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Feeding by a lotic mayfly grazer as quantified by gut fluorescence

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Abstract. It is often of interest in ecological research to quantify the feeding of study animals. Approaches used previously with grazing aquatic insects have been either labor-intensive (algal cell counts of gut contents), lacking in sensitivity (AFDM, relative gut fullness), or involve radioactive tracers (dual isotope method). A method developed and applied by marine biologists to study zooplankton feeding rates uses the amount of plant pigment in the gut as a feeding index. We have modified this technique to assess feeding by larvae of a grazing mayfly, *Baetis bicaudatus* (Ephemeroptera: Baetidae) in a western Colorado stream. Gut pigments include chlorophyll from ingested algae, which is rapidly degraded to phaeophorbide and passes unassimilated through the gut, thereby acting as a tracer of feeding activity. Our experiments showed that the optimum dissection and pigment extraction procedure entailed macerating whole *Baetis* individuals and extracting the gut pigments for 24 hr in 90% ethanol. Pigment levels (chlorophyll *a* and phaeopigment *a*) were quantified on a Turner Model 112 fluorometer and expressed as ng total pigment per animal. The technique was reliable using individual mayflies as the sample unit. Storage of intact animals in extraction bottles with a small amount of solvent for 8 hr before processing did not result in degradation of pigment, which added flexibility to the procedure. We foresee applications of this approach in studies of relative feeding rates (daily, seasonal, phylogenetic patterns) and feeding under experimental conditions involving manipulation of factors such as predation, competition, food availability, and the physical environment.

Key words: aquatic insect, grazer, gut analysis, feeding rate, *Baetis bicaudatus*, fluorometry, chlorophyll, stream ecology, Ephemeroptera.

Questions regarding the feeding ecology of target species arise frequently in studies of biological interactions within and among populations. Although in some species feeding ecology can be easily addressed, feeding by others may be difficult to quantify, and there are often disadvantages to even the most direct approach, such as gut content analysis (Peckarsky and Penton 1989). Recently, our research has generated questions about the feeding ecology of grazing mayfly nymphs, and has motivated us to develop a quick but reliable method of quantifying their gut contents. In this paper we discuss this method, developed by zooplankton biologists, and adapted for use with a lotic insect grazer, *Baetis bicaudatus* Dodds (Ephemeroptera: Baetidae).

There are numerous ways to quantify feeding by aquatic insect grazers, all of which have advantages and disadvantages. One such approach is the analysis of gut contents by microscopy (e.g., Allan et al. 1990), which is labor-intensive (up to 4 hr per animal; personal observations by authors) and may provide more information than is necessary to address the question at hand. For example, taxonomic composition of gut contents may be irrelevant when the parameter of interest is the gross feeding rate or quantity of material present in the gut upon sampling. In addition, number of algal cells without corresponding biomass measurements may be a poor indicator of volume of material ingested because of the great variability in algal cell sizes. Although conversion of algal cell counts to biovolumes is becoming common practice and remedies the problem of variable cell sizes, it is nonetheless a time-consuming process and may be prohibitive in studies with even moderate replication. Measurement of relative gut fullness as 0, 25, 50, 75 or 100% of maximum (Williams and Levens 1988) requires much less time and effort, but is not strictly quantitative and may be insensitive to small yet significant differences among animals. Measurement of ash-free dry mass of gut contents is similarly insensitive because complete separation of ingested food from other gut materials is difficult. Subtraction of background or average tare gut weight may introduce considerable error, particularly at low gut fullness (J. D. Allan, University of Maryland, personal communication). Yet another option, the dual

isotope method of Calow and Fletcher (1972), is among the most sensitive methods currently available. However, the technique is restricted to controlled environments and is not applicable to analysis of field collected animals. In addition, some researchers find the use of radioactive tracers undesirable.

