Introduction to getting started with fatty acids for use in trophic ecology and FAQs

Aaron W. E. Galloway
Assistant Professor (*Fall 2015)
University of Oregon
Oregon Institute of Marine Biology
http://www.aaron-galloway.com/
aaron.galloway@gmail.com
@awegalloway

Michael T. Brett
Professor
University of Washington
Civil and Environmental Engineering
http://www.ce.washington.edu/people/faculty/faculty.php?id=6
mtbrett@uw.edu

Preface

This could be a proper book section or entire website, but it's not. Not enough people are interested for that (yet). We do recommend a few in print and online resources (below) that are useful for more specific information. However, there is clearly a need for a more general 'nuts and bolts' intro for ecologists who are getting started with fatty acids. This document addresses many of the initial questions that people ask when they contact us. We'll update it when relevant and post it as a resource on our websites.

The goal of this document is to provide some information in a more informal context (i.e., **not** a scientific paper). Aaron wrote the first draft and invited Mike to add his perspectives since we both get contacted with similar questions; Mike was Aaron's fatty acid mentor.

Introduction

Why are ecologists interested in fatty acids? Ecologists are often trying to solve trophic mysteries for organisms and systems where direct observation is not possible or reliable. There are a diverse range of trophic-related questions that scientists and ultimately resource managers want answers for, and any method that can be brought to bear to help answer these questions will be used. Who is eating what? Are certain diets more nutritious than others, and why? Are there physiological indicators in creatures that predict future growth or reproductive success?

One of the most common broad goals that aquatic ecologists have is to understand what resources are supporting the creatures they study. For microscopic zooplankton or benthic macroinvertebrates, direct observation of foraging behavior and diet cannot be achieved in the field due to both the small size of the consumers, and the often inaccessible habitats they live in (deep subtidal ocean, under lake/ocean surface ice). Ecologists have therefore long used 'indicators' of all kinds to infer trophic relationships in aquatic consumers. For larger animals, stomach contents are one commonly used indicator.

Another indicator approach for trophic inference is the use of 'biomarkers' or 'tracers'. The basic idea is that we use various lab procedures to measure elemental content or complex molecules in both resources/prey and consumers, and then do some math to follow the biomarker 'signature' of resources/prey into the consumers. Commonly used trophic tracers include, but are not limited to, stable isotopes of common elements (C, N, S, H), elemental ratios, and complex biological molecules of both lipids (e.g., fatty acids, sterols) and proteins (e.g., amino acids). It's a pretty sexy idea to infer what some wild creature is eating based upon analysis of tracers in the tissues. There's a lot of interest in the

use of fatty acids for a myriad of questions in ecology related research, and the amount of publications in the field is growing quickly (Fig. 1).

In order for the tracer approach to be useful in trophic inference, researchers need to understand how the resource biomarker signature of diets are transferred and stored in the tissues of consumers. For example, if you (the human being reading this) ate nothing but corn, for every meal of your entire life, your tracer signature (regardless of the tracer being used) would not be equivalent to that of corn. Ecologists who work with tracers therefore rely heavily on both experimentation and good decision making about what assumptions to use and how those assumptions affect interpretation of the results.

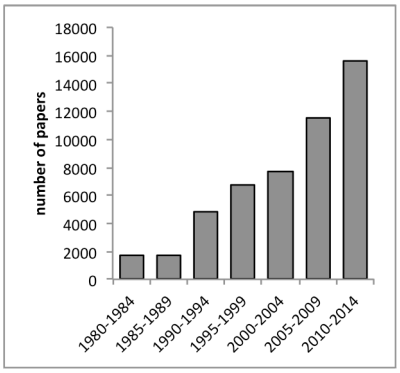

Fig. 1. Number of articles, reviews and book chapters published with subject of 'fatty acids' (Since 1980, n=49,759) in fields of Food Science Technology, Plant Sciences, Biology, Environmental Sciences, Marine Freshwater Biology, Fisheries, Zoology, Ecology, Water Resources, Oceanography, Limnology, (ISI Web of Knowledge, 15-Dec-2014).

Getting started with fatty acids

The first question that one should consider before starting down the path of using fatty acids (FA) in their research is: do you personally want to gain expertise working with FA or are you more interested in 'dabbling' in the field? There is nothing wrong with 'dabbling', but it requires that you are collaborating at some level with someone who does have the expertise necessary to assist with designing tractable FA questions and interpreting the FA results. See the 4 types of FA researchers below. Regardless of where you may fall on the FA personality type list, you will probably want to find a collaborator who knows the field who can you can lean on to either help interpret the data or guide you toward your desired level of expertise. There is kind of a steep learning curve with FA biomarkers, in part because there are many unique FA (n~20-50) in any given sample, many of which serve different physiological functions.

