only about 6% showed up on average, and nearly half of the cross-reactions were evidenced by just one line. The worst case, *Mya arenaria*, revealed 29% of the total possible number of cross-reacting lines, and the best case, *Nemertea* sp., only 4% (Fig. 2).

Extracts of, and antisera to, a particular organism may participate in different numbers of one-way cross-reactions. The directionality of this difference, i.e., whether the antiserum participates in more one-way cross-reactions than does the extract or vice-versa, was a significant factor in assessing specificity. For example, in the extreme case for *Hobsonia florida*, the antiserum had 13 of 19 possible cross-reactions, yet the extract had only 6 (Fig. 2). Thus, although the antiserum to *H. florida* also formed at least one precipitin line with 13 other extracts in the target community matrix, the extract itself was recognized only by the antiserum and 6 others. *Nemertea* sp. extract, on the other hand, participated in many more one-way cross-reactions than did its antiserum. Here, the extract cross-reacted with many other antisera in the matrix, and the antiserum formed against *Nemertea* sp. participated only in two-way cross-reactions. In Fig. 3 we classify the extracts in terms of their reactions with antisera. Phrased alternatively, Fig. 3 shows the ability of the antisera produced to discriminate the target taxa. Note that, using the extracts to define multidimensional axes, the antisera could be classified according to the extracts' abilities to discriminate them. Because the matrix of Fig. 2 is not symmetrical, a different dendrogram would result, but it would be of little interest in evaluating antiserum specificity. We discuss interpretations of this asymmetry below.

Immunological analysis of gut contents was successful (Fig. 4), and in many instances several different prey could be detected simultaneously in a single gut extract. In these gut analyses, 18% of the 209 possible links (11 predators and 19 potential prey) were confirmed. The algorithm eliminated a great many positive antiserum reactions which might have indicated trophic links. Nematode antiserum, for example, produced multiple precipitin lines with the gut contents of a variety of other taxa, but nematodes could not be confirmed as prey for any taxon. It is noteworthy that several deposit-feeders (*Hobsonia*

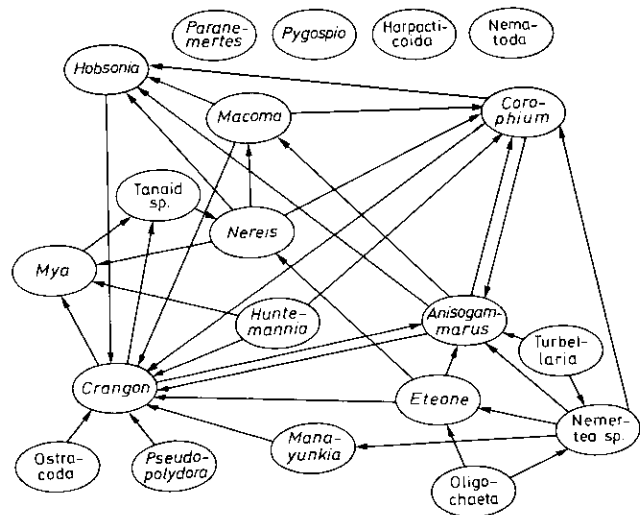


Fig. 4. Preliminary immunological identification of trophic connections among macro- and meiobenthic taxa of the Skagit River sand flat, northern Puget Sound, on 13 February 1978. The four taxa without links were not confirmed prey for any taxon tested. Full specific names are given in Table 2

*florida*, *Corophium salmonis*, *Macoma balthica*, and *Manayunkia aestuarina*, the latter a facultative deposit-feeder) contained animal antigens in their guts. Meiofaunal taxa, both hard- (Ostracoda, *Huntemannia jadensis*) and soft- (Oligochaeta, *Turbellaria*) bodied, were detected as prey of 6 different predators. Microscopic analysis of gut smears of comparable numbers of these same predators revealed the presence of amorphous material resembling detritus, but no discernible animal parts.