A technique that has proven to be both sensitive and efficient ($\leq$10 min per animal) was developed and applied by marine biologists studying zooplankton feeding rates, and uses the amount of plant pigment (chlorophyll and phaeopigment) in the gut as a feeding index (Mackas and Bohrer 1976). Chlorophyll from ingested algae is rapidly degraded to phaeophytin and ultimately phaeophorbide (Fig. 1) (Shuman and Lorenzen 1975, Welschmeyer and Lorenzen 1985, Ramcharan and Sprules 1988), which pass unassimilated through the gut and act as tracers of feeding activity. Since fluorometric analysis of gut contents quantifies both chlorophyll and phaeopigments, it reflects the amount of plant food present in the gut at a given time. The gut fluorescence technique has been applied successfully in studies of diel feeding patterns of marine zooplankton (e.g., Dagg and Wyman 1983, Baars and Oosterhuis 1984, Head et al. 1984), although its usefulness in studies of absolute feeding rates has been challenged because of possible losses of pigment through gut transit (Conover et al. 1986, Wang and Conover 1986, Lopez et al. 1988; see Discussion section of this paper). While this procedure measures only the "fullness" of the gut, the pigment content may be converted to average ingestion rate by employing formulae that take into account the gut evacuation time and the fractional loss of pigment (if any) in the gut (Mackas and Bohrer 1976, Kiørboe et al. 1982, Dagg and Wyman 1983). Mackas and Bohrer (1976) argue that if gut clearance and pigment loss are constant for a given species and sample set, then gut fullness (pigment content) is directly proportional to average ingestion rate, and the former may be used as an index of the latter. Accepting these assumptions, we have used gut fullness (ng pigment per individual) as an index of ingestion rate, as have other researchers (e.g., Boyd et al. 1980, Dagg and Grill 1982, Kiørboe et al. 1985), for assessment of feeding activity by a stream-dwelling grazing mayfly nymph, *Baetis bicaudatus*.

The objective of this study was to develop and test a procedure for determining recent feeding history or relative algal consumption by mayflies. To do this we had to:

1) determine how many mayflies should constitute the sample unit,

2) select the most effective and efficient method of gut removal and extraction, and

3) select from alternative response variables an index of *Baetis* feeding activity.

In addition, we have reviewed and summarized a large body of relevant marine literature. Detailed instructions for application of the method are presented as an appendix to the paper.

COMPOUND	MOLECULAR WEIGHT (relative to chl a)	FLUORESCENCE (relative to chl a)
Chlorophyllide	70 %	identical
Phaeophytin	97 %	lower
Phaeophorbide	67%	lower (same as phaeophytin)

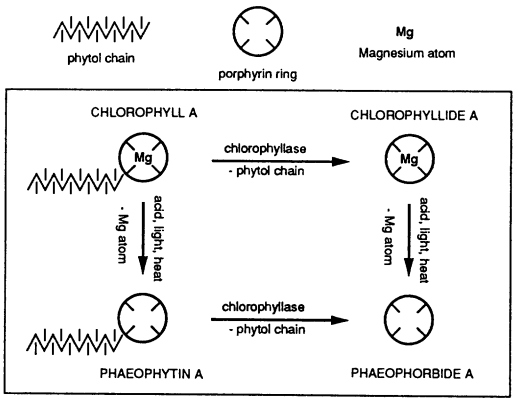

FIG. 1. The relative properties of chlorophyll *a* and its degradation products, the primary components of the molecules, and a schematic of the degradation of chlorophyll into its degraded forms. As a result of the similarities in fluorescence characteristics, chlorophyll and chlorophyllide cannot be distinguished fluorometrically, nor can phaeophytin and phaeophorbide. The convention among users of the gut fluorescence technique is to refer to phaeophytin and phaeophorbide collectively as phaeopigments.

Methods

1. Pilot study: determination of sample unit

In previous studies of gut pigment content, primarily with marine copepods, each replicate ranged from one animal (Lopez et al. 1988) to 100 animals (Wang and Conover 1986) extracted together. Typically, the amount of pigment per

