



Empirically Characterising Trophic Networks: What Emerging DNA-Based Methods, Stable Isotope and Fatty Acid Analyses Can Offer

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Abstract

Food webs in agricultural systems are complex and trophic linkages are difficult to track using conventional methodologies. Here, we review three alternative approaches that allow empirical assessment of feeding interactions: DNA-based techniques, and stable isotope and fatty acid analyses. DNA-based methods, namely multiplex PCR and next-generation sequencing, allow identification of food types and host–parasitoid linkages, resulting in taxonomically highly resolved feeding networks. Stable isotopes and fatty

acids reflect the assimilation of broader categories of resources, as metabolised into the consumers' tissue, together with the associated energy and nutrient fluxes in the food web. We discuss the strengths of the approaches but also highlight their limitations, providing practical advice on which technique is best suited to answer specific questions in examining food web interactions in agroecosystems. Future refinements of these techniques, especially when used in combination, could herald a new era in agricultural food web ecology, enabling management and environmental impact to be placed in the mechanistic context of trophic networks.



1. INTRODUCTION

Interest in food webs has increased considerably in recent years, with especially rapid progress being made via the development of new theoretical modelling and numerical simulation tools (e.g. [Dobson et al., 2009](#); [Ings et al., 2009](#); [Stouffer, 2010](#)). Although models and simulations have provided important new insights into food web structure and dynamics, it is still essential to validate trophic links empirically with real-world data ([Finlay-Doney and Walter, 2012](#)). However, it is often not known what, when and where specific trophic interactions occur in arable ecosystems, as feeding behaviour is affected by both the biotic and abiotic environment, which remains poorly understood. Consequently, our understanding of agricultural food webs is often still too fragmentary to understand fully how the community functions via the application of food web approaches. The current paucity of accurate dietary information is, in part, due to the difficulties of tracking trophic interactions in the field, and the lack of high-throughput techniques to record feeding interactions in communities is a longstanding obstacle for the construction of empirical food webs ([Cohen et al., 1993](#); [Mommott, 2009](#)).

These difficulties apply to ecological networks in general ([Ings et al., 2009](#)), but they are particularly acute in the agricultural context ([Bohan et al., 2013](#)), where the principal actors are typically small, invertebrates with cryptic and complex trophic behaviour, which are difficult to assess using classical techniques such as direct observation or morphological identification of prey remains in gut contents or faeces ([Sunderland et al., 2005](#)). In addition, many of the most common taxa in agricultural fields, such as carabid beetles, are opportunistic feeders, consuming a wide range of animal prey and plant material, which makes their diets difficult to characterise fully

for any given system (Holland, 2002). Many important consequences of generalist feeding behaviour, such as prey switching or intraguild predation, therefore remain difficult to measure in the field, despite their importance for the structure of food webs and the delivery of ecosystem services, such as pest control (e.g. Finke and Denno, 2004; Wilson and Wolkovich, 2011). Due to these difficulties, the most complex feeding networks measured in arable systems to date have dealt with relatively simple host–parasitoid communities (e.g. Bukovinszky et al., 2008; Tylianakis et al., 2007) or mutualistic, plant–pollinator webs (Burkle and Alarcón, 2011; Memmott, 2009). Although determining and quantifying host–parasitoid interactions is relatively easy, in contrast to assessing what generalist herbivores or carnivores consume, there are still significant methodological hurdles associated with the description of trophic interactions in host–parasitoid communities; for example, discriminating among cryptic interactions, such as multiparasitism and hyperparasitism, is impractical with conventional methods (Garipey et al., 2008a; Traugott et al., 2008) and requires novel approaches. Another particular problem is that, unlike many aquatic food webs, which tend to be dominated by engulfing, gape-limited consumers (e.g. see Gilljam et al., 2011; Ledger et al., 2013; O’Gorman et al., 2012), agroecosystems have large numbers of fluid-feeding consumers, whose gut contents are unidentifiable using traditional microscopy techniques.

In recent years, significant methodological advances have been made for studying feeding interactions in the field, opening up exciting new perspectives in trophic ecology. Stable isotope and fatty acid (FA) analyses provide new means to elucidate patterns of resource allocation (Boecklen et al., 2011; Ruess and Chamberlain, 2010), while DNA-based techniques allow feeding interactions to be characterised to a high level of taxonomic resolution (Garipey et al., 2007; Symondson, 2012). These approaches have been reviewed and their potential applications summarised elsewhere (e.g. Garipey et al., 2007; King et al., 2008; Martínez del Rio et al., 2009; Pompanon et al., 2012; Post, 2002; Ruess and Chamberlain, 2010; Sheppard and Harwood, 2005; Symondson, 2002), yet an integrated overview of the possibilities they offer for describing trophic networks is notable by its absence. Here, we address this gap by comparing DNA-based, stable isotope and FA analyses and providing guidance as to which tools are best suited to address specific questions in agroecosystem food web ecology (Fig. 3.1).

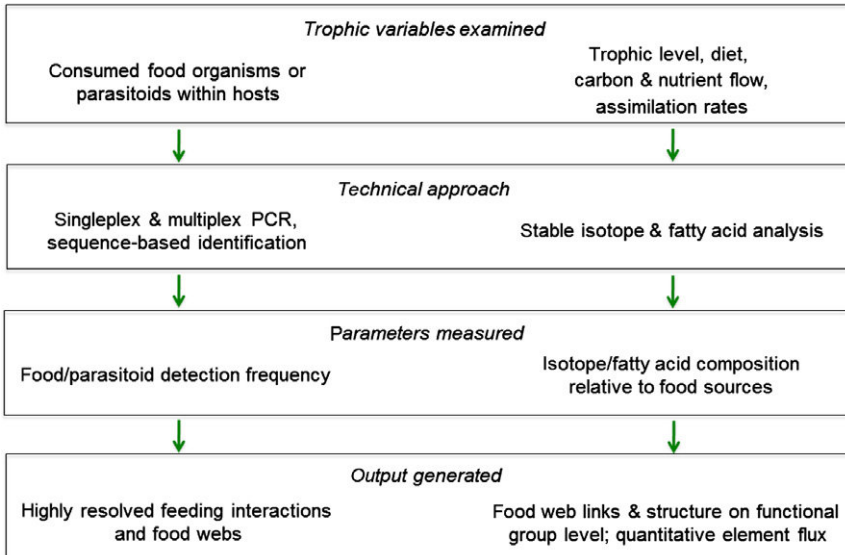


Figure 3.1 Utility of DNA-based approaches of food and parasitoid detection as well as of stable isotope and fatty acid analysis to empirically characterise trophic interactions and feeding networks in agricultural systems. For further details see text.



2. MOLECULAR APPROACHES TO ANALYSE TROPHIC INTERACTIONS

2.1. Methodological background

Isoenzyme electrophoresis (e.g. Traugott, 2003; Walton et al., 1990) and monoclonal antibodies (e.g. Hagler and Naranjo, 1994; Symondson et al., 1997) were the most commonly used molecular-based methods to assess invertebrate feeding interactions by targeting prey- and parasitoid-specific proteins during the 1980s and 1990s. While enzyme electrophoresis is relatively cheap and easy to conduct, this technique is often limited by low specificity and sensitivity to identify and detect specific prey and parasitoid enzymes within predators and hosts, respectively. Monoclonal antibodies, on the other hand, provide a highly specific and sensitive approach to prey and parasitoid detection, but it is a difficult and time-consuming process to generate antibodies for specific prey and parasitoid taxa in species-rich food webs (Chen et al., 2000; Symondson, 2002). This approach was therefore used primarily to track single pairwise predator-prey links, rather than attempting to characterise the full diet spectrum of consumers in complex networks.

In the late 1990s, it was demonstrated that prey DNA could be detected in the gut contents of invertebrate predators (Agustí et al., 1999; Chen et al., 2000; Zaidi et al., 1999). This provided a more flexible technology base to identify feeding interactions, and it now includes a wide range of rapidly evolving techniques. DNA-based techniques are used to study a wide range of trophic interactions, in aquatic and terrestrial systems, from consumption of plants and animals to the investigation of host–parasitoid interactions (Gariepy et al., 2007; King et al., 2008; Pompanon et al., 2012; Sheppard and Harwood, 2005; Symondson, 2002, 2012). Key factors in this success were the development and/or cheap availability of (1) polymerase chain reaction (PCR), allowing the specific amplification of minute amounts of target DNA from dietary/host samples; (2) DNA sequencing, enabling the design of specific PCR primers; (3) public sequence databases, allowing for sequenced-based DNA barcoding identification; (4) equipment and skills for DNA-based work; and (5) novel DNA techniques, fuelling the development of new approaches.

In general, there are two classes of DNA samples that can be differentiated: intact DNA from endoparasitoids and their host, and fragmented prey (food) DNA retrieved from regurgitates or gut contents or faecal samples, alongside intact and/or degraded consumer DNA. For the first class, DNA can be extracted using rapid and cheap methods that allow for easy analysis in downstream applications, such as diagnostic PCR and sequencing-based identification. In contrast, the detection and identification of food/prey species is typically restricted to short DNA fragments, which requires more sophisticated extraction and analytical methods. Due to its rapid technological development, the application of DNA technology to trophic ecology has been reviewed repeatedly over relatively short time steps (Gariepy et al., 2007; Greenstone, 2006; King et al., 2008; Pompanon et al., 2012; Sheppard and Harwood, 2005; Symondson, 2002, 2012). While these reviews have focused on specific trophic level interactions and/or technologies, our aim is to provide a more comprehensive network-based picture of the major techniques used for molecular assessment of trophic interactions in agroecosystems, including detritivory, herbivory, predator–prey and host–parasitoid interactions.