Phases of FA research

There are distinct phases of FA research, which we will refer to repeatedly in this document. The four phases to getting FA data are: 1) ***sample preparation***: isolate tissues/organisms to extract FA from, freeze samples, and then freeze-dry (lyophilize) samples, weigh the dry tissue samples; 2) ***FA extraction***: extract FA from dry tissue samples (series of chemical extraction steps that occur with reagents, a fume hood, centrifuge, etc.); 3) ***raw FA analysis***: run the purified FA samples through a gas-chromatograph (GC; equipped with either FID or MS) to obtain identity and quantity of the FA present in the sample; and 4) ***summarize FA data***: transform messy raw GC data into a simple multivariate data file.

The four FA researcher personalities

We have observed four general of approaches to FA work; listed are quick pros (+) and cons (-) to each:

1. Be a tenderfoot: Find a collaborator who you can ask to do steps 1-4. Your interest/investment in FA is minimal; you are concerned with other important things such as running a complex experiment or collecting raw samples using the Alvin sub's manipulator arm. You (or your minions) mainly collect/prepare raw samples.

   a. (-) This can be expensive if you really want the FA work done but you aren't finding FA collaborators to swoop in and save the day.
   b. (+) Alternatively, this can also be surprisingly cheap (because you pay nothing for someone to run your samples) if you do find a collaborator who is motivated.
   c. Note: for this to work, your idea has to be good enough that you can entice a do-it-yourselfer or crackerjack (see below) to do the work for co-authorship.
2. Be a dabbler: Do step 1 yourself and pay someone else to do steps 2-4.
   a. (-) Relatively expensive (~$US 100-125 per sample), and it is hard to find labs that do this (see below).
   b. (+) Relatively easy, relatively fast, get in and get out of the FA game quickly.
   c. Note: depending on the depth of your dabbling, you will still probably want a FA collaborator to help you interpret the results.
3. Be a do-it-yourselfer: Set up an extraction lab and do most of it yourself, *except* either send the extracted FA to a lab for just the GC work or arrange to do the GC work yourself in someone else's GC. (This is what Aaron did for Ph.D. work, in Mike's lab).
   a. (-) Kind of expensive to get the extraction lab set up (see below), lots of work
   b. (+) Much cheaper if you are going to do hundreds of samples and/or you find FA interesting and worthy of investing time and learning into.
   c. Note: you will most certainly need to collaborate closely with a FA expert (at least at first) in order to learn the ropes, but later you can let that mentor "drift away" if you want.
4. Become a crackerjack: Set up an extraction lab, get a GC, and do it all yourself (at least be in charge of a lab of students/researchers who help you get it done).
   a. If you are going to do this then you are seriously interested in FA, and you probably don't need to read my contrived pros and cons list. You get it.

Frequently asked questions and our musings

Below we elaborate on some of the details in each of the 4 general phases of FA analysis, organized into topical areas that have generally emerged as questions. This includes some of our musings (warning: these can come across as fairly opinionated). **This document is not designed to cover the actual technical methods used.** For this reason, we generally avoided adding citations to this document (except for a few references to our own papers that are relevant to particular issues). Details and justifications can be found both in individual FA papers and or lab protocol documents (which we can certainly share if requested).

Sample preparation – how to do it, generally?

Most ecologists can do step 1 in whatever university lab they have. You just need to be able to dissect/filter your sample organisms, clean them (if necessary), freeze the samples, freeze-dry the samples, weigh out dried samples, and put samples back in a freezer. A -80ºC freezer is great if it's available (particularly for the longer term storage), but a -20ºC freezer is fine for shorter term work. This is not a formal 'methods' description. People have empirically tested various effects of duration of preservation and temperature but this is beyond the scope of my current document. But in practice, we can say that we shoot for keeping unprocessed samples in the freezer for less than a couple of months and we don't worry much about not using a -80ºC in this relatively short time frame. If possible, for storing unprocessed samples, it is ideal to freeze dry and even vacuum pack samples. We would generally not hesitate to extract FA from a sample that we preserved carefully in a freezer for up to a

year. After that, it could still be legit, but you'd want to be more careful about trusting this without testing assumptions. More on sample preparation below.

Don't preserve your FA samples in ethanol, formalin or other chemicals, unless you have a thorough plan for justifying that (and we can't see what that plan could possibly be). Sample preservation in remote field settings (without access to a freezer) can be very challenging. Consider freezing in the field using liquid nitrogen, dry ice, or at least keep on ice for a short time (e.g., less than a few hours) before freezing in a freezer.