copepod was small and pigment extracts from single animals were too dilute for detection by a fluorometer. We conducted a pilot experiment in August 1988 to determine whether the amount of pigment per *Baetis* was within the range of sensitivity of our instrument, and if not, how many animals were needed to generate pigment extracts of sufficient concentration. Sixty *Baetis* from the East River, Gunnison Co., Colorado (see Peckarsky 1979 for study site description) were held for 24 hr in small circular flow-through plexiglass chambers (Peckarsky and Cowan, unpublished) containing periphyton-covered stones. The mayflies were then dissected, guts macerated (method DM of the following section) and extracted singly (n = 5 individuals) or in pooled sets of 5 (n = 5 sets of 5 individuals) or 10 (n = 3 sets) individuals. Chlorophyll *a* (chl *a*) and phaeopigment *a* (pheo *a*) were measured using a Turner Model 112 fluorometer, and ng pigment per animal compared among the three treatments. Data were analyzed by one-way MANOVA, with number of *Baetis* per group as the treatment effect, and four dependent response variables: chl *a* per individual, pheo *a*, total pigment (chl *a* + pheo *a*) and % chl (chl *a*/total pigment). Analysis of residuals indicated that a $\log(y + 1)$ transformation was necessary to satisfy the assumptions of normality and variance.

2. Gut dissection and exposure of pigments to solvent

In July 1989, the pigment yields of several potential methods of accessing *Baetis* gut contents were compared to address three questions: 1) to what degree must gut contents be exposed to the solvent; 2) is it necessary to completely remove the gut from the animal; and 3) must specimens be processed immediately or can processing be delayed for a short period of time? Sixty *Baetis* were sampled directly from a single riffle in the East River and 15 randomly assigned to each of four treatments: M = whole animal macerated (midsection opened with forceps and gut contents released into solvent) then contents (including animal) extracted; ML = whole animal placed in solvent for 8 hr, then processed as in treatment M; D = gut dissected from live animal, placed intact in bottle with solvent and extracted (without body); DM = gut dissected from live animal, macerated (opened with forceps, contents teased apart), and extracted (without body). Gut removal was only reasonable on live animals, as once the animal had been in preservative for approximately 60 s, the body tissues hardened and a clean dissection was impossible. Animals frozen then thawed presented a similar problem. Live dissection was performed at low power under a dissecting microscope by holding the body in place with one pair of forceps and tugging firmly on the head with another pair of forceps. The gut slid out cleanly with the head, which was then servered just before the foregut. Head capsule widths of experimental animals were measured, with the intention of keeping the animals within a preestablished size range (0.8–1.0 mm), and sex determinations of nymphs were made. Response variables (chl *a*, pheo *a*, total pigment, % chl) were compared among treatments using one-way MANOVA. Because treatments were not cross-classified, the four treatments (D, M, DM, ML) were analyzed as levels of one main effect. Finally, background fluorescence of *Baetis*, or fluorescence of anything other than gut pigments, was measured in July 1990. Fifteen *Baetis* were sampled from a productive riffle in the East River, the heads and guts removed and the remainder of the body extracted.

3. Extraction technique

Plant pigments in the guts were extracted and fluorescence measured on a Turner Model 112 fluorometer. The fluorometer was equipped with a high sensitivity door and calibrated with a standard chl *a* solution from *Anacystis nidulans* (Sigma Chemical no. C-6144) to a range of 0.05 to 100 μg per liter. The extraction technique was modified from Lorenzen (1967) and is detailed in the appendix to this paper. Notable deviations from standard procedure are the omission of a grinding step, boiling of extracts prior to extraction, and the use of ethanol rather than acetone. Grinding has been found to be unnecessary for diatoms and cyanophyta (the primary constituents of the East River flora) (Holm-Hansen and Riemann 1978, Sartory and Grobbelaar 1986) and is perhaps even detrimental to chl *a* yield (Burnison 1980, Cowan and Peckarsky, unpublished data). Very short-term boiling of extracts at the boiling point of the solvent (not

of water) has been shown to enhance chl *a* yield by as much as 28% (Burnison 1980, Sartory and Grobbelaar 1986).