2.2. Host–parasitoid trophic interactions

Traditionally, trophic interactions in host–parasitoid systems have been examined by rearing parasitoids from parasitised hosts or by host dissection to retrieve juvenile parasitoids (Henri and Van Veen, 2011; Sunderland et al., 2005), but these methods are, unfortunately, prone to many sources

of error. First, host and parasitoid mortality during rearing, resulting in parasitoid emergence failure, can be considerable and prolonged parasitoid post-emergence diapause can further complicate estimates of interaction rates made using this rearing approach (Garipey et al., 2007). Second, the morphological identification of parasitoids is often not possible for juvenile stages and may be confounded by the occurrence of unknown and/or cryptic species (Smith et al., 2006; Tylisanakis et al., 2007). Consequently, it can be very difficult to precisely state which species are interacting, and at what rate, in multi-parasitised and hyperparasitised hosts. In addition, rearing and host dissection also become impractical when large numbers of hosts need to be analysed (Garipey et al., 2008a). All these sources of error lead to unresolved host–parasitoid linkages, to biased estimates of percentage parasitism, and/or to a lack of replication in the host–parasitoid food webs analysed (Garipey et al., 2008a; Greenstone, 2006). The developments in DNA-based techniques have provided some solutions to these problems, which, for discussion, we classify into either diagnostic PCR or sequencing/barcoding approaches (Fig. 3.2).

In diagnostic PCR, the presence of a parasitoid species is searched for, or targeted, using specific primers that amplify a particular fragment of the

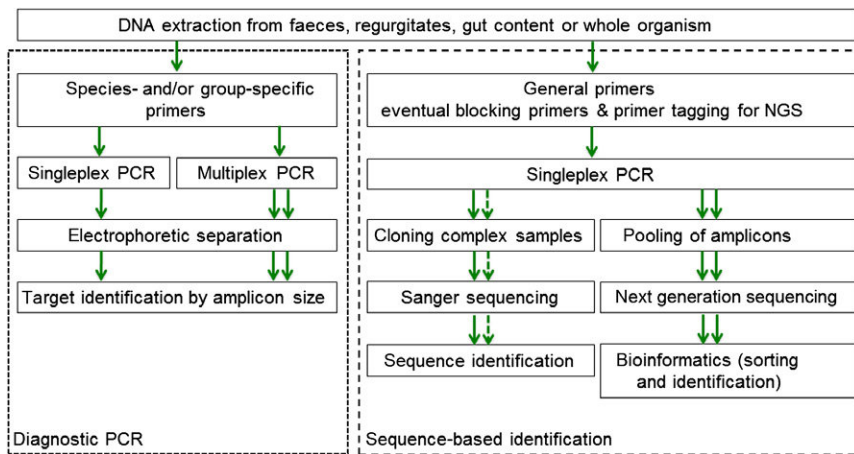


Figure 3.2 Overview on the different steps required for molecular analysis of diet and parasitism. The approaches can be broadly separated into diagnostic PCR (left panel) and sequence-based identification (right panel). Parallel double arrows indicate the simultaneous analysis of multiple food sources/parasitoids; dashed arrows show optionality for analysis of multiple prey/hosts. For further details see text.

parasitoid's total DNA, but does not amplify the DNA of the host. This approach has been used successfully to detect three species within the genus *Aphelinus* (Hymenoptera: Aphelinidae) as parasitoids of aphid hosts (Zhu and Greenstone, 1999), and to study parasitisation of the European corn borer, *Ostrinia nubilalis*, by tachinid flies (Agustí et al., 2005). Although these 'singleplex' PCR assays are highly sensitive and specific, allowing detection of a single parasitoid egg within a few minutes after oviposition (Traugott and Symondson, 2008), they require a separate reaction for each parasitoid species targeted. This makes the assessment of multiple parasitoid species a costly and time-consuming endeavour. Garipey et al. (2005) overcame this limitation by using multiplex PCR to detect, within a single reaction, three different species of *Peristenus* wasps (Hymenoptera: Braconidae) parasitising *Lygus* bugs (Hemiptera: Miridae). To date, up to eight different parasitoid taxa have been targeted within a single 'multiplex' PCR assay (Traugott et al., 2008). Multiplex PCR can also be used to confirm the identity of the host, by including host-specific primers, and to provide an internal, positive control, that is, a primer pair which amplifies a specific fragment of the host DNA, the presence of which indicates that the PCR was successful (Traugott et al., 2006). The latter becomes important where parasitism rates are low (e.g. Agustí et al., 2005). Otherwise, all samples testing negative for parasitoid DNA would need to be retested with general primers in order to exclude false-negative results. In order to maximise detection, it is important to balance the concentration of the host primers, within the multiplex reaction, to avoid negative effects on parasitoid detection sensitivity because the reaction can otherwise be distorted towards an amplification of host DNA (Traugott and Symondson, 2008). Multiplex PCR approaches have been used to provide precise information for trophic interactions in cereal aphid parasitoid communities (Traugott et al., 2008), host-parasitoid associations in classical biological control programmes (Garipey et al., 2008a), the effects of host plant identity on parasitoid species composition and parasitism rates (Garipey et al., 2008b) and the effect of farming type on parasitoid control of aphids (Macfadyen et al., 2009).

Although being a highly effective approach for screening large numbers of hosts for parasitoids, diagnostic PCR detects only the specific taxa targeted *a priori* by the primers. Hence, when it is the number and identity of parasitoid species which is unknown, this approach becomes inefficient. In such a situation, general invertebrate primers can be used to generate barcoding DNA sequences, allowing the identification and/or differentiation between parasitoid taxa (Fig. 3.2). DNA barcoding has been employed to detect 93 previously

unknown host–parasitoid links in the tropical rainforest of Papua New Guinea (Hrcek et al., 2011). Similarly, Kaartinen et al. (2010) compared leaf miner/gall inducer–parasitoid food webs derived by morphological identification with links derived from DNA–barcoded samples and found that trophic interactions were more specialised in the molecular–informed sample set than in the traditional one. DNA barcoding via classical ‘Sanger sequencing’, however, requires host and parasitoid tissue samples being analysed separately, as mixtures of DNA sequences are unreadable. Derocles et al. (2012b) overcame this problem by applying a primer pair specific to primary parasitoids of aphids, allowing for sequenced–based identification of the parasitoid. However, for six groups of closely related species, species–specific assignment was not possible, due to the common identity of the sequences, and sequences from two genes had to be combined to allow for identification of all species (Derocles et al., 2012a).

Although molecular detection of parasitoids provides an accurate and convenient means of recording host–parasitoid interactions, there are also drawbacks to this approach. Molecular–derived parasitism rates tend to overestimate parasitoid–induced mortality as some hosts might overcome parasitisation (e.g. via secondary endosymbionts, Vorburger et al., 2010). Moreover, the power of the taxonomic assignment in barcoding–based parasitoid and host identification is limited, largely, by the sequence information available in reference databases such as GenBank or BOLD. Constructing a database of barcoding DNA sequences from parasitoids that are relevant in a particular habitat is therefore highly recommended as a strategic priority in agricultural research to increase the chances for identifying the sequences recovered from field–collected samples.

2.3. Assessment of feeding on animals and plants

The detection and identification of consumed prey DNA is usually more challenging than the molecular examination of host–parasitoid feeding linkages. This is because the DNA is degraded during digestion and the amount of template molecules declines rapidly with increasing fragment size (Deagle et al., 2006). Hence, short DNA fragments, ranging in size from 80 to 450 bp, of multiple copy genes are targeted to increase the likelihood of successful DNA detection. The size of these short stretches of DNA poses two problems: (i) they complicate the combination of primers in multiplex PCR, as it can be difficult to create primer pairs that have amplicons of staggered length within a narrow amplicon size range; and, (ii) they provide less

information for discriminating and identifying species from the amplified sequences. Moreover, consumed prey DNA is often present in tiny amounts and it is embedded in a high concentration of consumer DNA, in whole body DNA extracts, or in faecal samples. Additionally, non-DNA compounds co-extracted with the consumed prey DNA can inhibit downstream molecular analyses (Juen and Traugott, 2006).

The selection of a DNA extraction method, which is the first working step towards analysing samples, depends on the type of sample used for dietary analysis. In general, complex samples, such as faecal material and whole-body DNA extracts of consumers, require highly efficient DNA extraction protocols, such as silica-based kits or CTAB-based protocols (Oehm et al., 2011; Zarzoso-Lacoste et al., 2013), whereas it is easier to retrieve dietary DNA from bolus and regurgitate samples (Poulakakis et al., 2005; Waldner and Traugott, 2012).

Isolated food remains such as bones, body parts of insects or plant remains are the easiest type of sample for molecular identification because the DNA can be extracted directly. For example, Kasper et al. (2004) sequenced mitochondrial 16S rDNA of masticated prey items to assess the prey overlap between native and introduced species of social wasps. More often, however, the food DNA has to be retrieved from complex dietary samples such as faeces or whole body extracts of consumers. In larger invertebrates, gut dissection has also been performed (Von Berg et al., 2012), achieving a dietary sample that is relatively straightforward to analyse in downstream applications (Juen et al., 2012). A non-invasive way to retrieve gut content material from invertebrates, such as carabid beetles, is to collect regurgitates. This allows for increased post-feeding prey DNA detection intervals and improved amplification success of larger prey DNA fragments (Waldner and Traugott, 2012), and makes regurgitates an ideal sample for sequence-based identification of consumed prey.