It is possible to preserve FA samples in the field in chloroform, but then you need to bring chloroform, the right glassware, and Teflon lids in the field too. Traveling internationally or anywhere through the air, or shipping samples with chloroform can be problematic. Try to freeze samples; it is much easier to ship frozen (and dry) samples in a way that they stay frozen. Samples for FA analysis should be 'clean' and frozen prior to freeze-drying as it is important to minimize oxidation of FA in the sample. We generally avoid drying samples in a drying oven, but in certain circumstances (e.g., algae?) this may be reasonable. Put careful thought into this based on literature review and discussions with people in the know.

I want to get in the game. How much should I budget for FA work?

This depends on whether you are going to be a FA tenderfoot, dabbler, or crackerjack (see the general FA personality types). In theory, you can do step 1 (described above) and send your dried tissue samples to a lab and pay them to do steps 2-4. There are a couple of labs that will process FA samples for a fee (Table 1), but we still don't know of a major throughput commercial lab (such as what you find with various stable isotope labs – if anyone knows of these labs please email me links and we will refer to them).

A lot of work (and maintenance of expensive equipment) goes into those steps, and therefore processing fatty acid samples is not cheap. You should expect to pay roughly ~$US 100-125 per sample. For comparison, when doing it ourselves, we budget about ~$US 10-20 per sample to cover reagents, GC gasses, and consumables. The extra cost can pretty much be attributed to personnel time in the lab.

That is so expensive! I can't handle that.

Some labs will reduce the cost if they will be a co-author on the work that comes out of it. If you go the route of arranging someone else to do steps 2-4, make sure you get advice from those experts in advance about how best to do step 1 for your particular experiment. An additional point we will make on this **is that it's not really that expensive**. Academics are not out there to make money off of other academics. Those of us in the FA game are really excited about FA and we'd like to help. But this work is time intensive and we all know that time isn't cheap.

Sample preparation – how much material is needed?

Requirements for sample preparation are context dependent. A common question we get about that is "how much material is required for FA analysis and how it should be preserved". There is no magic number but here is some 'ballpark' info, depending on sample type. THIS IS NOT A SCIENTIFIC METHODS SECTION. This point cannot be stressed this enough. **Read papers and determine details relevant to your approach with a collaborator who has successfully done this.** This is just an informal discussion to get you started.

In the FA extraction protocol we use in our labs (a modified Folch approach), we like to extract FA from clean, dry samples that weigh 5-10 mg. Not all labs do it this way. This means that we actually ideally want to have about 3-4 times that amount (in case we want to extract again later or if voucher material is needed for additional analyses). The ideal exact weight does depend on total lipid content of the sample. Samples rich in lipids require less total initial dry weight.

- For seston/particulate organic matter (POM), or phytoplankton: the standard approach is to filter samples onto clean, dry, pre-weighed GFF filters, (until there is enough material in the filter that it's visibly darkened), then store those filters in either eppendorf tubes or clean glass sample vials. Chloroform can be added immediately (as part of storage) to glass vials if you know what you are doing but filters can be frozen as is. Freezing as-is is what we have mostly done. It's harder to know the exact dry weight of the sample using the filters approach, unless you then dry them and re-weigh prior to analysis. When it's time to extract FA, the whole GFF can be added to the glass sample extraction vial. Seston/POM/phytoplankton can also be concentrated via centrifugation.
- For macrophytes (algae, seagrass, water plants): the standard we have used is to collect a few square centimeters of plant material, carefully clean it of epiphytes with filtered water and a gentle brush, then freeze the material. You want to have enough initially that you have plenty to do a couple of extractions – so the initial amount depends on how much it weighs after lyophilization. After conventional freezing, we freeze-dry (lyophilize) the material. This is the form we keep the sample in the freezer in until we are ready to start the extraction process. Prior to extraction, we use an acetone-cleaned stainless steel mortar and pestle to grind the frozen, dried sample, and 10 mg of ground material is added to the extraction vial.
- For zooplankton or small crustaceans (whole animal extractions): the amount needed for one sample extraction obviously depends on the size of the bugs; for our protocol, you want to isolate enough bugs that you have a sample that weights at least 10 mg when weighed after being lyophilized. For juvenile isopods or *Daphnia*, for example, this is approximately 5-15 animals. For adult isopods we could easily extract a sample from one animal. Consider whether your animals (especially in controlled feeding trials) should be starved for 24 hours or similar amount to enable them to clear their guts of the diet you have been feeding them. For wild animals this may not be feasible or desired. Our opinion is that the best approach (most humane) is to put the samples in small clean eppendorf tubes, freeze, then lyophilize. This is the form we keep them in the freezer until we are ready to start the extraction process. Prior to extraction, we grind in mortar and pestle, and weigh out 5-10 mg of material.
- For larger invertebrates or vertebrates (tissue specific extractions): with larger animals you need to decide if you want to extract FA from the whole, homogenized body or whether you are interested in the FA stored in certain tissues. Careful, think about this (and see the point about this below: "what tissue type to focus on"). Whatever you decide on, you will want to isolate that tissue (or homogenize it) and freeze, then freeze dry it. This is the form we usually keep the sample in, (freeze-dried) in the freezer until we are ready to extract FA. Depending on the animal and your particular lab and logistical constraints, you may need to freeze organisms whole and then dissect out specific tissues later. You may need to dissect animals while they are fresh. Prior to extraction, we grind in mortar and pestle, and weigh out 5-10 mg of material.