There is considerable disagreement in the literature over which solvent is ideal for extracting pigments from algae, although there is growing concern over the traditional use of 90% acetone, which has been demonstrated repeatedly to be inferior to most other solvents in extraction efficiency (Sartory and Grobbelaar 1986; also, see reviews by Marker et al. 1980, Nusch 1980, and Coveney 1988). Many alternatives have been explored, among them methanol (Marker 1972, Holm-Hanssen and Riemann 1978, Marker and Jinks 1982, Riemann and Ernst 1982), ethanol (Moed and Hallegraeff 1978, Nusch 1980, Sartory and Grobbelaar 1986), methanol + chloroform (Wood 1985, Lloyd and Tucker 1988), dimethyl sulfoxide (DMSO) (Shoaf and Lium 1976, Burnison 1980), and N-N-dimethyl formamide (DMF) (Speziale et al. 1984, Neveux 1988). We have selected ethanol from among these alternatives for use in our extraction procedures. Ethanol poses no problems of isomer or cation formation upon proper acidification (Moed and Hallegraeff 1978, Nusch 1980), is less toxic and hazardous than methanol (Marker et al. 1980), DMSO, or DMF (Speziale et al. 1984), and is a common component of aquatic research laboratories. In addition, Sartory and Grobbelaar (1986), in an elaborate controlled study, found 90% ethanol to be the most efficient extractant of chl *a* from diatoms, green algae, and blue-green algae, particularly when extracts were heated to solvent boiling point for 5 min before extraction.

Equations for determination of chl *a* and phaeopigments from fluorescence measurements were derived from Yunev and Berseneva (1986). Since we were interested in the amount of plant material originally ingested (presumably, but not necessarily, all chl *a* upon ingestion), we expressed concentrations of phaeopigments as molar equivalent weights, that is, as ng chl *a* from which the degradation product was formed (Coveney 1988, Dagg et al. 1989, Ellis and Small 1989). For ease of presentation, values in the figures and text are presented simply as ng pigment, but it should be understood that these quantities represent relative (molar) rather than absolute (mass) amounts of phaeopigments. Phaeophytin *a* and phaeophorbide *a* are 97% and 67% the molecular weight of chl *a*, respectively (Fig. 1), and therefore calculations of the concentrations of these compounds must be modified if they are to be expressed in absolute terms (Helling and Baars 1985, Conover et al. 1986). Values may be converted to ng/animal by multiplying phaeopigment by 0.67, assuming that all phaeopigment is phaeophorbide (which is likely; Dagg and Wyman [1983], Welschmeyer and Lorenzen [1985].)

Results and Interpretation

1. Determination of the sample unit

The pilot study showed that individual *Baetis* could be used as the sample unit. None of the values of pigment per animal (chlorophyll or phaeopigment) were less than 3 ng/animal, and most individuals contained between 10 and 200 ng chl *a*; the lower limit of resolution for our fluorometer was 0.50 ng per sample (in 10 ml solvent). Furthermore, estimates of mean levels of total pigment per animal did not differ between mayflies extracted singly or in groups of 5 or 10 (Table 1). However, the mean chlorophyll content for 10 pooled individuals was significantly lower than estimates based on 1 or 5 individuals. We suspect that this is an experimental artifact; because of the number of guts to be dissected in this treatment, exposure to the microscope lights was prolonged, which may have resulted in photodegradation of pigments, reducing the % chl but not total pigment. This conjecture is supported by the trend for lower % chl in the 10 *Baetis* treatment. Also noteworthy is that the variance was much lower in the pooled samples, indicating that greater replication of individuals would provide a better estimate of the mean. On the basis of the results of this pilot study we decided to use only one animal per replicate and to increase the replication in future experiments to $n = 15$.

2. Exposure of samples to solvent

Comparison of the four methods of sample preparation revealed differences in pigment yield (Fig. 2). When intact dissected guts were extracted without maceration (treatment D) we obtained a significantly lower yield of chl *a*, pheo *a* and total pigment per individual than

TABLE 1. Results of the pilot study determining number of individuals per replicate, comparing pigment yields of individuals (n = 5 replicates) and pooled animals (5 or 10 *Baetis* per replicate, n = 5, 3 replicates). The overall MANOVA was not significant ($F_{3,6\ df}$ = 1.17, p = 0.368); the outcome of individual ANOVAs for each response variable are presented. All pigment values are ng/individual.