As with the detection of parasitoids, the identification of consumed prey DNA can be classified into diagnostic PCR and sequence-based identification (Fig. 3.2). To date, diagnostic PCR has been the most commonly used technique to detect prey remains in dietary samples (King et al., 2008; Symondson, 2012), where consumed prey DNA fragments of a specific length are amplified and subsequently separated and visualised using electrophoretic techniques (Sint et al., 2011). Using different genes, PCR primers can be designed to target the food prey at the desired taxonomic level of resolution (King et al., 2008), including the evaluation of their specificity in multiple-species systems (Admassu et al., 2006).

Singleplex PCR, using a pair of prey-specific primers, is the simplest form of diagnostic PCR (Fig. 3.2). This approach has been used in several studies examining feeding interactions in agroecosystems. For example, Agustí et al. (2003) investigated collembolan prey choice by linyphiid spiders in wheat fields, using three PCR assays, each of which targeted a particular *Collembola* species. Aphid and collembolan-specific PCRs were used by Kuusk and Ekbom (2012) to track predation of both *Collembola* and cereal aphids by wolf spiders, in order to determine how densities of pest and alternative prey affect spider prey choice. Birkhofer et al. (2008) targeted DNA of the grain aphid *Sitobion avenae* to reveal that two species of cursorial spiders most frequently consumed the pest, highlighting that predator identity rather than predator diversity appears to drive biological control of cereal aphids. Greenstone et al. (2010) used singleplex PCR primers for the Colorado potato beetle, *Leptinotarsa decemlineata*, in order to identify its key invertebrate predators, while Szendrei et al. (2010) employed this assay to examine whether habitat manipulation affects pest detection frequencies in generalist predators. Singleplex PCR assays were also used to investigate the consumption of pest- and non-pest prey in below ground micro- (Heidemann et al., 2011; Read et al., 2006) and macro-arthropod predators (Juen and Traugott, 2007; Lundgren et al., 2009). The method also provides an effective tool to assess the feeding habits of herbivores, with Pumarino et al. (2011) and Staudacher et al. (2011) developed PCRs to detect DNA of specific plant species in plant- and soil-dwelling insects, respectively.

Testing animal consumers for the ingestion of several prey species requires running each sample in different singleplex PCR assays. Harwood et al. (2007) examined the pest- and non-pest feeding of anthocorid predators in soybean using three singleplex PCR assays, while Chapman et al. (2013) employed a set of four singleplex PCR assays to demonstrate dietary selectivity of linyphiid spiders for collembolans. This approach, however, soon becomes very time-consuming as the number of prey types increases. Harper et al. (2005) solved this problem by using multiplex PCR to screen for several prey taxa simultaneously (Fig. 3.3). This powerful approach has allowed the examination of carabid prey choice on different species and ecological groups of earthworms (King et al., 2010), assessment of which prey sustains overwintering predatory beetle larvae in arable land (Eitzinger and Traugott, 2011), tracking of intraguild predation of aphid parasitoids by generalist predators (Traugott et al., 2012) and determination of how frequently spiders are eaten by carabids (Davey et al., 2013). As in host-parasitoid systems, multiplex PCR can include primers

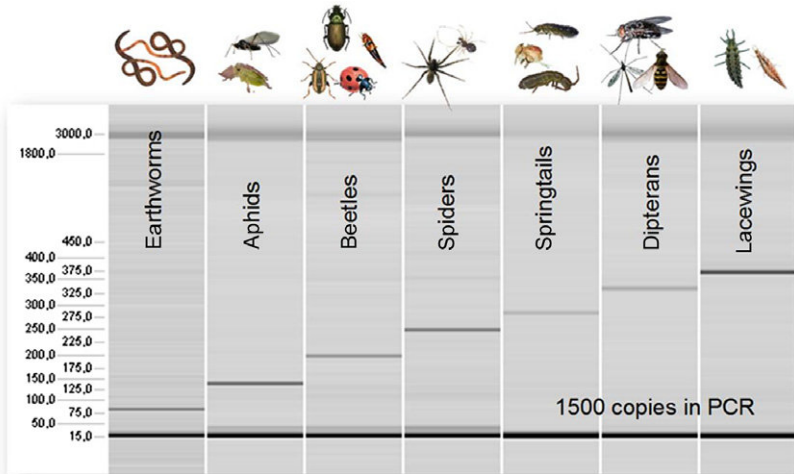


Figure 3.3 An example of a multiplex PCR assay targeting pest (aphids, dipterans), intraguild (spiders, lacewings, specific beetles) and alternative (springtails, earthworms) prey groups of generalist predators in temperate cereal agroecosystems. The assay generates short prey DNA fragments which show a specific size for each taxon. The primers used in this assay are group-specific which allows getting an overview on the diet of the predators. The advantage of the multiplex PCR system is that the dietary samples can be screened for several prey groups within one reaction. Size markers are depicted on the outer left-hand side and the thick band at the bottom of the gel is the 15 bp size marker; 1,500 template DNA copies were used to produce the PCR products. Gel picture provided by courtesy of K. Staudacher.

that target the consumer, providing an internal positive control to exclude false-negative results (Juen and Traugott, 2006) and a means to identify the consumer (Juen and Traugott, 2007). It should be noted, however, that the concentration of the consumer-specific primers needs to be lowered when analysing whole-body DNA extracts to achieve high sensitivity in prey detection (Sint et al., 2012). Taxon-specific primers for plants can also be simultaneously employed in this approach: Wallinger et al. (2012) developed multiplex PCR assays targeting two families and genera of plants as well as nine plant species commonly occurring in arable land. These assays revealed that root-eating click beetle larvae appear to feed preferentially on diverse plant mixtures in a maize cropping system (Staudacher et al., 2013).

In situations where the breadth of potential food sources and/or the prey spectrum of animals is to be examined, a sequence-based approach of food identification is required (Fig. 3.2). Typically, a DNA region that allows discrimination among food prey sources, by their sequences, is amplified by

primers that bind to a wide range of plant or animal taxa. In its simplest form, the prey DNA can be extracted from isolated, individual prey remains, which is then subjected to conventional DNA sequencing (e.g. [Clare et al., 2009](#)). The same approach can be employed where there is only one type of prey present in gut contents, such as for herbivorous mirid bugs where general chloroplast primers were used to identify the plant meal via DNA barcoding ([Hereward and Walter, 2012](#)). [Wilson et al. \(2010\)](#) employed DNA sequencing of pollen grains retrieved from the crops of solitary Hawaiian bees to examine their foraging behaviour. This work demonstrates the potential of DNA-based methods to study pollination and flower visitation networks, including the opportunity to measure pollen flow in native populations by employing microsatellite markers ([Albrecht et al., 2010](#)).

When dietary samples contain DNA of multiple foods, the PCR products may be cloned for dietary identification. This approach has been used to examine which arthropods were consumed by passerine birds ([Sutherland, 2000](#)) and bats ([Zeale et al., 2011](#)), and to identify the plant food taken by geese ([Stech et al., 2011](#)). However, sequencing of cloned-PCR products becomes impractical when many samples need to be analysed. Furthermore, as only a small fraction of the generated PCR products are sequenced, there is a risk of missing rare or less preferentially amplified food items. Next-generation sequencing (NGS) technologies have the potential to enhance sequence-based food identification significantly, as thousands of PCR products may be sequenced, in parallel, within a single reaction ([Glenn, 2011](#)). The dietary samples can then be meta-barcoded, allowing detection of a wide range of food items ([Pompanon et al., 2012](#)). Using this method, [Valentini et al. \(2009\)](#) demonstrated that parallel pyrosequencing of PCR-amplified chloroplast DNA allows identification of plants eaten by mammals, birds, insects and molluscs. [Bohmann et al. \(2011\)](#) employed NGS to examine the diversity of insect prey used by the two species of free-tailed bats, while [Brown et al. \(2012\)](#) subjected faecal pellets of the slow worm, *Anguis fragilis*, to 454 pyrosequencing to assess lumbricid consumption in different habitat types.

NGS-based techniques also provide a powerful tool for population-level assessment of diet choice, as multiple individual samples can be pooled and then sequenced (e.g. [Deagle et al., 2009](#)). NGS technology is less suited to analysing the dietary information of a large number of individual samples, however, because each sample needs to be amplified by a uniquely tagged pair of primers, increasing cost and potentially altering the sensitivity of the

PCR (Deagle et al., 2013; Pompanon et al., 2012). Moreover, food DNA can be excluded from amplification because general primers preferentially bind to the consumer's DNA which, depending on sample type, can be present in overwhelming excess to food DNA. It may be desirable, in such a case, to exclude the consumers' DNA from PCR by blocking oligonucleotides (Vestheim and Jarman, 2008). Furthermore, some DNA sequences, such as the cytochrome *c* oxidase subunit I (COI) gene that can be a useful barcode for many animals, cannot be amplified by one pair of 'general' primers covering a broad range of taxa. This makes the selection of the barcoding primer pair or the combination of several primer pairs (Deagle et al., 2009) a critical step in NGS-based diet analysis (Boyer et al., 2012). The current shortage of barcode sequences for many taxa in public repositories can limit the utility of NGS, as the DNA barcoding relies on reference sequences for identification (Purdy et al., 2010). As for the sequence-based parasitoid identification, it is important that researchers generate their own project-specific barcoding DNA sequence databases that include the most common prey found in the habitat of the consumer, and to substitute this information with what is provided in public repositories, to maximise sequencing-based identification power. With the increases in sequence information and rapid developments of the technology (Glenn, 2011), NGS-based dietary analysis will play an increasingly important role in the future of molecular analysis of trophic interactions (Purdy et al., 2010).