Sample preparation – what tissue type should I focus on?

If the question is about the FA of your study organism as food for something else, and you know that the predator will consume the body of your study organism whole, then you likely want to extract from the homogenized whole body. For many small organisms (e.g., zooplankton or larvae) the question of tissue type is irrelevant. They are too small for that; in fact a 'replicate' is not at the individual level, but is a pooled sample of multiple individuals.

If your question is about what resources supported your study organism, then you **still may** want to use the full body of your consumer. However, you may alternatively be interested in specific tissue types to put your work into context with other consumers. For example many researchers often extract FA from muscle tissue. The point is that different tissues (gills, digestive glands, muscle, brains,

gonads) often have distinctive FA profiles within any given organism. What you extract depends on your question, and this must be considered carefully.

How to learn FA skills?

If you are interested in doing it yourself but don't have the equipment and you don't want to deal with setting it all up, we recommend finding a collaborator who could train you, in their lab, and offer to pay a grad student or postdoc in their lab to teach you. This is how Aaron learned; under the mentorship of Mike and another of Mike's postdocs. Aaron paid a postdoc in the lab for their focused teaching time over the course of two weeks.

To our knowledge, there are not many specialized classes in FA ecology or lipids. Some programs do cover this well in the context of food science courses (e.g., see Sue Budge at Dalhousie University). There are certainly other people teaching in the field and specific courses out there that we don't know about; feel free to email Aaron info about those I will eventually post links to these on my website.

Reading papers and books is a challenging way to familiarize yourself with FA science, because at first you don't know which papers are a little more questionable than others. And in our opinion, there are some questionable papers out there. If we had to pick just 3 resources to start with, we would choose a book, a monograph, and a website. Aaron's choices for this are the book 'Lipids in Aquatic Ecosystems' a lot (Arts et al. 2009) (email us if you want a pdf of certain chapters). Aaron refers to it's chapters constantly. Aaron also often re-reads the excellent monograph/review by Dalsgaard et al. (2003) (email if you'd like a pdf of that). One of the more informative websites in the field is Lipid Library (http://lipidlibrary.aocs.org/).

Timeline for FA analyses?

It usually takes a couple of months to get from organisms in a vial to FA data in an excel file, obviously it really depends on number of samples and scope of project. If you are extracting yourself, you can extract 8-20 samples (step 2) in a full day. Most experiments, after replication, will end up with 50-100 samples, so for that I would budget ~2-3 weeks of focused lab and GC time. If you are shipping e.g., 100 samples to someone's lab, you could reasonably expect that something like this would take at least one to several months to get data back.

Co-authorship in exchange for FA analysis

Some researchers may be willing to exchange FA analysis (any range of the steps outlined above) for collaboration status and co-authorship on a paper. Whether this is appropriate for all involved obviously depends on the situation. Many ecologists (us included) have initially been trained to think that earning co-authorship must go beyond just running some specialized sample. We have two points to make about that. 1) in modern science, this is just part of doing business – we work now in a collaborative time when goals of research are more broad and specialized collaborators are needed… why not exchange co-authorship for someone to do work that you cannot do? 2) your FA collaborator should be expected to do more than just email you an Excel file of FA data… they will be critically involved in the FA analyses and interpretation.