	Mean ± 1 SE			ANOVA	
Variable	1 *Baetis*/rep.	5 *Baetis*/rep.	10 *Baetis*/rep.	$F_{2,10\ df}$	$p > F$
Chl *a*	43.4 ± 4.9	44.2 ± 5.5	24.7 ± 0.9	4.64	0.037
Pheo *a*	30.2 ± 14.0	23.2 ± 9.5	37.3 ± 3.8	0.65	0.543
Total Pigment	73.6 ± 16.4	67.4 ± 7.4	62.0 ± 3.1	0.06	0.938
% Chl	66.0 ± 9.8	68.3 ± 11.2	40.1 ± 3.1	2.13	0.169

all of the other treatments (Table 2). It appears that pigments became trapped in the gut, never being released into solution, and we conclude that if the gut is to be removed from the body, its contents should be thoroughly exposed to the solvent by maceration as well. There were no other differences among treatments. Notably, treatment ML, whereby individuals were placed in solvent and then prepared for extraction 8 hr later, was never significantly different from treatment DM, the procedure of maximum and most immediate exposure. In fact, mean pigment levels were almost indistinguishable for all four of the response variables tested (Fig. 2). We conclude that when intact animals were allowed to remain in the solvent for a short period of time (≤8 hr) before processing there was neither a decrease in the amount of pigment extracted per individual nor an effect on the relative abundance of chlorophyll and phaeopigment. One of our incentives for conducting this experiment was our concern that any delay in processing of intact animals would delay solvent penetration of the gut, while digestive activity, or at least degradation of pigments, continued to occur. Given this scenario, the % chlorophyll would have been lower in treatment ML than in either M or DM, which was not the case. We were also concerned that compounds present in animal remains would interfere with pigment fluorescence. Because there were no differences in any of the parameters between treatments with the carcass (M and ML) and without (DM), we found no reason to separate the gut from the body before extraction. Furthermore, mean background fluorescence (body alone) for *Baetis* was only 4 ng/animal, which was a very small portion (approximately 2%) of the total gut fluorescence, and varied little among individuals (SE = 0.23; range = 3–6 ng/animal).

3. *Selecting an index of Baetis feeding activity*

One of the goals of this study was to select one or more of the response variables as an index of *Baetis* feeding activity. The most commonly used index of zooplankton feeding has been total pigment per animal. The gut contains a mixture of chl *a* and its degradation products (Kiørboe and Tiselius 1987), the relative proportions of which may vary dramatically between individuals owing to differences in their recent feeding history. In studies where only the total amount of material ingested is of concern, total pigment is a good index because it is independent of the relative proportions of pigments, and of the state of the pigments when ingested.

Another potential feeding index is % chlorophyll. It might be expected that the amount of chlorophyll relative to phaeopigment would increase with the recentness of feeding activity and that, therefore, % chl would be a good indicator of how actively animals were feeding when sampled. The research of others (Nicolajsen et al. 1983, Ramcharan and Sprules 1988) and our own unpublished data suggest that this relationship is not straightforward. Ramcharan and Sprules (1988), when looking at gut filling rates, found that in actively feeding copepods chl *a* reached an equilibrium in approximately 30 min, whereas pheo *a* leveled off after 2 hr. Percent chl was therefore initially very high (entering an empty gut) but dropped as the gut filled and pigment began to degrade, the degraded pigment increasing in proportion to the incoming, non-degraded form. The result was