#### Discussion

The dendrogram of immunological similarities (Fig. 3) reveals two trends. First, it provides a convenient summary of which taxa will be most difficult to discriminate with the present antisera (e.g. *Eteone longa* from *Manayunkia aestuarina*). Second, it suggests as expected (e.g. Wright, 1974) that serological affinities may be in part a reflection of phylogenetic relationships. For example, peracarid crustaceans, bivalves, and spionid polychaetes all clustered as expected from conventional views of systematics (Barnes, 1974). The dendrogram, however, is by no means an accurate phylogenetic summary, viz., the *Nereis limnicola*-*Paraneimertes peregrina* and *E. longa*-*M. aestuarina* pairs. Although one could conclude that conventional phylogeny

might be incorrect in some instances, it is more likely that the immunological data supporting unusual relationships reflect the production of antisera to contaminating material in the guts of the organisms or on their body surfaces (Fig. 5). Our efforts to remove such materials may not have been entirely successful.

A weak hint as to food web relationships may be gained from the asymmetry of the observed cross-reactions. Because the precipitin reaction is more sensitive to antibody than to antigen concentration (Williams and Chase, 1971), the predator-prey cross-reactions due to a contaminated predator should, on the average, be stronger when the prey antiserum is used than when the contaminated predator antiserum is employed in double diffusion tests (see both one- and two-way cross-reactions of Fig. 5). *Paranemertes peregrina*, for example, is known to prefer nereids over alternative prey (Roe, 1976). *Nereis limnicola* antiserum produces 6 of 7 possible precipitin lines with *P. peregrina* extract, while *P. peregrina* antiserum produces 5 of 14 possible lines with *N. limnicola* extract. In spite of our decontamination procedure, it is thus likely that the *P. peregrina* extract we used still contained antigens from *N. limnicola*. We feel this is a likely possibility, as Davies (1969a) found that freshwater triclads could still be detected after 48 h in the guts of newts, fishes, and leeches. Using diffusion techniques similar to ours, Pickavance

(1970) also found that triclads could be detected in the guts of various starved invertebrate predators after nearly a week. Because the concentrations of individual antigenic components in whole-organism extracts are unknown in our procedure and because these components are likely to differ in the strength of the immunological responses they elicit, it would be imprudent to suggest that asymmetric cross-reactions provide a reliable indication of predator and prey identities. To give an unequivocal indication of predator-prey relationships, cross-reactions must be reduced to a level at which they cannot be confused with the self- or identity reaction.

Because of such cross-reactions, it is hazardous to employ immunological methods in predator-prey systems where all likely cross-reactions have not been (or cannot be) explored. If Fig. 2 did not contain all the abundant and frequent taxa at the Skagit site, the results in Fig. 4 could be questioned much more seriously; any one of the apparent predator-prey links might be due to cross-reactions with taxa missing from Fig. 2. We in fact have such reservations regarding "confirmed" prey of *Crangon franciscorum* and *Mya arenaria* (Fig. 4). *M. arenaria* may filter unassayed planktonic species from the water column, and mobile *C. franciscorum* individuals may forage in other communities. In both cases, unexplored cross-reactions might account for indicated food