As we pointed out above, there are many FA and they have differing roles / functions for organisms, and expertise in the field will be very beneficial for any kind of manuscript. If you choose to go it alone in your first FA papers, be aware you might make some mistakes you will likely later regret. There are papers in the literature by newbies that claim trees synthesize docosahexaenoic acid, copepods preferentially accumulate eicosapentaenoic acid, cladocera preferentially accumulate docosahexaenoic acid, and zooplanktivorous fish accumulate long chain saturated fatty acids (e.g., 24:0) even though their prey contain none of these FA.

On using a fatty acid mixing model (e.g., FASTAR) without measured trophic enrichment factors

We are getting more questions about using FASTAR or biomarker mixing models generally with FA to make inferences about resource use by consumers. We have a few papers on this (Galloway et al. 2014a; b, 2015), so most of the details and background justifying our opinions on this matter are detailed in those papers. Check those out; they are all on Aaron's website.

A common question folks email us to ask about is whether they can use a FA mixing model for a particular consumer that they haven't done feeding trials with. Feeding trials are used to help us know whether a dietary biomarker signal is transferred to the consumer you are working with. In a nutshell, it is our belief that without the trials, it's difficult to make meaningful conclusions, and we would probably be really skeptical of running the mixing model without any information about trophic modification of the fatty acids in the bugs.

For example, invertebrates are often quite enriched in essential fatty acids relative to their diets - but without knowing 'how much' and 'how variable' this enrichment is (from feeding trials), it would be hard to properly interpret the meaning of mixing model results. FASTAR or MixSIR (the Bayesian stable isotope mixing model that we used for FASTAR) or even newer models like MIXSIAR will be happy to run and give you pretty results, but we would worry quite a lot about how much stock one can put in such results without the feeding trials.

A little terminology: accounting for biomarker trophic modification is commonly referred to as 'fractionation' or 'trophic enrichment factors' in the stable isotope literature, and sometimes referred to as trophic modification or dealt with using 'calibration coefficients' in the FA literature, depending on the project. All these terms are generally trying to describe the same thing: accounting for the difference between the biomarker signature of the prey and the subsequent biomarker signature of some consumer that has eaten and incorporated that prey into it's own tissues.

For fatty acids, it makes sense to refer to this generally as FA trophic modification. As we have pointed out in many conversations (and our papers on this topic), the issue of mixing models being sensitive to trophic modification is NOT only relevant for FA – stable isotope mixing models have also been shown to be very sensitive to these assumptions. Moreover, it has been shown that stable isotope fractionation is not as consistent as many assume it is (see discussion in my papers referenced above, for links to the research). The feeding trials we are advocating for more of will be useful for any biomarker approach for dietary inference.

I'm not going to run those trials! That's too hard! Is there a way to use FASTAR without FA modification data from experiments?

For those who really want to use mixing models without this trophic modification information anyway (there are lots), we see 3 potential ways to approach running the models in a way that would still have the potential to make a useful contribution towards advancing the field of dietary inference using a FA mixing model:

1. Tough love answer: stop complaining about how it's difficult to run the feeding trials. Set up experiments where the major basal sources are fed to your consumers. Let us get this straight: it's too difficult to run an experiment but it's somehow better to use taxpayer money to do an analysis (based on untested assumptions) that may be meaningless at best but potentially misleading at it's worst? We totally do understand that some animals are really challenging to do feeding trials with: big, slow-growing marine mammals, for example. We have a bit more sympathy for the mammal folks. However, it may still be possible to leverage information gathered from aquarium partnerships (animals in aquaria are usually fed known diets and may even be operated on periodically – this would be a chance to get tissue samples from the consumer).

2. Make assumptions based on other published feeding trials from a related creature. "Gather" FA diet-to-consumer data from other published studies involving your bug or a close relative by finding other papers that show FA contents of diets and bugs as data tables within other papers, where the goal wasn't running FASTAR. Several researchers have reported this kind of data for some benthic inverts; then use this other existing data to build a 'defensible' FA library. Maybe you could reach out to someone like that and see if they would collaborate and provide additional unpublished data from feeding trials.
3. Modeling FA modification with robust sensitivity analysis of assumptions. If you can't find much published work that you would base the #2 approach on, then you could consider, as a last ditch effort, to make a major part of your analysis a formal sensitivity test to a range of 'possible' FA modification coefficients for each FA in your model. You might want to still do this even if you take the approach in #2. How sensitive are the model results to uncertainty about FA modification? (I suspect they will be very sensitive). But still, directly addressing this quagmire would be a form of progress, and it also be possible to do this for the isotope fractionation factors as well (I think the isotope people are too quick to just use the standard published fractionation in their mixing models).

Thanks for reading this "working document". Feel free to suggest new sections, new questions, or provide feedback if you disagree with something on here!

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