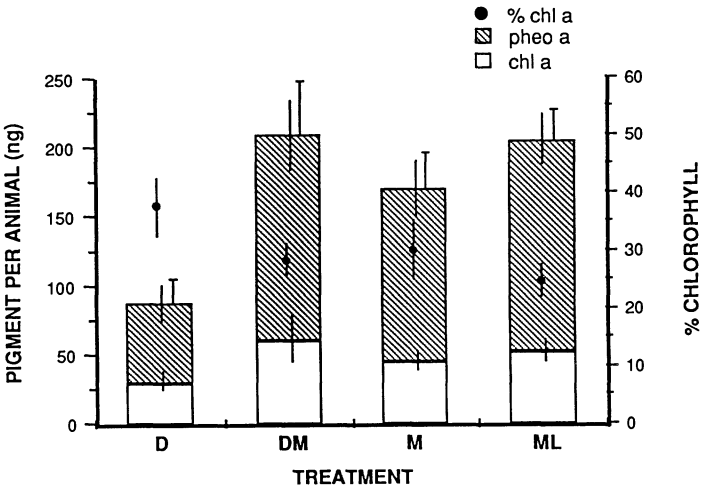

FIG. 2. Results of the exposure study, comparing gut pigment yield among four methods of processing animals. Treatment categories are: M = whole animal macerated (midsection opened with forceps and gut contents released into solvent) then contents (including animal) extracted (n = 11); ML = whole animal placed in solvent for 8 hr, then processed as in treatment M (n = 13); D = gut dissected from live animal, placed intact in bottle with solvent and extracted (without body) (n = 15); DM = gut dissected from live animal, macerated (opened with forceps, contents teased apart), and extracted (without body) (n = 15). Values ae means ± 1 SE. T-shaped error bars at tops of columns are for total pigment; plain error bars are for individual pigments. Several replicates were discarded from treatments M and ML because individuals were immediately pre-emergent, as was indicated by large black wing pads and empty guts.

an inverse relationship between % chl and total pigment.

When considered in the context of gut evacuation, however, the interpretation becomes more complex. In gut clearance studies we found a strong negative relationship between total pigments and % chl (Spearman's $r = -0.662$, $p = 0.0001$; Cowan and Peckarsky, unpublished data), a relationship that has appeared consistently in our descriptive and experimental studies. Peckarsky (in press) reported a consistently low, almost non-existant, fraction of pheo *a* on East River stones; thus it is improbable that % chl in guts is a function of incoming (ingested) phaeopigment. We suspect that the explanation is related to digestive activity, which may cease or decrease when feeding stops (Nicolajsen et al. 1983), leaving the material in the anterior of the gut in a less digested form than in its posterior counterpart. Upon evacuation the contents of the hind gut (primarily phaeopigment) are eliminated leaving a gut mass that is higher in % chlorophyll than before defecation. This hypothesis is supported by the results of Nicolajsen et al. (1983) who found that % chl was consistently lowest at night when feeding of the copepod *Pseudocalanus* sp. was greatest. The findings of the present study argue even further for this hypothesis, in that a strong negative relationship was observed between % chl and total pigment (Spearman's $r = -0.594$, $p = 0.0001$). Essentially, high values of % chl could indicate either a filling or an emptying gut, two distinctly different physiological and ecological states. We conclude that % chl is not a reliable indicator of recentness of feeding and cannot be interpreted without additional information, such as rate of pigment degradation in the gut, and rates of gut filling and evacuation. In the absence of such data we suggest that total pigment alone provides a consistent and easily interpretable index of algal consumption.

Discussion

We have presented a procedure for measuring gut fluorescence of the grazing mayfly *Baetis bicaudatus*, that should be broadly applicable. However, constraints may be imposed on the application of the technique by the natural history of the study organism and its environment, and/or the nature of the questions being asked.

TABLE 2. Summary of data analyses of experiments to determine optimum specimen preparation for extraction. For the MANOVA, sum of squares is Wilk's criterion, and the df are the numerator and denominator degrees of freedom. Treatment categories are M = whole animal macerated (midsection opened with forceps and gut contents released into solvent) then contents (including animal) extracted (n = 11); ML = whole animal placed in solvent for 8 hr, then processed as in treatment M (n = 13); D = gut dissected from live animal, placed intact in bottle with solvent and extracted (without body) (n = 15); DM = gut dissected from live animal, macerated (opened with forceps, contents teased apart), and extracted (without body) (n = 15).

Variable	Source	Sum of Squares	df	F	$p > F$	Paired Comparisons
MANOVA	treatment	0.679	9, 117	2.24	0.024	
Chl *a*	model	7.986	3	3.92	0.014	D < DM
	error	33.925	50			
Pheo *a*	model	18.651	3	7.16	0.0004	D < M; D < DM; D < ML
	error	43.389	50			
Total Pigment	model	13.399	3	6.87	0.0006	D < M; D < DM; D < ML
	error	32.514	50			
% Chl	model	0.900	3	1.55	0.214	
	error	9.692	50			

We will briefly address considerations that should be made when attempting to employ this technique, and that should guide the selection of specific aspects of the methodology.