It is not straightforward to compare prey-food DNA detection rates derived from gut content samples among different consumer and prey taxa, as both consumer and food identity can affect detection success. For instance, heteropterans, spiders and centipedes can retain the prey DNA for significantly longer after feeding than coleopteran predators (Greenstone et al., 2007; Sheppard et al., 2005; Traugott and Symondson, 2008; Waldner et al., 2013). This might suggest prey DNA detection rates could be adjusted to account for the higher probability of detecting prey in those consumers that digest their food slowly (Gagnon et al., 2011; Greenstone et al., 2010; Szendrei et al., 2010; Traugott et al., 2012). Several other factors, however, such as feeding frequency, diet composition (Weber and Lundgren, 2011), ambient temperature (McMillan et al., 2007; Von Berg et al., 2008a) and changing rates of digestion over the lifetime of a consumer (Lundgren and Weber, 2010) will also affect food DNA detection probability, making these adjustments challenging. However, these same limitations also apply to the more traditional microscopy-based approaches to food web construction. Additionally,

taxon-specific effects on food-prey DNA detection success have been found for both animal (e.g. [Gagnon et al., 2011](#)) and plant ([Wallinger et al., 2013](#)) food sources, complicating comparisons between different food types. Finally, methodological factors such as the sensitivity of PCR assays for detecting DNA fragments of different food sources affect relative detection success. Fortunately, protocols have been established to measure and adjust the sensitivity of PCR primers to minimise methodological error variation in molecular-derived trophic data ([Sint et al., 2012](#)).

The amount of food DNA present in a gut content sample can be measured by quantitative PCR (e.g. [Lundgren et al., 2009](#); [Weber and Lundgren, 2011](#)). This measure usually does not allow calculation of how much food was consumed because it is not possible to determine the digestion time for samples collected in the field ([Symondson, 2002](#)). Faecal samples are better suited to quantification of prey consumption as they are the end-product of the digestive process, which allows estimates of the proportion and frequency of different prey types consumed, giving semi-quantitative information on diet composition ([Deagle and Tollit, 2007](#)). However, these estimates currently have wide confidence intervals and are affected by a variety of parameters related to the particular methods that were used ([Deagle et al., 2013](#)).

When setting up a study where molecular methods are applied to examine trophic interactions, it is important to consider all the steps from sample collection, storage and DNA extraction, to the detection of the food DNA. For example, suction sampling cannot be recommended for collecting invertebrates for PCR-based gut-content analysis because the collected invertebrates cross-contaminate each other with their own and potential gut content DNA during the sampling process ([Greenstone et al., 2011](#); [King et al., 2012](#)). It may also be necessary to clean invertebrate consumers, removing environmental DNA that can contaminate the outer body surface, before subjecting them to DNA extraction ([Greenstone et al., 2012](#); [Remen et al., 2010](#); [Staudacher et al., 2013](#)). [King et al. \(2008\)](#) provide a general overview of which aspects should be considered in molecular prey detection, including guidelines for best practices and the interpretation of the molecular-derived trophic data. However, with rapid improvement in methodology, the latest, pertinent papers also need to be taken into account and reviewed regularly (e.g. [Deagle et al., 2013](#); [O'Rorke et al., 2012](#); [Pompanon et al., 2012](#); [Simonelli et al., 2009](#); [Sint et al., 2011, 2012](#)).

Despite their many advantages, DNA-based methods also have disadvantages. Probably the most critical disadvantage is the double-edged sword of

high sensitivity of PCR; PCR approaches will not just detect food-item and parasitoid DNA but also DNA which has been introduced due to contamination, for example, when collecting the sample. Hence, great care needs to be exercised to avoid DNA contamination when collecting samples, during DNA extraction and when subjecting the samples to PCR. Highly sensitive techniques such as NGS exacerbate this problem further as a wide range of contaminants can be readily detected.



3. STABLE ISOTOPE ANALYSIS

3.1. Methodological background

The principles of stable isotope analysis have been known since the beginning of the twentieth century (Högberg, 1997). In the second half of the century, palaeontologists and plant physiologists in particular adopted this method increasingly to address diverse topics, including characterising the diets of extinct animals and nitrogen-fixing efficiency of plants (deNiro and Epstein, 1978; Gannes et al., 1998). Animal ecologists first began to use the approach in the early 1970s (deNiro and Epstein, 1978), but it took until the beginning of the 1990s for stable isotope analysis to be more regularly applied in the field (Gannes et al., 1997). The method has since then proven to be a valuable tool for food webs studies (reviewed by Boecklen et al., 2011; Ehleringer et al., 1986; Fry, 2008). Its main value can be ascribed to two distinct properties of the technique (Hussey et al., 2012). First, natural isotope ratios are widely conserved between food sources and consumers, which is particularly the case for the $^{13}\text{C}/^{12}\text{C}$ ratio (Fig. 3.4). This ‘we-are-what-we-eat’ property allows us to distinguish the actual source of food from other potential ones, provided that their ratios differ (Gannes et al., 1998). For example, Mollot et al. (2012) used this approach to show that the addition of a cover crop, as new primary resource, led to a dietary change in an ant species that can act as a biological control agent of a weevil. Second, for other elements, such as nitrogen, fractionation leads to an enrichment of the heavier isotope from diet to consumer (Fig. 3.4). The reasons for this bioaccumulation have been discussed in detail by Martínez del Río et al. (2009). Among the first to use this property in terrestrial systems were Scheu and Falca (2000), who studied the soil fauna food web of two beech forests. Earlier studies on the bioaccumulation of ^{15}N have suggested an average enrichment of 3.4‰ per trophic step, theoretically allowing identification of the trophic level of the organism, at least relative to a reference species (Eggers and Jones, 2000). One key advantage this approach can offer over traditional measures

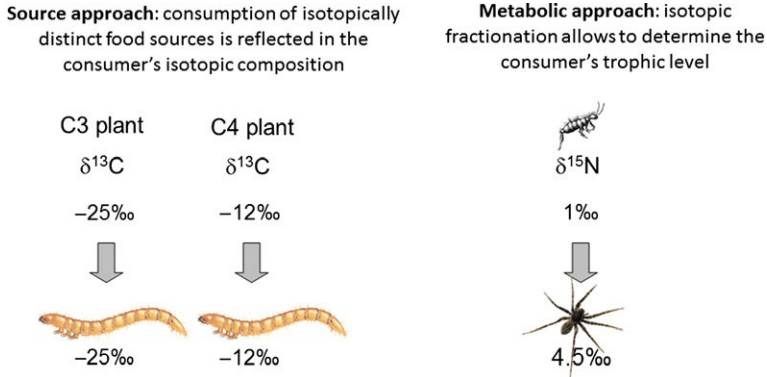


Figure 3.4 Stable isotope analysis allows identifying the food sources (source approach, left panel) and the trophic position (metabolic approach; right panel) of consumers. In the left-hand example, herbivorous click beetle larvae feed either on C3 or C4 plants (differing in their carbon isotopic composition) which is reflected in their isotopic signature. In the right-hand example, a spider is enriched by 3‰ in $\delta^{15}\text{N}$ compared to its collembolan prey. The bioaccumulation in heavy nitrogen reflects its predatory nature and can be used to determine its trophic position in the food web.

is that it allows trophic status to be measured on a more realistic continuous scale, as it integrates over all the food chains entering a consumer, rather than using crude binary steps (e.g. maximum food chain length) used in early food web research (Ings et al., 2009). However, fractionation is a complex process and although diet and trophic position are main factors influencing isotopic ratios in animals, other sources of variation have to be considered to avoid misleading conclusions (reviewed by Boecklen et al., 2011; Jardine et al., 2006; McCutchan et al., 2003; Vanderklift and Ponsard, 2003). To summarise, the main factors are: (1) the type of nitrogen assimilation and excretion; (2) taxonomic affiliation; (3) habitat type (terrestrial vs. freshwater vs. marine); (4) tissue type; (5) intra-specific variability and seasonal variation in fractionation rates; (6), isotopic baseline variations; (7) omnivory; and (8) movement of animals and nutrients between food webs. Several authors such as McCutchan et al. (2003), Vanderklift and Ponsard (2003) and Caut et al. (2009, 2010) have discussed the consequences of these factors on the fractionation rate of ^{15}N in different taxa and tissues. However, many studies still cite the originally suggested enrichment of 3.4‰ as the *de rigueur* division between 'trophic levels'. To avoid repeating this common error in the future, we will discuss the most important sources of variation in agroecosystems and possible solutions below.

Stable isotope values result not only from trophic interactions, but also from a large variety of biological and chemical processes. Therefore, when assessing the trophic level of an animal, two important facts have to be considered. Firstly, the amount of carbon and nitrogen isotopes varies between sites and across time, causing primary producers to differ in their isotopic ratios within and across systems (Jardine et al., 2006). Therefore, the assignment of an organism to the correct trophic level requires the definition of an isotopic baseline. Ignoring this possible spatio-temporal variation may lead to misinterpretation of the results (Chouvelon et al., 2012; Ramos and González-Solís, 2012). To establish this correct baseline, it is necessary to measure the isotopic signatures of all carbon sources at the base of the food web, both in all localities and time periods under investigation (Casey and Post, 2011). This problem has still no obvious single solution when the number of potential basal resource pools is high (Layman et al., 2012). Secondly, the process of fractionation is complex and may be influenced by numerous factors rendering interpretations of isotopic signature difficult, such as whether the consumer is sated or starving. Wilson et al. (2011), for example, showed that aphids were highly depleted in ^{15}N , contrary to expectations. Tibbets et al. (2008) observed enrichment in ^{15}N in adults relative to larvae in five out of six insect species, which were not due to a diet switch but to protein metabolism during metamorphosis. Changes in isotopic enrichment in relation to food quality have been documented, too, both in phytophagous (aphids, Sagers and Goggin, 2007) and arthropod predators (spiders, Oelbermann and Scheu, 2002; Rickers et al., 2006a). Spence and Rosenheim (2005) observed large variation in ^{15}N enrichment from plants to herbivorous insects concluding that enrichment needs to be measured for each trophic link investigated to avoid misleading conclusions. Recently, compound-specific stable isotope analysis has been developed where the trophic position is assessed from differences in isotopic ratios of different compounds (Boecklen et al., 2011, see also Section 4). This approach can provide more robust and accurate results than an analysis based on bulk tissues, because it greatly increases the resolution of stable isotope signatures. For example, Chikaraishi et al. (2011) successfully applied this approach to a range of terrestrial insects to investigate their trophic position.