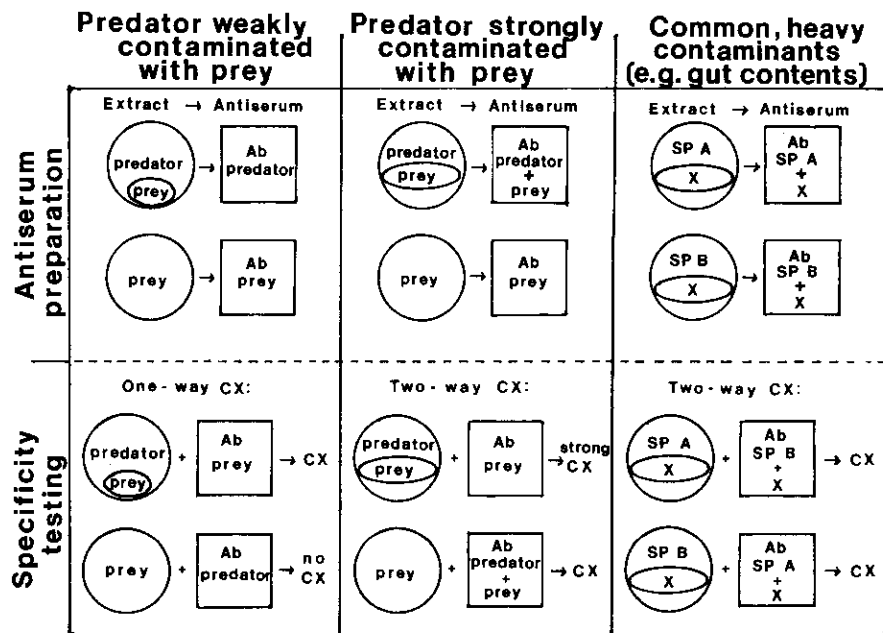


Fig. 5. Schematic showing both antibody (Ab) production from immunizations with contaminated extracts and cross-reactions (CX) expected in specificity testing of the resultant antisera

web links. A more subtle problem with the algorithm we use is that species close to each other in the dendrogram (Fig. 3) are difficult to distinguish. Confirmation of *Pseudopolydora kempii japonica* as a prey item, for example, all but precludes confirmation of *Pygospio elegans*. Other notable problem pairs in this category are *Harpacticoida-Huntemannia jadensis* and *Nereis limnicola-Paranemertes peregrina*. Thus, the actual food web surely has more links than we have indicated. The feasibility of using immunological methods to test hypotheses regarding selective predation of congeners or even members of the same family should be tempered with the knowledge of this potential problem.

The ideal immunoassay would utilize only species-specific antisera (i.e., no cross-reactions among target taxa), but such antisera are not easily obtained. Thus, to achieve additional discriminatory power, it is necessary to approach the problem of cross-reaction in one of three ways: (1) prepare species-specific antisera using decontaminated or immunologically pure organism extracts (*sensu* Caldwell et al., 1975); (2) use cross-reacting antisera as they are but identify which of their components cross-react; or (3) eliminate the cross-reacting antibodies from the antisera after production. The rationale for using any or a combination of these approaches must rest with the particular problem at hand.

Production of species-specific immunogens, substances which provoke antibody formation, could thus be approached in two basic fashions: feed the organisms to be used to make the extract some exotic food which is known not to cross-react with antisera to other target organisms, or isolate single antigenic components known to be unique to the target species. The first technique, if successful, may enhance the gut clearance process (as might elevated temperatures, or glass beads for deposit-feeders) and yield immunogens uncontaminated with gut contents, thereby reducing the probability of secondary antibody production. Cannibalistic organisms may be ideal in this sense. Extension of the isolation period may also be helpful, provided mortality can be minimized. Our animals suffered high mortalities when we tried to starve any of them for more than a week. However, phylogenetic similarities may appear among organisms in any target community, especially when whole-organism extracts are used as immunogens. The second technique, e.g. the excision of specific precipitin lines from two-dimensional immuno-

trophograms and subsequent injection of this material as an immunogen (Caldwell et al., 1975; Vestergard, 1975; Alexander and Kenny, 1977), would be highly useful since the resulting antiserum would contain only those antibodies specific to an individual major antigenic component of the target species. This method is time-consuming, since it requires extensive antigenic analysis.

Alternatively, if it were known exactly which components cross-reacted between different taxa and which did not, our ability to discriminate predator-prey relationships would be greatly improved. Several techniques are routinely used for identifying cross-reacting components. By employing two-dimensional immunoelectrophoresis and rocket-line immunoelectrophoresis (Axelson and Bock, 1972; Thirkill and Kenny, 1974, 1975), we identified the antigenic determinants responsible for cross-reactions between the bivalves *Macoma balthica* and *Mya arenaria*. Other methods for identifying cross-reacting antigenic components include the enhancement and suppression of precipitin lines in immunoelectrophoresis (Axelson and Bock, 1972). If we are successful in identifying other cross-reacting components among the target community taxa, it may then be possible to reveal even more trophic links than are possible with our present, strictly numerical algorithm. Interpretation becomes increasingly more difficult and time-consuming the more cross-reactions there are, but unambiguous identification of food web connections using electrophoretic techniques is nonetheless possible. The time and volume of antiserum expended in this approach preclude its routine application in our system.