The data reported here provide a useful reference point for investigators wishing to employ this technique. If the target species is of similar or greater size than *Baetis*, individuals should suffice as the sample unit; smaller species or earlier instars should be tested to determine the sample unit, taking into consideration the sensitivity of the instrument and the lower range of pigment content per study animal. In addition, the feeding periodicity of the study population should be known, as is demonstrated by a discrepancy in the results of the exposure study reported herein and an identical study conducted in August 1987 (Cowan, unpublished data). The same procedural comparisons from the 1987 study, conducted at a time of day when *Baetis* gut fullness was very low (1:00 P.M.), revealed no differences among treatments. Only when the experiment was repeated at a time when *Baetis* gut fullness was highest (this study: 6:00 A.M.; fullness = 4× that of the 1987 study) did differences among treatments become clear (Fig. 2). Finally, the information we collected about dissection and exposure techniques should be universally applicable, unless the anatomy of the species deviates from *Baetis* in a way that would interfere with pigment extraction. In that case, further experimentation may be necessary, including a determination of background (body) fluorescence.

The types of algae in the grazer's diet will influence the choice of solvent and extraction technique (Speziale et al. 1984). Of principal concern is whether the algae contain predominantly chl *a*, or if there is an abundance of other pigments, particularly chlorophyll *b*, that will interfere with fluorescence analysis (Holm-Hansen and Riemann 1978, Gibbs 1979, Coveney 1982). The extraction technique detailed in this paper is applicable to grazers with a diet of predominantly diatoms and incidental amounts of bacteria and fine particulate detritus. When green algae are present in appreciable amounts, a different extraction procedure may be necessary. These algae, rare in the East River flora (Peckarsky and Penton 1990), are relatively difficult to extract, particularly in acetone, often requiring grinding of samples; and they contain considerable amounts of chlorophyll *b* (Marker 1972). In systems where they are abundant, investigators may find it useful to explore alternatives proposed for circumventing chlorophyll *b* interference in fluorescence analysis (Loftus and Carpenter 1971, Boto and Bunt 1978, Gibbs 1979, Coveney 1982, Neveux and Panouse 1987).

Potential limitations to the application of this

method and the interpretation of results mean that researchers considering its use should weigh these constraints against their application before setting out. Conover et al. (1986), Wang and Conover (1986), Kiørboe and Tiselius (1987) and Lopez et al. (1988) tested the assumption that conversion of chl *a* to pheo *a* is 100% efficient, with no loss of pigment to digestive processes, and concluded that it can occur and to different degrees among copepod species. Pigment loss, if and when it occurs, limits the usefulness of plant pigments as conservative tracers of feeding activity, particularly when the method is being employed to assess absolute ingestion rates which, for example, are being extrapolated to whole system models predicting grazer impacts on energy flow (e.g., Baars and Fransz 1984). Similarly, pigment loss is of concern when plant pigment fluxes in the environment (i.e., changes in the relative abundance of degraded and non-degraded chlorophyll) are used as indicators of grazer feeding (e.g., Welschmeyer and Lorenzen 1985, Barlow et al. 1988, Bianchi et al. 1988). Pigment loss is less critical in studies of relative feeding rates, particularly if pigment loss is a constant fraction. For instance, when feeding is compared over temporal (daily, seasonal) or spatial (micro- or macro-distributional) scales, or contrasted between control animals and those under experimentally manipulated conditions of factors such as predation, competition, resource availability, and physical environment, pigment loss may result in an underestimate of feeding rate. However, as long as this underestimate is slight and is uniform across all treatments, it should be of little concern. Lopez et al. (1988) provide a thorough review of this topic, and some researchers are now including correction factors when absolute rates of ingestion or egestion are of interest (e.g., Dam and Peterson 1988).