Lipids tend to be depleted in heavier isotopes of carbon relative to carbohydrates and proteins by a magnitude of about 5‰ (Newsome et al., 2010). Consequently, variation in lipid content can be confounded with variation in diet (Post et al., 2007). To solve this problem, two strategies have been proposed: either applying an appropriate arithmetic correction

based on the C:N ratio to estimate the lipid content of the sample and the contribution to isotopic ratio (Sweetings et al., 2006) or, alternatively, to extract lipids before analyses in order to normalise samples (Boecklen et al., 2011). Boecklen et al. (2011) reviewed the main factors influencing the usefulness of lipid extraction and the reliability of arithmetic corrections. They emphasised the potential impact of lipid extraction on nitrogen isotopic ratios, potentially biasing conclusions.

The diet of an animal often changes according to season and to its age or size. Consequently, the dynamics of isotopic incorporation have received considerable attention in order to assess the temporal scale over which consumer isotopic signatures integrate diet. Isotopic incorporation is also tissue-specific (Martínez del Río et al., 2009) and depends on the fractionation rate and the metabolic activity of the tissue (Jardine et al., 2006). Typically, in mammals, bones integrate the isotopic signal over years, hair over months, muscles over weeks and blood over days (Boecklen et al., 2011). Comparing isotopic signatures between tissues can highlight ontogenetic or seasonal switches in diet, such as have been reported in ladybird beetles (Gratton and Forbes, 2006).

The isotopic approach lacks the taxonomic resolution for identifying different food items where their diversity is high. Theoretically, at least, resolution could be improved by combining isotopes from multiple elements. In addition to C and N, hydrogen (H) and oxygen (O) are most commonly used in ecological studies while strontium (Sr) and sulphur (S) are rarely employed (Hood-Nowotny and Knols, 2007). While the assessment of isotopic signatures from multiple elements {e.g. C, N, O, S, Sr} is used, primarily for food authentication and isotopic provenancing (Oulhote et al., 2011), this approach remains to be widely explored in the analysis of agroecosystem food webs.

3.2. Identifying food sources and quantifying their dietary contribution

Isotopic analyses integrate diets over time and they can be carried out at the individual level, provided the sample has a dry mass >2 mg, which is usually the minimum amount of sample which can be measured accurately. As isotopic signatures of specific elements are transferred from the base to the top of the food web, the relative contribution of isotopically distinct basal resources can be determined at higher trophic levels (Fig. 3.4). Such variation can be natural or artificially induced by isotopic labelling of specific food

sources, with the required isotopic difference between food sources depending on consumer sample size and variation (Traugott et al., 2007).

There is a rich literature on using differences in isotopic signatures of C3 and C4 plants to assess their relative contribution to recent and ancient consumer diets (Hobbie and Werner 2004). Interestingly, in arable systems, the determination of diet has not been the primary goal for C3/C4 assays. Rather isotopic signatures were used to assess aspects of the life cycle of phytophagous insects, such as the origin of aphids colonising crop fields (Vialatte et al., 2006), colonisation behaviour of ladybird beetles (Prasifka et al., 2004), dispersal capacity of adult (Schallhart et al., 2009) and larval click beetles (Schallhart et al., 2011), and the rate of hybridisation in host races of European corn borer, *O. nubilalis* (Malausa et al., 2005). Several studies have, though, employed stable isotope analysis to determine food sources of agrobiont invertebrates. Carbon isotopic analysis was applied to assess the contribution of weeds, maize and soil organic matter in the diet of soil-dwelling click beetle larvae (Traugott et al., 2008), while $^{15}\text{N}/^{14}\text{N}$ ratio was used to detect a switch from detritivorous to herbivorous diet in a collembolan species in the presence of plants (Endlweber et al., 2009). The same ratio helped to reveal seasonal changes in the trophic levels of generalist predators (Birkhofer et al., 2011) and to demonstrate the impact of adding maize mulch on aboveground trophic cascades and pest control in wheat fields (Von Berg et al., 2010). Wise et al. (2006) compared the carbon and nitrogen isotope ratios between generalist predators and their aphid and collembolan prey to estimate the effectiveness of generalist predators in biological control.

Isotopic labelling provides an opportunity to study the pathways of organic matter in arthropod food webs (Elfstrand et al., 2008; Seeber et al., 2009). For example, Ke and Scheu (2008) examined the interactions between plant growth and insect pest performance, in a wheat-aphid model system utilising ^{15}N labelling, and Nienstedt and Poehling (2004) employed ^{15}N labelling to study predation on aphids by carabid beetles and spiders under semi-natural field conditions. By tracing the ^{13}C signal applied by pulse labelling, Seeber et al. (2012) showed that recently photosynthesised carbon was quickly incorporated in the tissues of root-feeding mites and collembolans in a subalpine grassland. Schallhart et al. (2012) used individual labelling of plants to examine how plant identity and diversity affects the feeding choice of root-feeding click beetle larvae. Crotty et al. (2011) used bacteria which were heavily enriched in ^{13}C and ^{15}N to track the flow of these elements through bacterial and higher trophic faunal feeding channels

in a soil food web, revealing that collembolans, mites and nematodes were the principal feeders of the labelled bacteria while the microarthropod predators were nourished in particular by the collembolans.

Stable isotope analysis can be readily used to quantify consumption between two food sources that exhibit contrasting isotopic signatures, but it is challenging (or even impossible) when the contribution of several food sources needs to be disentangled. In principle, the number of contributing food sources cannot exceed the number of isotopes measured plus 1 (Bearhop et al., 2004). However, several approaches have been suggested to address situations where the number of potential sources is higher (Phillips and Gregg, 2003; Phillips et al., 2005). Recently, Bayesian frameworks have been developed which are well suited to deal with multiple sources of uncertainty in estimates (Moore and Semmens, 2008; Parnell et al., 2010), though they do not allow coping with uncertainty in mixing models, that is, a situation where the number of food sources exceeds the number of measured isotopes plus 1 (Boecklen et al., 2011).

3.3. Trophic level, niche differentiation and food web structure

Trophic level assessment is based on: (1) determining a relevant baseline; and, (2) determining a discrimination value (i.e. the isotopic shift) for each trophic transfer (Layman et al., 2012). The discrimination value allows the conversion of isotope values into trophic positions relative to the baseline, and this is generally obtained through feeding experiments. While this approach has been widely used in aquatic systems (e.g. Layer et al., 2011), it has been less frequently applied in terrestrial arthropod food webs (for reviews, see Caut et al., 2009, 2010; McCutchan et al., 2003). Bennett and Hobson (2009) found that the isotopic assignment of trophic levels supported prior expectations about likely foraging niches, based on direct observations, by measuring $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in a broad range of arthropod taxa from boreal forests. In agroecosystem studies, the ratio $^{15}\text{N}/^{14}\text{N}$ has been used to determine the trophic level of click beetle larvae in arable soils (Traugott et al., 2008), to assess the trophic structure of an ant community in an organic citrus grove (Platner et al., 2012) and to study generalist arthropod predators and their linkage to detrital and grazing food webs (McNabb et al., 2001). Oelbermann and Scheu (2010) used $^{15}\text{N}/^{14}\text{N}$ ratios to identify trophic guilds of generalist feeders in a forest-meadow transect, suggesting that commonly used trophic guilds, such as detritivores and predators, consist of subsets of organisms which use various resources; so-called

sub-guilds. Furthermore, stable isotope analysis may be used to reveal intraguild predation (Rickers et al., 2006b).

Bearhop et al. (2004) proposed the use of variance in stable isotope signatures as a proxy for trophic niches. Following the same rationale, Newsome et al. (2007) defined the isotopic niche, mirroring the ecological niche definition of Hutchinson (1957), as an area with isotopic values as coordinates. Hence, isotopic ratios and their intra-specific range might be directly used to assess the degree of diet overlap in potentially competing species. However, Flaherty and Ben-David (2010) suggested that the use of isotopic niches as a proxy of ecological niches could be deceptive, stressing the influence of habitat in isotopically heterogeneous landscapes. Despite this ongoing debate, the utility of isotopic niches for answering questions in trophic ecology has been demonstrated (Rodríguez and Gerardo Herrera, 2013), and it has been used recently to distinguish five trophic groups of soil-dwelling oribatid mites (Maraun et al., 2011). Isotopic analyses helped to assess the niche overlap and hence the potential resource competition between desert locusts and domestic herbivores and showed few trophic interactions between locusts and livestock (Sánchez-Zapata et al., 2007). Finally, stable isotope signatures have also been used to examine whether closely related species use different feeding niches, such as the case of two carabid species within the genus *Amara* (Sasakawa et al., 2010).