Many techniques are available for increasing antiserum specificity after its production, among them dilution and absorption. Since precipitin lines at equivalence are composed primarily of antibodies (Williams and Chase, 1971) and form with varying intensity depending on antigen and antibody concentration, antiserum dilution allows precipitin line formation only with those antigenic components in highest concentrations and having the highest affinity. In preliminary double-diffusion tests with various dilutions of antisera run against their homologous whole-organism extracts and cross-reacting extracts, we have found in many cases that cross-reacting precipitin lines no longer form, but that the self-identity lines continue to form, although in reduced numbers. We are evaluating this method of increasing antibody specificity fur-

ther because of its inherent simplicity. Davies (1969a, and personal communication) was able to reduce the number of cross-reactions using absorption. Given sufficient antigenic material to permit its expenditure, this method entails addition of cross-reacting antigens to an antiserum in excess of the amount required to completely bind homologous antibodies. The resulting precipitate is removed by centrifugation, leaving a more specific antiserum available for use.

Finally, the immunogen dose could be reduced in hopes of stimulating production of antisera containing antibodies to some major and specific component of the species. Whole-organism extract of *Corophium salmonis* was injected into rabbits at concentrations of 0.04, 0.4, and 4.1 mg protein ml<sup>-1</sup>. No detectable antibodies were found at the lowest level, but antibodies were produced against both of the higher extract concentrations. The antiserum from the 4.1 mg protein ml<sup>-1</sup> injection series gave the greatest number and most intensely stained precipitin lines in the self-reaction, but we are now determining whether the intermediate dosage may have produced a more species-specific antiserum.

Thus, while several approaches are available for increasing the specificity of our antisera or for preparing new antisera of greater specificity, our immunoassay in its present state is a powerful tool for elucidating trophic interactions. We would like to emphasize, however, that quantification of prey consumption by predators is not now possible with our immunoassay. The micro-Ouchterlony technique (Ouchterlony, 1968) cannot be used as a quantitative assay of antigen concentration in predator-prey applications because the numbers, relative positions, and curvatures of precipitin lines are functions of rates of diffusion of antibodies and antigens. These diffusion rates are affected by concentration gradients, molecular weights, pH, temperature, and agarose gel properties. At least the first two parameters in this list are altered by digestion, and the antigen concentrations must be extremely sensitive to predator and prey sizes. Although a variety of quantitative immunological techniques are available, many are inapplicable because our antisera are not taxon-specific. Techniques which would allow us to use our antisera to identify and quantify non-cross-reacting antigenic components, e.g. rocket-line immunoelectrophoresis (Axelson and Bock, 1972), would require a relatively large

expenditure of antisera. Such an expenditure would be justified if the antigen concentration in the gut could be easily translated into mass or number of prey consumed. However, this translation would require knowledge of gut detection times ("contact times" *sensu* Peterson and Bradley, 1978), feeding periodicities, prey size selection, and further trophic considerations. Obtaining this information for even a few predator-prey combinations is extremely time-consuming. Since our primary goal is to document the trophic links of an entire community, expending such an amount of time on only a few predator-prey combinations was not deemed appropriate.

We have been most encouraged by the results of our preliminary evaluation of immunological methods for analysis of predator-prey interactions in our target mudflat community. Collection difficulties aside, we were indeed able to produce antisera of sufficient specificity to positively identify many unexpected trophic links among these benthic invertebrates. Many of these links would go undetected using traditional visual gut content analysis. Having had such success using relatively unspecific antisera in a preliminary analysis of only a few predators, we are led to believe that production of even more specific antisera will provide the methodology necessary to document the breadth of trophic connections in a marine benthic food web.

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