While there is a tremendous wealth of information about the use of fluorescence to analyze gut contents of marine zooplankton, to our knowledge the present study is the first application of the gut fluorescence technique to an aquatic insect. It clearly remains to be tested whether the generalizations made after extensive testing with marine invertebrates may be extended to grazing aquatic insects as well. For example, the physiology and anatomy of insect digestive systems may differ from crustaceans in such a way that concerns over potential digestive loss of pigments are not relevant to insect biologists. Until such experimental data become available, researchers should make methodological decisions on a case-by-case basis. If the procedure withstands rigorous experimentation, we feel that it represents a powerful tool for aquatic and terrestrial entomologists alike.

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APPENDIX 1.

Instructions for the gut fluorescence technique and extraction of pigments

Processing of specimens

1. Sample animals, blot excess water, and place in 30-ml amber bottles containing 2–3 ml 90% ethanol. At this point, animals may be kept up to 8 hr at room temperature in sealed bottles without degradation of pigments. If longer term delay of processing is desired, immediate freezing of animals is an alternative (Baars and Oosterhuis 1984, Dam and Peterson 1988, Ramcharan and Sprules 1988; however, see Nicolajsen et al., 1983).

2. Empty contents of bottle into a small dissecting dish with enough 90% EtOH to just cover the animal. Under dim light and with expedience, split the ventrum along the longitudinal axis so that the gut and its contents are revealed, and tease apart the gut material to ensure that the contents will be thoroughly exposed to the solvent. At this point, preferably just before the dissection, size or sex determinations, if desired, should be recorded. It is important to keep the duration and intensity of exposure to light at a minimum, to prevent photodegradation of pigments. In addition, the amount of solvent used at each step should be kept small, so that the final pigment concentration remains relatively high.

3. Using a small funnel and a rinse bottle of 90% EtOH, return the contents of the dissecting dish to the amber bottle; rinse the dish and funnel thoroughly, and secure the bottle cap.

Fluorometric analysis

4. To extract pigments, first prepare a water bath at the boiling point of the solvent (78°C), and include a stirring magnet to enhance flow around and between bottles. Place groups of bottles in the water bath for 5 min. Remove and allow extraction to continue at room temperature for 6 to 24 hr.

5. After extraction, filter the extracts to remove the animal remains. We filtered through a Whatman GF/F glass fibre filter (25 cm diam.) housed in a Gelman filtration unit, into a graduated 50-ml centrifuge tube. Centrifugation is another possibility for clarification of extracts. Bring the final volume up to the nearest whole ml and record it. Ideally, extract volumes should be 10–20 ml (or lower). Extracts of as much as 30 ml should still be readable; however beyond this point one runs the risk of diluting extracts beyond the sensitivity of the fluorometer.

6. Read the fluorescence of each sample; instructions may vary with fluorometer type. The following applies to a Turner Model 112 fluorometer fitted with a high sensitivity door and calibrated to a range of 0.05 to 100 μg/L of standard chl *a* solution. The lamp/filter combination were a GE® F4T5/B lamp, with a Corning CS 2-64 emission filter and Corning CS 5-60 excitation filter. Fluorescence was read in a 75 × 12-mm cuvette. The sample was then acidified with 2N HCl (0.08–0.13 ml per 10 ml extract; final pH = 2.6–2.8) and allowed to stand for at least 2 min. Fluorescence was read again and recorded.

Calculation of chlorophyll a and phaeopigment a

7. The formulae employed to determine the concentrations of chl *a* and phaeopigment are as follows:

$$\text{chl } a = [(K_x/(K_x - 1))(F_o - F_a)(m) - \text{int}]V_{ex}$$

$$\text{phaeo } a = [(K_x/(K_x - 1))(K_xF_a - F_o) \cdot(m) - \text{int}]V_{ex}$$

where

F_o = fluorescence of sample before acidification
F_a = fluorescence of sample after acidification
m = slope of regression line from calibration curve
int = intercept of regression line
K_x = maximum acidity factor from calibration (F_o/F_a for pure chlorophyll)
V_{ex} = final extract volume.

The units of measurement are ng chl *a* per individual, and molar equivalents phaeo *a* per individual. See methods section for discussion of units, and correction factor for conversion of phaeo *a* to ng per animal.