4. FATTY ACID ANALYSIS

4.1. Methodological background

Fatty acid (FA) analysis is a well-established tool for studying trophic interactions in many different ecosystems. In marine environments the utility of FAs as bottom-up dietary tracers in food webs is reflected in recent reviews on arctic mammals (Thiemann et al., 2008), sea birds (Williams and Buck, 2010), plankton (Perhar et al., 2012) and the benthos (Kelly and Scheibling, 2012). FA use in arable and soil ecology has lagged behind. Starting a decade ago with laboratory investigations of binary links between fungi and nematodes (Chen et al., 2001; Ruess et al., 2002), FA methods were subsequently extended to higher trophic levels, including, for example, omnivorous Collembola (Ruess et al., 2004). It is only recently that the predatory soil fauna have been considered (Ferlian et al., 2012; Pollierer et al., 2010) and the first review on FAs as trophic biomarkers in soil food webs was published (Ruess and Chamberlain, 2010).

FA signatures can be used as dietary tracers due to the diverse array of specific FAs that originate in bacteria, fungi, algae and plants that animals are not capable of synthesising. These marker FAs are assimilated as entire molecules into consumer tissues and are subsequently transferred up the food web. The enzymatic capabilities in the lipid metabolism of micro-organisms have long been recognised and employed in chemical taxonomy (e.g. [Lechevalier and Lechevalier, 1988](#); [White et al., 1996](#)). More recently, FA profiling was applied to determine feeding strategies of invertebrates ([Chamberlain et al., 2004, 2005](#); [Ruess et al., 2002, 2005a](#)). FAs are assimilated from the diet and are preferentially directed into the neutral lipids of consumers ([Haubert et al., 2006](#); [Ruess et al., 2004](#)). These neutral lipid fatty acids (NLFAs) build the majority of animal lipids, whereas in prokaryotes phospholipid fatty acids (PLFAs) in membranes form the dominant lipid fraction ([Alvarez and Steinbüchel, 2002](#)). This allows separation of viable cells (i.e. PLFAs) from microbial FAs assimilated (i.e. NLFAs) in the animal consumer, thereby distinguishing bacteria transported via the gut or cuticle from microbial carbon incorporated into consumer tissue ([Ruess et al., 2005a](#)).

For trophic interactions in soil food webs, two types of biomarkers have been used: absolute and relative markers ([Ruess and Chamberlain, 2010](#)). Absolute marker FAs are absent from consumer lipid metabolism, whereas relative marker FAs are biosynthesised by the consumer, but increase in proportion if resources rich in these lipids are eaten. Absolute markers are available for bacterial diets, with methyl-branched (iso, anteiso) FAs as indicators for consumption of Gram-positive and cyclic FAs of Gram-negative taxa ([Haubert et al., 2006](#); [Ruess et al., 2005a](#)). Additionally, monoenoic bacterial FAs of the vaccenic type, with the double bond located at the ω 7 carbon, are useful bacterial markers. Unfortunately, in decomposer systems many FAs are non-specific and common across resources and consumers. This is caused by uniform mechanisms of FA biosynthesis in eukaryotes, that is, animals, plants and fungi, yielding palmitic acid as the major lipid in the pool ([Weete, 1980](#)). Nevertheless, relative marker FAs such as 18:2 ω 6,9 and 18:1 ω 9, which are accumulated from the diet, allow assignment of fungal and plant consumption, respectively ([Chamberlain et al., 2005](#); [Ruess et al., 2005a](#)).

The lack of uniqueness of FAs to a specific food source can be overcome by the combination of lipid profiling with stable isotope probing (FA-SIP), that is, compound-specific analysis of the $^{13}\text{C}/^{12}\text{C}$ ratio in FAs ([Chamberlain et al., 2004, 2006a,b](#); [Ruess et al., 2005b](#)). When food is

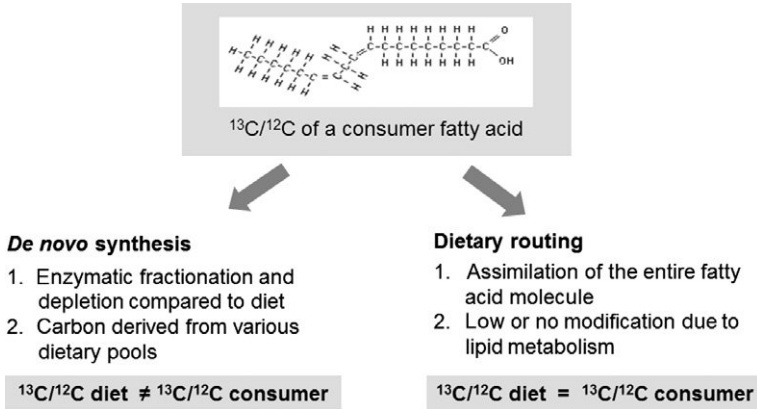


Figure 3.5 The fractionation in $^{13}\text{C}/^{12}\text{C}$ of biomarker fatty acids between diet and consumer can be used to assign specific trophic links in a food web.

plentiful, tissue lipid composition and $\delta^{13}\text{C}$ values are dominated by assimilated dietary FAs (Gaye-Siesseger et al., 2004). In turn, the $^{13}\text{C}/^{12}\text{C}$ ratios of the same FAs in resource and consumer display comparable values (Fig. 3.5). On the other hand, if lipids are derived from other resources, the $^{13}\text{C}/^{12}\text{C}$ ratio of FAs in proposed diet and consumer will vary (Haubert et al., 2009; Ruess et al., 2005b). This also applies to a diet low in lipids but abundant in energy, where consumers may store the available energy in *de novo* synthesised lipids (Gaye-Siesseger et al., 2003) (Fig. 3.5). In sum, for both marker FAs as well as their $^{13}\text{C}/^{12}\text{C}$ pattern, consumer FA signatures will never entirely match dietary FA composition because of *de novo* synthesis and modification of FAs in lipid metabolism.

4.1.1 Qualitative fatty acid profiling

The major prerequisite for assignment of feeding strategies, by qualitative FA analysis, is the specificity of markers to a certain resource or organism. However, FA patterns are often available only for some of the biota in the ecosystem of interest, predominantly in basal resources, such as micro-organisms in soil and photosynthetic eukaryotes in marine environments. Potential markers for trophic transfer along the food chain include FAs with the double bond at the $\omega 3$ carbon, such as γ -linolenic acid detected in protozoa (Lechevalier and Lechevalier, 1988) or eicosapentaenoic acid in Collembola (Chamberlain et al., 2005), which are generally not synthesised by animals occupying higher trophic levels.

A list of characteristic ester-linked FAs of common soil biota is given (Ruess and Chamberlain, 2010). In particular, FA patterns of soil nematodes are comprehensively ascribed ranging from early studies on free-living and plant parasitic species (e.g. Fletcher and Krusberg, 1973; Krusberg, 1967; Sivapalan and Jenkins, 1966) to recent work on bacterial and fungal feeders (Chen et al., 2001; Ruess et al., 2002, 2004). This large database for nematodes has resulted in a great advance in FA profiling and for the differentiation and identification of species by their lipid composition (Sekora et al., 2009). Such determination of species-specific FA patterns is promising, as it may ultimately allow identification of a particular nematode prey in a predator.

Overall, the use of lipid signature biomarkers for trophic interactions in agroecosystems is still under development and a broader knowledge of FA patterns in major food web biota as well as variation in this pattern due to environment or diet or life cycle is needed. This applies in particular to cases where FA profiles are more strongly related to species or environmental factors than to their diet, which may mask the signature of a trophic niche indicated by FA composition and thus hamper the value of the approach in food web studies. Abiotic factors such as temperature as well as biotic factors such as life stage were shown to affect *Collembola* lipid pattern, whereas, for example, starvation did not alter the lipid profile (Haubert et al., 2004, 2008). This underlines the need for a comprehensive screening of the lipid pattern of major faunal groups in agroecosystems in order to broaden this approach to higher trophic levels of the food web.

4.1.2 Quantitative fatty acid analysis

While qualitative aspects of trophic transfer in FAs along food chains are now relatively well understood, in order to fully understand and model ecosystems, quantitative information is necessary on the synthesis and turnover rates of FAs at different levels of the food web. In contrast to terrestrial ecosystems, FAs in marine organisms are extremely diverse with high levels of long-chain polyunsaturated FAs (Bell and Tocher, 2009). The value of such FAs in trophic ecology has resulted in detailed knowledge of the FA composition of various organisms and tissues, which permits both qualitative and quantitative dietary inferences to be made (reviewed in Budge et al., 2006). Quantitative FA signature analysis (QFASA) was developed as a tool to estimate predator diets in marine mammals by Iverson et al. (2004) and successfully applied to assess the diet in grey seals, polar bears and seabirds (Iverson et al., 2004; Thiemann et al., 2008; Williams and Buck, 2010),

underscoring the potential value of the QFASA model for other predators and ecosystems. The primary assumption of this approach is that differences in FA signatures are a function of diet rather than a reflection of predator metabolism. QFASA assumes that the specific (lipid pathways) or current (life cycle, starvation) metabolism of the predator is predictable and quantifiable. For invertebrates such information often can only be guessed at, with the exception of Collembola, which were surveyed comprehensively in regard to the impact of food quality, environmental factors and metabolic constraints on their lipid pattern (e.g. Haubert et al., 2004, 2008; Holmstrup et al., 2002; van Dooremalen and Ellers, 2009). More controlled experiments that measure the size of such effects are needed now, to ensure that FA data are interpreted correctly. After such validation and the development of calibration coefficients to account for predator lipid metabolism, for example, QFASA could be applied to a wide range of predators in above- and belowground ecosystems.

This note of caution applies to the quantification of carbon flow in the food web by FA-SIP, as certain metabolic pathways of organisms can hamper this approach. Besides fractionation occurring in *de novo* synthesis (see Section 3.1 and Fig. 3.5), the $\delta^{13}\text{C}$ values of individual FAs depends upon: (i) the ratios of precursor molecules used in FA synthesis relative to other biosynthetic activities, (ii) the isotopic fractionation by, for example, chain elongation, desaturation and (iii) the balance of direct assimilation from the diet relative to *de novo* synthesis within an organism (Ruess and Chamberlain, 2010). Hence, the $\delta^{13}\text{C}$ values of a specific FA can considerably vary with the carbon pool as well as the degree of unsaturation and chain length. Therefore, care must be taken when estimating trophic carbon flux at natural $^{13}\text{C}/^{12}\text{C}$ levels, when diet and consumer are not substantially isotopically different to avoid drawing misleading conclusions on trophic linking (Bec et al., 2011).

4.2. Determining food sources

4.2.1 Fatty acids as trophic markers

In soil ecosystems, Collembola populations have been ascribed to feeding guilds (i.e. fungivores, bacterivores, herbivores, predators) by trophic marker FAs across a range of forest (Ruess et al., 2005a, 2007) and arable soils (Haubert et al., 2009; Ngosong et al., 2009). These field surveys revealed that changes in the PLFA composition at the food web base, that is, within microbial communities, were closely mirrored by the lipid profiles of the fauna. This bottom-up transfer of FAs allowed indicating dietary

changes in Collembola related to farming practice such as altered amount and type of fertiliser (Ngosong et al., 2009). The study further revealed that arbuscular mycorrhizal fungi, although a frequent fungal resource in that arable site, were not consumed by Collembola grazers. FAs have also been applied to assess feeding behaviour as well as the gut microbiota of earthworms (Dungait et al., 2008). Both, the gut flora and the microbial assemblages in earthworm casts can vary with the type of resource (Gómez-Brandón et al., 2011). Trophic transfer of FAs further revealed specific microbial communities in the gut, from which the earthworm can derive energy, and nutrients in addition to microbiota present in the ingested soil (Sampredo et al., 2006).

4.2.2 ^{13}C in fatty acids as dietary tracers

Most ecological investigations using stable isotope analysis have involved the investigation of whole tissue or organisms, that is, 'bulk isotopic analysis', but more detailed information can be gained from FA-SIP (Fig. 3.5) approaches, where the determination of $^{13}\text{C}/^{12}\text{C}$ ratios in FAs in prospective diets and consumers allows attribution of binary trophic links as well as carbon flow in food webs (Budge et al., 2008; Hammer et al., 1998; Haubert et al., 2009). In addition, the application of FA-SIP also permits the determination of sources of lipids absorbed in archaeological material (Copley et al., 2003) and palaeodietary reconstruction (Stott et al., 1997).

Natural ^{13}C signals in terrestrial ecology mainly derive from $\delta^{13}\text{C}$ differences due to isotopic fractionations during C3 and C4 photosynthesis. Litter or sugars derived from C3 and C4 plants can be used to investigate translocation of carbon from micro-organisms or fungi to consumers, as demonstrated with Collembola grazers in laboratory FA studies (Chamberlain et al., 2006b; Ruess et al., 2005b). Besides natural isotopic differences, a signal can be introduced via $^{13}\text{CO}_2$ pulse labelling of plants or by ^{13}C marked resources (e.g. plant tissue, micro-organisms). For example, Drigo et al. (2013) used $^{13}\text{CO}_2$ labelling to investigate the rhizosphere carbon flow into microbial communities and protozoan grazers, with the latter expressed as excess ^{13}C incorporation into arachidonic acid (20:4 ω 6,9,12,15) as a marker FA.

The first complete C budget for the trophic link between bacteria and protozoa based on FA-SIP was quantified by Kupphardt et al. (2010), who found that nine FAs (20:0, i20:0, 22:0, 24:0, 20:1 ω 9c, 20:1 ω 9t, 22:1 ω 9c, 22:1 ω 9t, and 24:1) were derived from the protist *Uronema* sp., with 18% of bacterial biomass being incorporated in this consumer and

11% being mineralised, although not all of the FAs that were chosen matched those generally assigned to protozoan origin (see [Section 4.1.1](#)).

Only a few studies have applied FA-SIP in the field environment across multiple trophic levels. PLFA-SIP has been used for quantitative and chemotaxonomic information on resource allocation in microbial communities ([Chen et al., 2008](#); [Evershed et al., 2006](#); [Lu et al., 2004](#)), and more recent field studies in arable soil have identified trophic links up the food chain by FA-SIP of resources (plants, bacteria, fungi), grazers (Collembola) and predators (spiders) ([Haubert et al., 2009](#); [Ngosong et al., 2011](#)). Overall, these studies indicate that FA-SIP provides a high-resolution method to define feeding strategies of invertebrates *in situ*.



5. WHICH APPROACH TO CHOOSE, HOW TO START AND HOW TO INTERPRET THE DATA?

The three different methodological approaches we have outlined have different strengths and weaknesses, which are summarised in [Table 3.1](#). In general terms, the molecular techniques allow identification of highly taxonomically resolved interactions to be identified, which can produce very detailed, complex ecological networks of different types (e.g. host–parasitoid networks, food webs, plant–pollinator networks). Stable isotopes and FAs reflect broad pathways of biomass that is assimilated into consumers' tissues, together with the associated energy and nutrient fluxes in the food web. Hence, the appropriateness of a particular technique depends on the questions being asked and on the scale of the study.

Besides field studies, all of the three methodologies are well suited to disentangling feeding interactions in laboratory, mesocosm and cage experiments, which can help in obtaining better mechanistic understandings, or ground-truthing, of field-derived observations ([Stewart et al., 2013](#)). For example, [Von Berg et al. \(2008b\)](#) employed DNA-based gut content analysis to examine how rain affects predation of cereal aphids by ground-based generalist predators, and the development and dynamics of the aphid population. In a mesocosm experiment, [Schallhart et al. \(2012\)](#) used isotopic labelling of individual forbs, grasses and legumes to show that plant diversity rather than abundance modulates the feeding choices of root-eating click beetle larvae. FAs were applied to assess trophic interactions between bacteria and their protozoan grazers in closed microcosms, which can serve as model systems to link microbial and faunal food webs ([Kupphardt et al., 2010](#)).

Table 3.1 Overview on the advantages and disadvantages of DNA-based, stable isotope and fatty acid analysis to assess trophic interactions as well as the potential applications in food web ecology

	Advantages	Disadvantages	Application
1. DNA-based trophic analysis	<ul style="list-style-type: none"> • Assessment of ‘difficult’ trophic interactions • High sensitivity and specificity • Taxonomic resolution of food can be adjusted according to research needs • Cost-effective and rapid screening of large numbers of dietary samples 	<ul style="list-style-type: none"> • Sensitive to DNA cross-contamination • Cannibalistic interactions not yet addressed • No differentiation between active predation and scavenging • Comparisons across different consumer and prey taxa not straightforward 	<ul style="list-style-type: none"> • All studies where food sources and parasitoids are to be specifically identified
1.1. Diagnostic PCR	<ul style="list-style-type: none"> • Rapid screening of large numbers of samples • Detection of multiple taxa within multiplex PCRs 	<ul style="list-style-type: none"> • Detects only DNA of food/parasitoid taxa for which assays have been designed 	<ul style="list-style-type: none"> • Assessment of single (singleplex PCR) and multiple (multiplex PCR) trophic links at required taxonomical resolution • Individual-based diet analysis
1.2. Sequence-based identification	<ul style="list-style-type: none"> • Broad coverage of a wide range food sources and parasitoids 	<ul style="list-style-type: none"> • Identification of sequences dependent on reference data base • Sequencing errors can corrupt analysis • Not yet suited for examining large numbers of individual samples 	<ul style="list-style-type: none"> • Assessment of complex diets/host–parasitoid systems • Characterisation of diet breath/parasitoid diversity • Population-based diet analysis

Table 3.1 Overview on the advantages and disadvantages of DNA-based, stable isotope and fatty acid analysis to assess trophic interactions as well as the potential applications in food web ecology—cont'd

	Advantages	Disadvantages	Application
2. Stable isotope analysis	<ul style="list-style-type: none"> • Assessment of feeding history • Integrative view on metabolised food sources 	<ul style="list-style-type: none"> • Low resolution for consumption of multiple food sources • Non-trophic effects can corrupt conclusions 	<ul style="list-style-type: none"> • Determination of food sources and trophic position • Examination of nutrient flow through food webs
2.1. Natural isotope abundance	<ul style="list-style-type: none"> • Differentiation between main food sources • Determination of trophic level 	<ul style="list-style-type: none"> • Discrimination power depends on isotopic differences between food sources 	
2.2. Isotopic labelling	<ul style="list-style-type: none"> • Quantitative assessment of the consumption of specific food sources 	<ul style="list-style-type: none"> • Only a small number of food sources can be labelled • Homogenous isotopic labelling can be difficult • Leaching of label and unintended labelling of other food sources 	<ul style="list-style-type: none"> • Quantification of metabolised diet

Continued

Table 3.1 Overview on the advantages and disadvantages of DNA-based, stable isotope and fatty acid analysis to assess trophic interactions as well as the potential applications in food web ecology—cont'd

	Advantages	Disadvantages	Application
3. Fatty acid analysis	<ul style="list-style-type: none"> • Widely distributed and diverse markers • Indicates ingestion and assimilation of diet 	<ul style="list-style-type: none"> • Intermediate specificity • Metabolic modification of markers in the consumer 	<ul style="list-style-type: none"> • Determination of trophic groups • Estimation of carbon flow in food webs
3.1. Fatty acid profiling	<ul style="list-style-type: none"> • Simplicity of chemical analysis and high sample processing rate • Qualitative and quantitative trophic measure 	<ul style="list-style-type: none"> • Food sources without unique markers • Biosynthesis in consumers hampers quantification 	<ul style="list-style-type: none"> • Assignment of feeding strategy • Quantification of predator diets
3.2. Fatty acid SIP	<ul style="list-style-type: none"> • Resolution on the level of binary links • Quantification of carbon flux through food webs 	<ul style="list-style-type: none"> • Considerable pool size in the trophic cascade necessary • Carry-over effects in labelling experiments 	<ul style="list-style-type: none"> • Ascribing binary links • Carbon transfer along a food chain

Combining the different methodologies provides a synergy of their respective strengths and such an integrated approach is a powerful means of characterising food web interactions, in terms of both ingestion and assimilation. For example, [Hardy et al. \(2010\)](#) combined molecular prey identification with stable isotope analysis to identify food–consumer dynamics. Within a field experiment examining the potential to control soil insect pests via vegetational diversification, [Staudacher et al. \(2013\)](#) combined carbon isotope with DNA-based gut content analysis. While the former technique was employed to quantify the consumption of the C4-crop maize, diagnostic PCR was used to measure the consumption of C3 plants on a species-specific level, which was not possible using isotopic analysis. [Drigo et al. \(2013\)](#) used ^{13}C pulse labelling and subsequent RNA-SIP and FA-SIP in a study investigating a 3-year impact of elevated CO_2 , as a tool to assign rhizosphere carbon flow into micro-organisms and associated microfauna.

When interpreting the trophic information obtained by these very different methodologies, care needs to be taken to ensure meaningful conclusions are drawn. For instance, DNA-based methodologies do not allow differentiation between the consumption of dead and living food resources ([Juen and Traugott, 2005](#); [Wallinger et al., 2013](#)). Disentangling scavenging from predation, however, can be critically important when top-down trophic effects are to be identified, such as in biological control studies, as the consumer–resource dynamics involved are very different. Behavioural studies on the preferences for living or dead prey (e.g. [Von Berg et al., 2012](#)) as well as the assessment of the availability of carrion prey need to be taken into account in such a situation. Stable isotope analysis, on the other hand, can identify the proportion of dead organic material in the diet of a consumer. Soil organic matter typically differs significantly in its isotopic signature when compared with plant litter and living plants, enabling the proportional contribution of each to the diet of animals such as click beetle larvae to be determined ([Traugott et al., 2008](#)). Comparably, the $^{13}\text{C}/^{12}\text{C}$ ratio of FAs in consumers varies with the predominant plant resource, allowing assessment of the translocation of carbon from dead plant litter ([Chamberlain et al., 2006b](#)) and living plant tissue ([Haubert et al., 2009](#); [Ngosong et al., 2011](#)) into the faunal food web.

Secondary predation represents another potential source of error when binary trophic relationships are characterised, and it can create strange anomalies in food webs, whereby prey that can be well outside the consumer's normal range appear in its diet, due to this 'Russian dolls' effect ([Woodward et al., 2010b](#)). Secondarily predated prey, where food that

was originally consumed by an intermediate predator is then ‘accidentally’ eaten by a second predator, can lead to errors in food chain assignment and mismatches between the predictions of structural food web models and empirical data (Woodward et al., 2010b). This type of error is especially relevant in DNA-based food detection, although the possibility of detecting secondarily predated prey via molecular techniques depends on the specific predator–prey system investigated (Sheppard et al., 2005). Multiplex PCR and NGS, which allow screening the consumer for food sources from different trophic levels, can provide a good estimate of the fraction of secondarily consumed food. For a putatively omnivorous animal, the proportion of secondarily consumed plant material acquired by feeding on herbivores can be estimated by the detection frequency of that herbivore prey. Stable isotope analysis, at natural isotopic abundance levels, is usually not affected by this type of error as the secondarily consumed food is unlikely to result in a significantly different contribution to the overall isotopic composition of the consumer—as it is usually a tiny biomass relative to the typical diet. However, in isotopic labelling, where labels are usually used at high concentrations, the ingestion of secondarily predated prey can corrupt the assignment of trophic links. Secondary predation also affects the interpretation of lipid patterns as marker FAs can be transferred across trophic levels, from basal resources (bacteria, fungi) via first-order consumers (nematodes, Collembola) to omnivores or predators (Collembola, Chilopoda, Arachnida) (Pollierer et al., 2010; Ruess et al., 2004). However, besides the problems of assigning binary links, tri-trophic level shifts of marker molecules have the distinct advantage of indicating the dominant carbon pathways in the food web, such as those trophic connections along bacterial, fungal and root energy channels.

Another challenging trophic interaction is cannibalism, which is common in many food webs (Ings et al., 2009). DNA-based prey detection has not yet been used to track cannibalistic interactions, although it might be possible to identify conspecific prey by genetic fingerprinting techniques. Isotopic enrichment can be used to assess the magnitude of cannibalistic interactions, but it is usually difficult to discriminate it from other forms of intraguild predation (e.g. Greenwood et al., 2010).

For more comprehensive information on how to interpret the data derived by the three different methodologies, we refer the reader to several other recent review articles that have focused on each in turn (e.g. Boecklen et al., 2011; King et al., 2008; Pompanon et al., 2012; Ruess and Chamberlain, 2010).

The three techniques reviewed here offer the exciting possibility of understanding better community functioning in arable systems via a food web approach (Memmott, 2009). We expect that future methodological developments will further augment the power of these techniques, allowing characterisation of feeding networks at unprecedented resolution and to measure nutrient fluxes through complete food webs. This toolkit also provides the opportunity to measure, empirically, important aspects of food webs such as their spatio-temporal heterogeneity (Olesen et al., 2010), response to environmental stressors such as climate change (Woodward et al., 2010a), their long-term dynamics (Layer et al., 2011), their vulnerability to biodiversity loss (Bohan et al., 2013) and the linkage between webs in different habitats, such as those in below- and above-ground agroecosystems (Mulder et al., 2013) and within the landscape mosaic (Massol and Petit, 2013). The increased information on empirically derived food web data generated by these techniques will also complement insights obtained from machine learning approaches (Tamaddoni-Nezhad et al., 2013), provide a solid data base for modelling of interaction networks in agroecosystems (Tixier et al., 2013), and fuel theoretical and concept-based research on food webs, such as trait-based approaches (Railsback and Harvey, 2013) or eco-evolutionary perspectives (Loeuille et al., 2013; Melian et al., 2011). This will feed a better mechanistic understanding of agroecosystems function and how to optimise their management to maximise ecosystem services (Bohan et al., 2013).

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GLOSSARY

DNA-based techniques

Blocking primer unique primer specifically designed to prevent the amplification of particular DNA sequences. Widely applied in NGS-based diet analysis, to avoid preferential amplification of consumer DNA over DNA from food remains.

Diagnostic PCR a PCR assay which is used to test (dietary/host) samples for the presence of DNA from a specific species or a group of organisms.

DNA cloning process where individual PCR products are inserted into a plasmid to generate many identical copies which can then be sequenced. Cloning allows sequencing mixtures of PCR products derived from different taxa.

Meta-barcoding the identification of taxa derived from a bulk sample (environmental DNA) via high-throughput sequencing and bioinformatic analysis.

Next-generation sequencing (NGS) a suite of technologies which parallelise the sequencing process and generate millions of sequences concurrently, a process which is also called high-throughput sequencing. This approach allows revealing many different food items by identifying their sequences.

Primers short fragments of single-stranded DNA which are complementary to the target sequence. The new DNA strand is synthesising from the 3'-end of the primer. The specificity of the primers can be adjusted to species or higher taxonomic levels (species- and group-specific primers).

Quantitative PCR a PCR assay which allows determining the starting quantity of template DNA molecules which are present in a sample.

Sample cross-contamination unintended carryover of DNA between samples.

Singleplex PCR/multiplex PCR a polymerase chain reaction (PCR) where one pair of primers is used to amplify one specific PCR fragment is called a singleplex PCR, whereas in multiplex PCR more than one primer pair is employed to simultaneously amplify several PCR fragments within one reaction.

Tagging a short sequence added to the 5'-end of a primer allowing to recognise sequences of specific samples after a high-throughput sequencing of PCR products from pooled samples.

Template molecule the sample DNA molecule that contains the target sequence the primers bind to.

Stable isotope analysis

Compound-specific stable isotope analysis determination of the isotopic composition in specific organic analytes present in the sample matrix such as lipids.

Delta notation the difference in isotopic composition between a sample and a reference which is expressed in per mil (‰).

Fractionation/discrimination a process within the consumer which causes stable isotopic abundance variation between food source and consumer. An example is ^{15}N which is usually enriched along the food chain.

Isotopic baseline the isotopic composition of primary producers in a given system which needs to be taken into account for calculation of diet or trophic position.

Isotopic niche an area within an isotopic space where the isotopic values are used as coordinates.

Fatty acid analysis

Absolute markers fatty acids a consumer cannot synthesise *de novo* and therefore only appear in its lipids when feeding on the respective diet.

Dietary routing incorporation of dietary fatty acids without modification and transfer of the entire molecules into consumer tissue.

FA-SIP (fatty acid stable isotope probing) determination of the $^{13}\text{C}/^{12}\text{C}$ ratio in individual fatty acids in order to assess trophic carbon flow.

- Omega (ω) notation** classification of unsaturated fatty acids according to the number of carbon atoms from the terminal methyl group (ω end) to the nearest double bond.
- Quantitative fatty acid signature analysis (QFASA)** a statistical model providing estimates of proportions of prey species in the diets of individual predators using fatty acid signatures.
- Relative markers** fatty acids that are synthesised in consumer metabolism but additionally are highly accumulated from the diet.

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